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Comparative study on the oxidative stress of commercially important fish species from localities with different ecological conditions along the Bulgarian Black Sea coast

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Abstract

The aim of the present study was to perform a pilot assessment and analysis of the oxidative stress (OS) level in four commercially important fish species (round goby, red mullet, sprat and horse mackerel) from different localities of the Bulgarian Black Sea coast. The fish were sampled during trawl selectivity experiments. The OS level in the fish was assessed by measuring lipid peroxidation (LPO), glutathione concentration (GSH), activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST), as well as acetylcholine esterase (AChE) in gills and liver. Round goby and red mullet caught in the Nessebar Bay showed clear signs of OS with the highest levels of LPO and GST activities, accompanied by the lowest AChE activities in both liver and gills. On the contrary, round goby caught near Maslen Nos (a region with good ecological conditions) were least affected by OS with low LPO and high GSH concentrations and SOD activity. There were no significant differences in the OS bioindicators of horse mackerel from the different localities. Sprat caught in Nessebar Bay, compared to those caught from the other localities, showed presence of OS indicated by lower GSH levels and relatively higher CAT, GPx and GST activities, accompanied by low AChE activity in gills. It can be concluded that round goby and red mullet were more vulnerable to OS induced by marine environmental factors than the horse mackerel and sprat. However, their antioxidant defense system allows them to tolerate and adapt to the environment of their habitats. Further studies are needed for the assessment of OS in important fish species in the Bulgarian part of the Black Sea.

Keywords
Bulgarian Black Sea, horse mackerel, oxidative stress, red mullet, round goby, sprat

Introduction

Fish are a major component of aquatic ecosystems and the good state and health of their populations is crucial for ecosystem stability. Marine fish populations are subjected to increasing environmental pollution and also to overfishing pressure (Daskalov 2003; Radu et al. 2011; Ţoţoiu et al. 2018). At present, Black Sea fish species with significant economic and ecological importance, also in the Bulgarian part, are sprat, turbot, red mullet, bluefish, horse mackerel and round goby (Raykov et al. 2019). The assessment of the condition of the exploited fish species (Descriptor D3) in the Bulgarian Black Sea part showed that there are no species in "Good condition", and sprat, whiting, horse mackerel, mullet, turbot and others are even classified in "Bad" condition (Panayotova and Todorova 2017).

The sustainable management of marine fish stocks requires not only regulation of the fish populations, but also knowledge regarding the ability of the fish to respond and adapt to multiple environmental stressors (Hagger et al. 2006, 2008). A general reaction of marine organisms to environmental pressures (abiotic, biotic and anthropogenic) involves the activation of oxidative processes in their cells where the generated reactive oxygen species (ROS) play the role of signaling molecules that trigger the organisms' adaptative responses to environmental changes (Birnie-Gauvin et al. 2017). However, under prolonged or acute exposure, excess ROS generation cannot be compensated by the antioxidant defense system and provokes oxidative stress (OS) (Villarreal et al. 2014; Chowdhury and Saikia 2020). The OS in marine fish can be induced by multiple stress factors which were proved to adversely affect the growth, development and reproduction of fish species (Stoliar and Lushchak 2012; Vinagre et al. 2012; Dolci et al. 2013; Mozhdeganloo and Heidarpour 2014; Florescu et al. 2021). All these stressors (and related stresses) can result in community-scale effects (community structures) and may ultimately drive species coexistence (Steinberg 2012).

The aim of the present study was to provide a pilot assessment and analysis of the oxidative stress level of four commercially important fish species from representative localities of the Bulgarian Black Sea coastal area.

Materials and methods

Sampling

The fish species selected for this study were: the demersal round goby (*Neogobius melanostomus* Pallas, 1814), two benthopelagic species – red mullet (*Mullus barbatus* Linnaeus, 1758) and horse mackerel (*Trachurus mediterraneus* Steindachner, 1868), and

the pelagic species – sprat (*Sprattus sprattus* Linnaeus, 1758). The fish species were identified in accordance with BSFishList (2020). The fish individuals were randomly picked from several trawl selectivity experimental catches by pelagic Midwater otter trawl (7 × 7 mm mesh size of the codend) from 3 localities of the Bulgarian Black Sea aquatory (Table 1). On board, random samples of 9–12 fish individuals of every species were shock frozen and transported to the laboratory.

