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Is the Marine Environment of the Black Sea Stressful for Organisms: A Pilot Assessment of Oxidative Stress in Bulgarian Coastal Fish Species

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Abstract. The present study is the first assessment of oxidative stress (OS) in fish species inhabiting the Bulgarian Black Sea coastal zone. The fish were caught during trawl selectivity experiments from different localities of the northern and southern coastal regions. The pro/antioxidant status of fish individuals was assessed by measuring standard OS biomarkers (lipid peroxidation, glutathione concentration, activities of superoxide dismutase and catalase) in gills and liver. Differences in the concentration and activity of OS biomarkers in the studied organs were clearly demonstrated. It was found that the level of OS in the studied fish species differed depending both on the species and on the coastal region they inhabit. Our results demonstrated for the first time the presence of OS in fish inhabiting coastal ecosystems of the Bulgarian Black sea sector with different quality of the marine environment. Obviously, further studies are needed for the assessment of multiple stressor effects on the ecology of Bulgarian Black Sea fish populations.

Key words: Black Sea, Bulgaria, coastal fish, oxidative stress.

Introduction

Marine ecosystems are under the increasing influence of numerous stressors, including climatic, oceanographic, environmental and anthropogenic, causing significant changes in their functioning and the services they provide. The Black Sea is a unique semi-exclosed basin accepting a high river inflow from the rivers Danube, Dnieper, Dniester, and Southern Bug (Zaitsev & Mamaev, 1997). Because of the significant ecological deterioration, the Black Sea has been declared as a highly polluted sea (Oguz & Velikova, 2010; Makedonski et al., 2017). However, intensive pollution does not affect the entire Black Sea but, rather, its northwestern part and also the marginal habitats where marine, terrestrial, and freshwater organisms interact (Zaitsev, 2008).

Fish inhabit a broad range of ecosystems where they are subjected to many different aquatic contaminants. Fish responses to stress can be polymorphic depending on the species, age, diet and on the stressors severity (Pimentel et al., 2015;

Hamilton et al., 2017; Zielinski & Pörtner, 2000). Different biomarkers are recently becoming an integral part of health assessments and management of marine ecosystems, in addition to the more routine water chemical analyses (Valvanidis et al., 2006; Hook et al., 2014). Fish are traditionally used as bioindicators and play an important role in marine monitoring programs. In many cases, the detrimental effects of water contaminants have been connected to induction of oxidative stress in fish (Lushchak, 2016). The adaptive response of marine fish to environmental changes can be expressed at the cellular level through changes in their pro/antioxidant status. Oxidative stress (OS), as a disturbance of the oxidation/reduction balance in the cell, is characteristic of all aerobic organisms where reactive oxygen species (ROS) are generated together with the antioxidant processes in which they are neutralized (Birnie-Gauvin et al., 2017). The Black Sea ecosystems are especially vulnerable to pressures by various anthropogenic activities and fish, as a key component of these ecosystems, are exposed to multiple stressors of the changing marine environmental conditions. Application of biomarkers for assessing the biological impact of pollutants and xenobiotics, and the relationship between antioxidant responses and susceptibility to oxidative stress in different species of Black Sea fish were studied by a number of authors (Rudneva et al., 2010; Kovyrshina & Rudneva, 2016; Skuratovskaya et al., 2017; Chesnokova et al., 2020; Sigacheva et al., 2020).

At present no research has been carried out on the OS in marine fish in Bulgaria. The aim of this preliminary study was to make an initial assessment of the activities of a battery of OS biomarkers in several common fish species from the Bulgarian Black Sea coastal zone.

Material and Methods

Sampling

The fish selected for the study are common demersal species, including three benthic forms - *Platichthys flesus* (Linnaeus, 1758), *Neogobius melanostomus* (Pallas, 1814), *Trachinus draco* (Linnaeus, 1758), and two bentho-pelagic forms - *Mullus barbatus* (Linnaeus, 1758) and *Merlangius merlangus* (Linnaeus, 1758) (BSFishList, 2020; FishBase ver. 2021). Fish were randomly sampled from trawl catches using pelagic Midwater otter trawl (7x7 mm mesh size of the codend) from 4 localities of the northern and 3 localities of the southern Bulgarian Black Sea coast (Table 1).

Table 1. Trawling localities along the Bulgarian Black Sea coast with geographical co-ordinates and sampled fish species. *Legend:* *N – northern locality; S – southern locality.