In the laboratory, fish individuals were measured with a caliper and weighed on scales with an accuracy of 0.01 g. The Fulton's condition factor (K) of the fish individuals was computed according to Nash et al. (2006): $K = 100 * TW/TL^3$, where TW is the total body wet weight in grams and TL is the total length in cm; the factor 100 is used to bring K close to a value of one.

Tissue preparation

The fish were dissected and their liver and gills were extracted. The organs were homogenized in 0.1 M potassium phosphate buffer (pH 7.4) and centrifuged at 3000 g for 10 min to obtain a post-nuclear fraction, used for determination of lipid peroxidation and glutathione levels. A portion of post-nuclear fraction was re-centrifuged at 12000 g for 20 min at 4 °C for obtaining a post-mitochondrial supernatant, used for measurement of the antioxidant enzymes activities.

Measurement of oxidative stress biomarkers

The OS biomarkers were measured spectrophotometrically using commercially available kits: Lipid Peroxidation (MDA) Assay Kit MAK085, Glutathione Assay Kit CS0260, SOD Assay Kit-WST 19160, Catalase Assay Kit CAT100, Glutathione Peroxidase Cellular Activity Assay CGP1, and Glutathione-S-Transferase Assay Kit CS0410 (Sigma-Aldrich Co. LLC, USA). The manufacturer's working instructions were strictly followed.

Acetylcholinesterase (AChE) activity was assayed by the method of Ellman (Ellman et al. 1961). The reaction mixture contained 0.1 M K-PO4 buffer pH 8.0, 0.045 M acetylthiocholine iodide, 0.008 M 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and the appropriate amount of fish's tissue homogenate. The enzymatic hydrolization of acetylthiocholine produced thiocholine. The latter reacted with DTNB and yield a yellow colored product, 5-thio-2-nitrobenzoic acid with absorbance pick at 412 nm. The enzyme activity was calculated as U/mg protein. Protein concentration

Table 1. Trawling localities along the Bulgarian Black Sea coast with depth and bottom temperature.

Code	Trawling locality	Trawling start point	Trawling end point	Depth	T_{bottom}
		N, E	N, E	[m]	[°C]
S1	Maslen Nos	42.312452°N, 28.054628°E	42.296292°N, 28.089651°E	37	7.7
S2	St. Oryahovo	42.993074°N, 27.951590°E	42.960442°N, 27.948072°E	23	7.9
S3	Nessebar	42.599613°N, 27.791345°E	42.619495°N, 27.826710°E	20	7.4

was measured according to Lowry et al. (1951) and was computed from a standard curve, obtained using bovine serum albumin (CAS Number: 9048-46-8, Merck KGaA, Darmstadt, Germany).

Statistical analyses

For statistical analyses the software package STATISTICA 10 (StatSoft Inc.) was used. The Kruskal–Wallis test was used to assess the significance of differences among the values of the OS biomarkers. Post hock comparisons between variables were made using Mann-Whitney test. Multidimensional Scaling (MDS) was carried out to detect patterns of similarities among the studied objects.

Results

Data from the measurements of the OS biomarkers in the liver and gills of the studied fish species from the selected localities of the Bulgarian Black sea coast are presented in Table 2. Our data showed that the measured OS bioindicators significantly varied among the fish species, the studied organs and the localities. In general, the two fish organs studied showed different susceptibility to oxidative changes, due to its unique composition and metabolic profile. Significantly higher lipid peroxidation (LPO) was found in the gills compared to the liver of the fish species, with the exception of the round goby, where no differences were observed (Table 2). The gills of all studied fish species had lower glutathione-S-transferase (GST) and higher acetylcholine esterase (AChE) activities than the liver. The activity of superoxide dismutase (SOD) in the liver was approximately twice as high as in the gills for all fish species studied, and the activity of catalase (CAT) was two to five times higher in the liver of round goby, red mullet and sprat, compared to gills. Glutathione peroxidase (GPx) activity was significantly higher in the gills than in the liver only in sprat. The glutathione (GSH) level in the gills of red mullet was significantly lower than in the liver, while the opposite was observed in sprat. In the round goby and horse mackerel no difference in GSH between the two organs was present (Table 2). The gills of the studied fish species were more affected by OS as a whole, showing high levels of LPO, with the exception of round goby. In the liver of the studied fish species lower activity of CAT, SOD (except for the fish species from Maslen Nos) and GST were present, compared to the gills (Table 2).