Code	Trawling locality	Trawling start point	Trawling end point	Fish species
*N1	Tyulenovo	43.521269 28.728255	43.481384 28.717535	*Platichthys flesus, Merlangius merlangus*
N2	Kaliakra cape	43.365251 28.428852	43.371263 28.405309	*Platichthys flesus, Neogobius melanostomus*
N3	Batova bay	43.378802 28.157542	43.343525 28.142787	*Platichthys flesus, Neogobius melanostomus, Mullus barbatus*
N4	Shkorpilovtsi	42.976214 27.966946	42.952405 27.940966	*Platichthys flesus, Merlangius merlangus*
S1	Nessebar bay	42.599613 27.791345	42.625721 27.832556	*Neogobius melanostomus, Trachinus draco, Mullus barbatus*
S2	Pomorie bay	42.569317 27.791159	42.609556 27.796726	*Trachinus draco, Mullus barbatus*
S3	Sozopol bay	42.439217 27.914862	42.433054 27.887407	*Platichthys flesus, Merlangius merlangus, Neogobius melanostomus, Trachinus draco*

Tissue preparation

The fish samples were shock frozen on board for best preservation (Secci & Parisi, 2016) and transported to the laboratory. The fish were dissected and their liver and gills were extracted following available protocols (Stoyanova et al., 2020 a,b). The organs were homogenized in 0.1 M potassium phosphate buffer (pH 7.4) and thereafter centrifuged at 3000 g for 10 min. The post-nuclear fraction was used for determination of lipid peroxidation (LPO) and glutathione (GSH) levels. For obtaining a post mitochondrial supernatant used for measurement of the antioxidant enzymes activities, a portion of the post-nuclear fraction was re-centrifuged at 12 000 g for 20 min at 4°C.

Measurement of oxidative stress biomarkers

Lipid peroxidation was determined using MDA assay kit (Catalog No: MAK085), purchased from Sigma-Aldrich Co. LLC (USA). The assay is based on the reaction of thiobarbituric acid (TBA) with end-products of the LPO. The absorption of the formed malone dialdehyde (MDA) was read at 532 nm and was calculated as nmoles/mg protein using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Glutathione concentration was measured according to Rahman et al. (2006). The reduced glutathione (GSH) reacted with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) giving a color compound with absorption peak at 412 nm. The GSH amount was calculated using a reference standard and expressed as ng/mg protein.

Superoxide dismutase activity was measured according to Peskin & Winterbourn (2017). The inhibition of water-soluble tetrazolium (WST-1) reduction by superoxide radicals is a measure for enzyme activity. The values were expressed in U/mg protein as one unit is defined as the amount of enzyme needed to inhibit the WAT reduction by 50%.

Catalase Activity was assayed by the method of Aebi et al. (1984), based on the decrease of absorption at 240 nm that corresponds to the enzymatic decomposition of H_2O_2. Enzyme activity was expressed as U/mg protein.

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

Statistical analyses

The software package used was STATISTICA (Data analysis software system), StatSoft Inc. (2010), Vers. 10. The non-parametric Kruskal–Wallis test was used to confirm the presence of differences among the values of the OS markers in the different groups studied. Post hock comparisons between variables were made using Mann-Whitney test.

Results

In this preliminary study the differences of the OS biomarkers were measured both among the selected fish species and among the localities along the Bulgarian Black Sea coast from which they were sampled. The overall results of the analyses are summarized in Table 2. Kruskal–Wallis test indicated significant differences of OS indicators among sampling sites, fish species and organs which were post hock tested by Mann-Whitney statistics.

The first observation in this study was the presence of significant difference between values of the studied OS indicators in the gills and the liver of all fish species. This differences and their statistical significance are summarized in Figure 1. The LPO and SOD were higher in gills of fish from both northern and southern localities. The SOD activity was significantly higher in gills of fish from the southern localities. In contrast to SOD, the activity of CAT was significantly higher in the liver of fish from both northern and southern localities. Despite the individual differences in GSH concentration in the fish species, no significant differences were found between the two organs of the fish from northern and southern localities.

The analysis of the level of the OS indicators in the fish species from different

localities (Table 2) showed that round goby (*N. melanostomus*) had the highest LPO level in the liver of individuals from Tulenovo (N1). The highest LPO in gills was observed in greater weever (*T. draco*) individuals from Nessebar bay (S1). In gill high LPO was found in flounder (*P. flesus*) from Shkorpilovtsi (N4) and Sozopol bay (S3), in round goby (*N. melanostomus*) from Tyulenovo (N1) and in greater weever *(T. draco)* from Pomorie bay (S2) and Sozopol bay (S3).

The lowest GSH concentration in liver was observed in round goby from Nessebar bay (S1) and Sozopol bay (S3) and in gill – in round goby from Tyulenovo (N1). The highest GSH values were measured in whiting from Shkorpilovtsi (N4) in both organs liver and gill. SOD activity in red mullet was high in liver and gills from the localities N3, S1 and S2. High values of SOD were observed in gills of greater weever from the southern areas (S1, S2 and S3) and in round goby from Nessebar bay (S1) and in liver of flounder from Tyulenovo (N1), Pomorie bay (S2) and Sozopol bay (S3). The activity of CAT was in general higher in the liver of all fish species analyzed in comparison to gills. The highest values were detected in the liver of whiting from Shorpilovtsi (N4). The lowest values were obtained in gills of round goby from Tyulenovo (N1).

In the studied individuals of European flounder higher LPO was measured in the gills in comparison to liver, as the highest values were measured in fish from Shorpilovtsi (N4) and Sozopol bay (S3), accompanied by relatively low GSH concentrations (Table 2). These observations indicated stronger oxidative stress in these locations. The relatively low LPO in the flounders from Pomorie bay (S2) was probably due to higher SOD activity in liver (the highest one) and gills. The very high activities of SOD and CAT reported in the gill of flounders caught near Tyulenovo (N1) and the high SOD activity in liver were probably responsible for the lower OS, as indicated also by relatively lower LPO level. It could be assumed that the flounders from the Batova bay (N3) were least stressed, judging by the relatively lower levels of LPO, higher GSH concentrations and the lack of antioxidant enzymes induction in both studied organs.

In whiting, higher LPO was found in the gills. The lowest LPO levels (in both organs) were measured in individuals from Shkorpilovtsi, probably due to the antioxidant defense represented by high levels of GSH and SOD activity in both organs and high CAT activity in liver (Table 2). It seemed that the whiting inhabiting the littoral near Tyulenovo (N1) were exposed to higher OS stress, indicated by the higher LPO in liver, accompanied by low GSH content and lower CAT activity.

Round goby sampled from Tyulenovo (N1) demonstrated extremely high LPO in liver, accompanied by high activity of SOD compared to the individuals from other localities. The highest LPO level in gills of gobies was also measured in the samples from Tyulenovo (N1), along with the lowest GSH content.

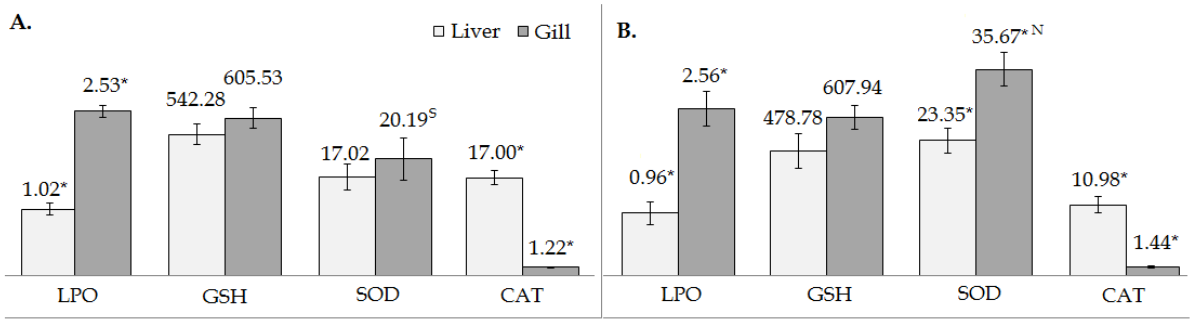
It should be mentioned the low level of GSH in liver of round goby from southern locaties, Nessebar bay (S1) and Sozopol bay (S3), compared to the northern localities. In general, these findings suggested that most probably round goby individuals inhabiting the marine waters near Tyulenovo (N1) were exposed to higher OS stress.

In greater weever the highest LPO both in liver and gills was measured in the specimens sampled from Nessebar bay (S1), together with comparatively lower GSH concentration (Table 2). SOD activities were lowest in the samples from Sozopol Bay (S3) both in liver and gills. Most probably, the marine habitat conditions in Nessebar bay (S1) were more stressful for the greater weever as suggested by the higher LPO in both organs and the low GSH concentration in gills.

In mullet the highest LPO, accompanied by low concentrations of GSH, were found in the liver of the fish from Nessebar bay (S1),

suggesting that the mullets in this location were exposed to higher OS stress (Table 2).

In general, the content and activity of the measured OS biomarkers were found to vary not only among fish liver and gills in general, but they varied significantly also among the fish species depending on the different localities they inhabit (Table 3).



(LPO is measured as nmoles MDA/mg protein; GSH is in ng/mg protein; SOD and CAT activities are in U/mg protein)

Fig. 1. Measured OS biomarkers in liver and gills in total fish samples from northern (A) and southern (B) localities (* - difference of indicator between the two organs significant at $p<0.05$; N and S mark significance of difference ($p<0.05$) between indicator from the northern and southern locality correspondingly.

Table 2. Values of the measured OS biomarkers in gills and liver of the studied fish species from different localities of the Black Sea coast (N – northern localities; S – southern localities). *Legend:* *statistical significance of differences at $p<0.05$: N_n or S_n indicate significant differences of the OS indicator in fish species between sites as N=northern sites and S=southern sites; letters (G, L) indicate significance of differences of the OS indicator between organs as G=gill and L=liver.

Organ	**Liver**				**Gills**			
Location/ biomaker	**LPO**	**GSH**	**SOD**	**CAT**	**LPO**	**GSH**	**SOD**	**CAT**
				Platichthys flesus				
N1	$*0.54^{N2,G}$	340.00	$34.49^{N2,N3,N4,S2,G}$	$13.79^{N2,G}$	2.64^{L}	431.35	24.34^{L}	5.73^{L}
Tyulenovo	±0.07	±6.45	±1.45	±1.11	±0.49	±39.26	±1.11	±0.26
N2	$1.04^{N1,N4,S2,G}$	597.09	$2.98^{N1,S2,S3,G}$	$21.48^{N1,N3,N4,S2,S1,G}$	$3.28^{S2,L}$	513.87	12.13^{L}	0.77^{L}
Kaliakra cape	±0.21	±77.67	±0.35	±1.56	±0.09	±119.54	±1.01	±0.31
N3	0.61^{G}	694.38	$5.12^{S2,S3,G}$	$14.94^{N2,G}$	2.49^{L}	585.25	12.96^{L}	1.10^{L}
Batova bay	±0.23	±134.82	±1.18	±3.76	±0.40	±106.34	±0.88	±0.11
N4	$0.45^{N2,G}$	343.77	$3.00^{S2,S3,G}$	$14.23^{N2,G}$	$3.62^{S2,L}$	379.12	11.03^{L}	1.76^{L}
Shkorpilovtsi	±0.03	±43.76	±0.77	±1.32	±0.32	±38.20	±0.86	±0.23
S2	0.65	515.05	$47.22^{N1,N2,N3,N4,G}$	$12.75^{N2,G}$	$1.74^{N2,N4,S3,L}$	481.11	19.49^{L}	1.03^{L}
Pomorie bay	±0.08	±88.14	±2.47	±1.57	±0.31	±94.64	±5.01	±0.17
S3	0.87^{G}	443.65	$39.63^{N2,N3,N4,G}$	$10.43^{N2,G}$	$3.81^{S2,L}$	313.00	17.78^{L}	0.71^{L}
Sozopol bay	±0.25	±142.30	±17.09	±4.78	±0.42	±144.80	±5.14	±0.35
				Merlangius merlangus				
N1	$2.11^{N4,S3}$	734.24^{N2}	27.61^{G}	$17.53^{N4,S3,G}$	2.97^{N4}	$899.11^{N4,L}$	$5.12^{N4,L}$	1.42^{L}
Tyulenovo	±0.08	±41.61	±2.8	±2.10	±0.65	±36.14	±1.18	±0.84
N4	$0.70^{N1,G}$	1082.62^{N1}	30.26^{G}	$30.13^{N1,G}$	$1.33^{N1,S3}$	$1673.19^{N1,S3,L}$	$10.45^{N1,L}$	1.18^{L}
Shkorpilovtsi	±0.12	±112.52	±4.10	±4.33	±0.19	±314.48	±0.45	±0.61
S3	$0.92^{N1,G}$	958.53	22.31^{G}	$27.10^{N1,G}$	2.58^{N4}	910.69^{N4}	7.57^{L}	1.38^{L}
Sozopol bay	±0.26	±263.65	±1.92	±0.60	±0.22	±148.07	±1.45	±0.22
				Neogobius melanostomus				
N1	$12.19^{N2,N3,S1,S3,G}$	359.75	$19.36^{N2,N3,S1,S3}$	$6.75^{N3,S3,G}$	$4.18^{N2,N3,S3,L}$	$293.08^{N2,S1,S3}$	27.13^{N2}	$0.55^{N3,L}$
Tyulenovo	±0.94	±63.50	±7.93	±0.63	±0.32	±37.64	±5.16	±0.03

N2	1.68^{N1}	394.30	6.02^{N1}	5.92^{N3,G}	1.84^{N1}	495.33^{N1,S3}	1.54^{N1,N3,S1,S3,L}	0.82^{N3,L}
Cape Kaliakra	±0.45	±53.52	±0.54	±2.18	±0.42	±32.83	±0.25	±0.09
N3	1.25^{N1}	518.07	4.92^{N1,G}	23.07^{N1,N2,S1,G}	2.18^{N1}	427.17^{S3}	31.69^{N2,L}	1.73^{N1,N2,S1,S3,L}
Batova bay	±0.74	±87.28	±0.92	±1.36	±0.73	±108.66	±1.72	±0.05
S1	2.23^{N1}	238.19^{N3,G}	6.46^{N1,G}	4.48^{N3,G}	2.63^{N1}	415.67^{N1,S3,L}	43.36^{N2,L}	0.84^{N3,L}
Nessebar bay	±0.32	±29.55	±1.76	±1.00	±0.31	±28.87	±3.47	±0.13
S3	0.93^{N1,S1}	287.09^{N3,G}	1.98^{N1,G}	15.58^{G}	0.85^{N1,N2,N3,S1}	929.68^{N1,N2,N3,S1,L}	35.49^{N2,L}	0.83^{N3,L}
Sozopol bay	±0.25	±55.74	±0.52	±3.73	±0.16	±199.64	±2.47	±0.37
				Trachinus draco				
S1	1.08^{S2,G}	571.21^{G}	7.09^{G}	5.87^{G}	4.96^{L}	469.13^{S2,S3,L}	45.08^{S2,L}	2.36^{S2,L}
Nessebar bay	±0.08	±26.77	±0.75	±0.73	±0.25	±9.52	±4.12	±0.29
S2	0.47^{S1,G}	307.86^{S3,G}	7.58^{G}	4.75^{G}	3.81^{L}	756.43^{L}	58.93^{S1,S3,L}	1.60^{S1,S3,L}
Pomorie bay	±0.14	±39.98	±0.45	±0.57	±0.25	±36.72	±1.83	±0.23
S3	0.72^{S1,G}	628.05^{S2,G}	5.17^{G}	6.98^{G}	3.76^{L}	897.50^{L}	39.08^{S2,L}	2.51^{S2,L}
Sozopol bay	±0.08	±24.49	±0.66	±0.72	±0.28	±52.57	±1.71	±0.13
				Mullus barbatus				
N3	0.55^{S1}	483.54	44.39	22.09^{S1,S2,G}	0.73	357.87^{S2}	51.66^{S2}	1.46^{L}
Batova bay	±0.13	±195.73	±6.49	±4.7	±0.27	±22.25	±6.21	±0.28
S1	0.96^{N3}	390.37	50.05	11.22^{N3,G}	0.61	354.62^{S2}	49.99^{S2}	1.21^{L}
Nessebar bay	±0.02	±20.71	±5.02	±0.82	±0.02	±29.37	±2.60	±0.24
S2	0.72	447.28	38.09	11.18^{N3,G}	0.74	504.63^{N3,S1}	39.89^{N3,S1}	1.95^{L}
Pomorie bay	±0.08	±82.98	±10.27	±0.87	±0.10	±34.35	±0.41	±0.09

Table 3. Results of OS biomarker content and activity measured in the different species and localities. *Legend:* *statistical significance of differences at p<0.05: N or S indicate significant differences of the OS indicator in fish species among northern (N) sites and southern (S) sites; G (gills) or L (liver) indicate significance of differences of the OS indicator between organs.

	Liver		**Gills**	
	Northern	**Southern**	**Northern**	**Southern**
		Platichthys flesus		
LPO	0.66±0.28^{G}	0.76±0.21^{G}	3.01±0.89^{L}	2.77±1.09^{L}
GSH	495.23±176.12	479.34±123.62	477.39±115.48	397.00±148.24
SOD	6.40±3.35^{G}	43.42±2.78^{*G}	15.11±5.45^{L}	18.64±5.15^{L}
CAT	16.12±3.83^{G}	11.30±3.66^{G}	1.09±0.48^{L}	0.87±0.32^{L}
		Merlangius merlangus		
LPO	1.41±0.71	0.92±0.20^{G}	2.16±0.94	2.58±0.17^{L}
GSH	908.43±193.75	958.53±124.00	1286.15±447.10	910.69±120.00
SOD	24.90±3.57^{G}	30.26±3.39^{G}	7.79±2.81^{L}	7.75±1.19^{L}
CAT	23.83±7.16^{G}	27.10±0.48^{G}	1.62±0.76^{L}	1.38±0.18^{L}
		Neogobius melanostomus		
LPO	3.05±1.48	1.59±0.71	2.73±1.16	1.74±0.92
GSH	424.04±97.26	267.62±50.86^{G}	405.19±108.75	672.68±119.59^{L}
SOD	10.10±4.01*	4.22±1.30*^{G}	20.12±7.32^{S}	39.43±5.02^{N,L}
CAT	11.92±4.02^{G}	10.03±6.19^{G}	1.03±0.51^{L}	0.84±0.27^{L}
		Mullus barbatus		
LPO	0.56±0.15	0.84±0.15	0.73±0.31	0.73±0.15
GSH	483.54±113.00	418.83±73.22	357.87±25.70	429.62±89.31
SOD	44.39±7.49	44.07±11.01	51.66±7.17	44.94±5.90
CAT	22.09±5.43^{*G}	11.20±0.93^{*G}	1.46±0.32^{L}	1.58±0.45^{L}

Among the studied fish species, a general pattern in the total level of biomarkers in organs was demonstrated, i.e. in flounder - higher LPO and lower CAT activity in gills than in liver; in whiting higher SOD and CAT activities in liver than

in gills; in round goby – higher SOD activity in gills than in liver and in contrast lower CAT activity in gills than in liver; in mullet – higher CAT activity in liver than in gills (Table 3). Additionally, significant variations in the OS biomarkers in gills and liver between the fish species from the northern and southern localities were also observed. These variations could have induced the antioxidant enzyme complex. The flounders inhabiting southern localities had higher average SOD activity in liver than those of northern localities; the round goby inhabiting northern localities had higher average SOD activity in liver and lower in gills, and the mullet individuals from the northern localities had a higher average CAT activity in liver.

Discussion

In this preliminary study, biomarkers of oxidative stress in the gills and liver of five marine fish species of the Bulgarian Black Sea part were analyzed as indicators of the stressfulness of the marine environment.

The presence and level of OS in the fish species studied cannot be determined only by the level of a separate marker, rather the interrelation of all markers should be taken into consideration. Both the gills and the liver are considered target organs sensitive to oxidative damage. The gills are the organ in direct contact with the marine environment and the pollutants in it. Thus, gills are exposed to higher concentrations of pollutants than other organs (Heath, 1987). Therefore, our results on the significant differences of the OS markers in fish liver and gills in particular localities are logical and expected. A number of authors note that gills are more sensitive to oxidative damage than the liver and may respond earlier to oxidative challenges induced by pollutants (Ahmad et al., 2004; Guilherme et al., 2012). In general, in the gills of the studied fish the LPO, as a marker for the prooxidant effect of adverse environments, was significantly higher than those in liver. Specifically, we found that the activities of CAT in the gills of the studied fish were significantly lower than those in liver. This finding is consistent with the observations of other authors (Jos et al., 2005; Cazenave et al., 2006; Ballesteros et al., 2009).

Regardless of the variations among the studied fish species our results showed that the fish from the northern localities (Tyulenovo) were more strongly exposed to oxidative stress - the highest LPO in liver of round goby among all tested fish and also high LPO in gills, accompanied by the lowest GSH. Similar pattern of high levels of LPO in liver and gills and low GSH concentrations in whiting from Tulenovo was also present. These findings are in line with the fact that the most northern region of the Bulgarian Black Sea is known to be under the strong influence of contamination by the River Danube inflow (Dineva, 2011). Sewage effluents, even treated, and other pollutants are also known to compromise the health of aquatic organisms (Hébert et al., 2008; Kamel et al., 2012, Yancheva et al., 2020). Localities, exposed to higher coastal inputs of pollutants, due to their proximity to anthropogenic sources, such as the resorts Slanchev Bryag, Nessebar and Sozopol with high touristic flow, were found to cause oxidative stress in the studied fish species from these localities, i.e. flounder (relatively high LPO, low GSH, increased SOD activity), round goby (low GSH, activated SOD) and mullet (low GSH, activated SOD). Changes in the oxidative status of the same or similar fish species caused by anthropogenic contamination in different regions of the Black Sea were reported by other authors (Kovyrshina & Rudneva, 2012, 2016; Sigacheva et al., 2020; Chesnokova et al., 2020; Bozcaamutlu et al., 2020).

Changes in the activity of antioxidant enzymes as biomarkers of the response of organisms to environmental conditions are well accepted in environmental monitoring systems (Winstin & Di Giulio, 1991; Oruc et al., 2004; Jebali et al., 2013; Bozcaarmutlu et al., 2020). Their peculiarity is in the fact that, depending on the duration and strength of the effect, they can be either activated (as an adaptive reaction) or inhibited (Ballesteros et al., 2009). Thus, the activity of antioxidant enzymes changes

following a bell-shaped curve and it is difficult to assess the state of organisms by assessment of enzyme activity alone. It is more accurate to observe a set of biomarkers to obtain a more reliable picture of environmental impacts. The SOD and CAT activities in fish liver indicate also the activity of species as the more mobile fish species were found to have higher enzyme activity compared with the low mobile forms (Filho et al., 2007; Rudneva et al., 2010). The higher activity of liver antioxidant enzymes in the more mobile fish species is correlated with the higher oxygen consumption and metabolic rate (Martinez-Alvarez et al., 2005; Filho, 2007). This leads to higher free radical production rates that cause induction of antioxidant defense mechanisms (Zelinski & Portner, 2000). Our results showing higher activity of SOD and CAT in liver of *M. barbatus* and *M. merlangus* are in line with these observations.

Conclusion

The preliminary assessment of the oxidative status of common marine fish species, inhabiting different localities along the Bulgarian Black Sea coast was carried out. Significant variation of the biomarkers of oxidative stress were established which indicated the presence of different effects of anthropogenic pressure and the presence of corresponding response of the studied fish species. All the fish species studied were subjected to different levels of oxidative stress caused by the ecological state of the marine environment in the localities they inhabit. Changes in the oxidative stress biomarkers in fish species from the Bulgarian Black Sea coastal zone can be used in marine monitoring surveys as integral measure of the effects of multiple stressors. Obviously, further studies are needed.

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References

Aebi, H. (1984). Catalase in vitro. *Methods of Enzymology, 105*, 121-126. doi: 10.1016/s0076-6879(84)05016-3.

Ahmad, I., Pacheco, M. & Santos, M. A. (2004). Enzymatic and nonenzymatic antioxidants as an adaptation to phagocyte-induced damage in *Anguilla anguilla* L. following *in situ* harbor water exposure. *Ecotoxicology and Environmental Safety, 57*(3), 290-302.

Ballesteros, M. L., Wunderlin, D. A. & Bistoni, M. A. (2009). Oxidative stress responses in different organs of *Jenynsia multidentata* exposed to endosulfan. *Ecotoxicology and Environmental Safety, 72*(1), 199-205. doi: 10.1016/j.ecoenv.2008.01.008.

Birnie−Gauvin, K., Costantini, D., Cooke, S. J. & Willmore, W. G. (2017). A comparative and evolutionary approach to oxidative stress in fish: a review. *Fish and Fisheries, 18*(5), 928-942.

Bozcaarmutlu, A., Sapmaz, C., Kaleli-Can, G., Turna, S., Aygun, Z. & Arinc, E. (2020). Monitoring of pollution in the western Black Sea coast of Turkey by striped red mullet (*Mullus surmuletus*). *Environmental Monitoring Assessment, 192*(9), 586. doi: 10.1007/s10661-020-08509-6.

"BSFishList". (2020). Retrieved from blacksea-commission.org

Cazenave, J., Bistoni Mde, L., Pesce, S. F. & Wunderlin, D. A. (2006). Differential detoxification and antioxidant response in diverse organs of *Corydoras paleatus* experimentally exposed to microcystin-RR. *Aquatic Toxicology, 76*(1), 1-12. doi: 10.1016/j.aquatox.2005.08.011.

Chesnokova, I. I., Sigacheva, T. B. & Skuratovskaya, E. N. (2020.). Comparative Analysis of Hepatic Biomarkers of Black Scorpionfish *Scorpaena porcus* Linnaeus, 1758 from Sevastopol Water Areas (the Black Sea) with Different Pollution Levels. *Water Resources, 47*, 486-490. doi: 10.1134/S0097807820030045.

Cheung, C. C., Zheng, G. J., Li, A. M., Richardson, B. J. & Lam, P. K. (2001). Relationships between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of marine mussels, *Perna*

viridis. *Aquatic Toxicology, 52*(3-4), 189-203. doi: 10.1016/s0166-445x(00)00145-4.

Dineva, S. I. (2011). Water Discharges into the Bulgarian Black Sea. International Symposium on Outfall Systems. Mar del Plata, Argentina. Retrieved from osmgp.gov.ar.

Filho, D. W. (2007). Reactive oxygen species, antioxidants and fish mitochondria. *Frontiers in Bioscience, 12*, 1229-1237. doi: 10.2741/2141.

"FishBase". (2021). Retrieved from fishbase.in

Guilherme, S., Gaivao, I., Santos, M. A. & Pacheco, M. (2012). DNA damage in fish (*Anguilla anguilla*) exposed to a glyphosate-based herbicide - elucidation of organ-specificity and the role of oxidative stress. *Mutation Research, 743*(1-2), 1-9. doi: 10.1016/j.mrgentox.2011.10.017.

Hamilton, S.L, Logan, C.A., Fennie, H.W., Sogard, S.M., Barry, J.P. & Makukhov, A.D. (2017). Species-Specific Responses of Juvenile Rockfish to Elevated pCO_2: From Behavior to Genomics. *PLoS ONE, 12*(1), e0169670. doi: 10.1371/journal.pone.0169670.

Heath, A. G. (1995). Water pollution and fish physiology (2nd ed.). CRC press. Retrieved from routledge.com.

Hébert, N., Gagné, F., Cejka, P., Bouchard, B., Hausler, R., Cyr, D. G., Blaise, C. & Fournier, M. (2008). Effects of ozone, ultraviolet and peracetic acid disinfection of a primary-treated municipal effluent on the immune system of rainbow trout (*Oncorhynchus mykiss*). *Comparative Biochemistry and Physiology, 148*(2), 122-127.

Hook, S. E., Gallagher, E. P. & Batley, G. E. (2014). The Role of Biomarkers in the Assessment of Aquatic Ecosystem Health. *Integrated Environmental Assessment and Management, 10*(3), 327–341.

Jebali, J., Sabbagh, M., Banni, M., Kamel, N., Ben-Khedher, S., M'Hamdi, N. & Boussetta, H. (2013). Multiple biomarkers of pollution effects in *Solea solea* fish on the Tunisia coastline. *Environmental Science and Pollution Research, 20*(6), 3812-3821. doi: 10.1007/s11356-012-1321-2.

Jos, A., Pichardo, S., Prieto, A. I., Repetto, G., Vazquez, C. M., Moreno, I. & Camean, A. M. (2005). Toxic cyanobacterial cells containing microcystins induce oxidative stress in exposed tilapia fish (*Oreochromis* sp.) under laboratory conditions. *Aquatic Toxicology, 72*(3), 261-271. doi: 10.1016/j.aquatox.2005.01.003.

Kamel, N., Jebali, J., Banni, M., Ben Khedher, S., Chouba, L. & Boussetta, H. (2012). Biochemical responses and metals levels in *Ruditapes decussatus* after exposure to treated municipal effluents. *Ecotoxicology and Environmental Safety, 82*, 40-46. doi: 10.1016/j.ecoenv.2012.05.008.

Kovyrshina, T. B. & Rudneva, I. I. (2012). Seasonal dynamics of activity of oxidative modification of proteins and oxidation-inhibiting enzymes in the blood of goby Neogobius melanostomus inhabiting the Black Sea and the Sea of Azov. *Journal of Ichthyology, 52*(4), 277-283. doi: 10.1134/S0032945212020099.

Kovyrshina, T. B. & Rudneva, I. I. (2016). The effects of coastal water pollution of the Black Sea on the blood biomarkers of the round goby *Neogobius melanostomus* Pallas, 1811 (Perciformes: Gobiidae). *Russian Journal of Marine Biology, 42*(1), 58-64.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*(1), 265-275.

Lushchak, V. I. (2016). Contaminant-induced oxidative stress in fish: a mechanistic approach. *Fish Physiology and Biochemistry, 42*(2), 711-747. doi: 10.1007/s10695-015-0171-5 .

Makedonski, L., Peycheva, K. & Stancheva, M. (2017). Determination of heavy metals in selected black sea fish species. *Food Control, 72*, 313-318.

Martínez-Álvarez, R. M., Morales, A. E. & Sanz, A. (2005). Antioxidant defenses in fish: biotic and abiotic factors. *Reviews in Fish Biology and fisheries, 15*(1), 75-88.

Oguz, T. & Velikova, V. (2010). Abrupt transition of the northwestern Black Sea shelf ecosystem from a eutrophic to an alternative pristine state. *Marine Ecology Progress Series, 405*, 231-242.

Oruc, E. O., Sevgiler, Y. & Uner, N. (2004). Tissue-specific oxidative stress responses in fish exposed to 2,4-D and azinphosmethyl. *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology*, *137*(1), 43-51. doi: 10.1016/j.cca.2003.11.006.

Peskin, A. V. & Winterbourn, C. C. (2017). Assay of superoxide dismutase activity in a plate assay using WST-1. *Free Radical Biology and Medicine, 103*, 188-191. doi: 10.1016/j.freeradbiomed.2016.12.033.

Pimentel, M.S., Faleiro, F., Diniz, M., Machado, J., Pousão-Ferreira, P. & Peck, M.A. (2015). Oxidative Stress and Digestive Enzyme Activity of Flatfish Larvae in a Changing Ocean. *PLoS ONE, 10*(7), e0134082. doi: 10.1371/journal.pone.0134082.

Rahman, I., Kode, A. & Biswas, S. K. (2006). Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nature Protocols, 1*(6), 3159-3165. doi: 10.1038/nprot.2006.378.

Rudneva, I. I., Kuzminova, N. S. & Skuratovskaya, E. N. (2010). Glutathione-S-transferase activity in tissues of Black Sea fish species. *Asian Journal of Experimental Biological Sciences, 1*(1), 141-150.

Secci, G. & Parisi, G. (2016). From farm to fork: lipid oxidation in fish products. A review. *Italian Journal of Animal Science, 15*(1), 124-136. doi: 10.1080/1828051X.2015.1128687.

Sigacheva, T. B., Chesnokova, I. I. & Gavruseva, T. V. (2020). Characterization of Some Hepatic Biochemical Indicators in Three Demersal Black Sea Fish Species. *Journal of Evolutionary Biochemistry and Physiology, 56*, 55-62. doi: 10.1134/S002209302001007X.

Skuratovskaya, E. N., Kovyrshina, T. B. & Rudneva, I. I. (2017). A comparative study of long-term pollution effects of marine waters on the blood biomarkers of two benthic fish species: the scorpion fish *Scorpaena porcus* (Linnaeus, 1758) and the round goby *Neogobius melanostomus* (Pallas, 1814) from the Black Sea. *Russian Journal of Marine Biology, 43*(3), 232-238. doi: 10.1134/S1063074017030105.

Stoyanova, S., Georgieva, E., Velcheva, I., Iliev, I., Vasileva, T., Bivolarski, V., Tomov, S., Nyeste, K., Antal, L. & Yancheva, V. (2020a). Multi-Biomarker Assessment in Common Carp (*Cyprinus carpio*, Linnaeus 1758) Liver after Acute Chlorpyrifos Exposure. *Water, 12*(6), 1837. doi: 10.3390/w12061837.

Stoyanova, S., Nyeste, K., Georgieva, E., Uchikov P., Velcheva I. & Yancheva V. (2020b). Toxicological impact of a neonicotinoid insecticide and an organophosphorus fungicide on bighead carp (*Hypophthalmichthys nobilis* Richardson, 1845) gills: a comparative study. *North-Western Journal of Zoology, 16*(1), 64-73.

Valavanidis, A., Vlahogianni, T., Dassenakis, M. & Scoullos, M. (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicology and Environmental Safety*, *64*(2), 178-189. doi: 10.1016/j.ecoenv.2005.03.013.

Zaitsev, Y. & Mamaev, V. O. (1997). *Biological diversity in the Black Sea: a study of change and decline* (Vol. 3). United Nations Publications.

Zaitsev, Y. P. (2008). *An introduction to the Black Sea ecology*. Smil Editing & Publishing Agency Ltd. Retrieved from oceandocs.org.

Zielinski, S. & Pörtner, H. O. (2000). Oxidative stress and antioxidative defense in cephalopods: a function of metabolic rate or age? *Comparative Biochemistry and Physiology, 125*(2), 147-160.

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