Significantly higher LPO and GST activities were established, together with the lowest AChE activity in both liver and gills of round gobies and mullets from Nessebar Bay, compared to those from Maslen Nos and Staro Oryahovo (Table 2), thus indicating clear signs of OS. Round gobies from Maslen Nos had the lowest level of OS as indicated by the lowest LPO levels and GST activities, and the highest concentrations of GSH and SOD activity, together with highest activity of AChE (Table 2). No significant differences were present in the measured OS biomarkers between mullets caught from Maslen Nos and from Staro Oryahovo. The OS markers in sprat from

Table 2. Values (ME±SD) of the measured OS biomarkers in gills and liver of the studied fish species from the different localities of the Black Sea coast (*statistical significance of differences at p<0.05: S^n indicate significant differences of the OS indicator in fish species between sites; letters (G, L) indicate significance of differences of the OS indicator between organs – G = gills and L = liver).

Site	Liver							Gills						
	*LPO	GSH	SOD	CAT	GPx	GST	AChE	LPO	GSH	SOD	CAT	GPx	GST	AChE
							Neogobius melanostomus							
	4.83	**701**	**16.29**	**1.54**	**19.72**	**37.1**	**31.27**	**2.90**	**457**	**16.00**	**1.05**	**20.20**	**38.99**	**42.7**
S1	±1.42 *$s^{2,3}$	±134 *$s^{2,3}$	±4.85 *$s^{2,3}$	±0.02	±3.22	±12.1 *s^{3}	±0.64 *$s^{2,3}$L	±0.18 *$s^{2,3}$	±99	±2.8 *$s^{2,3}$	±0.02 *s^{3}	±1.43 *s^{3}	±0.87 *$s^{2,3}$	±1.43 *$s^{2,3}$G
	13.81	**295**	**3.60**	**1.35**	**19.73**	**40.3**	**17.94**	**15.49**	**287**	**1.15**	**1.09**	**16.44**	**49.09**	**28.4**
S2	±2.01 *s^{1}	±7 *s^{1}	±0.26 *s^{1}	±0.08	±2.86	±1.8 *s^{3}	±0.59 *s^{1}L	±1.68 *s^{1}	±39	±0.16 *s^{1}	±0.15 *s^{3}	±1.21	±1.22 *$s^{1,3}$	±0.33 *s^{1}G
	19.26	**304**	**3.50**	**1.84**	**23.76**	**264.3**	**13.86**	**20.92**	**276**	**1.87**	**0.54**	**14.23**	**68.10**	**24.8**
S3	±0.65 *s^{1}	±23 *s^{1}	±1.05 *s^{1}	±0.03	±10.7	±40.6 *$s^{1,2}$L	±1.06 *s^{1}L	±0.04 *s^{1}	±33	±0.38 *s^{1}	±0.04 *$s^{1,2}$	±1.84 *s^{1}	±4.14 *$s^{1,2}$	±0.85 *s^{1}G
Site Mean	**12.63**	**433**	**7.80**	**1.58**	**21.07**	**113.9**	**21.02**	**13.10**	**340**	**6.34**	**0.89**	**16.96**	**52.1**	**31.9**
	±1.36	±54	±2.05	±0.04 *G	±5.59	±18.2 *G	±0.76 *G	±0.63	±57	±1.13	±0.07 *L	±1.49	±2.08 *L	±0.87 *L
							Mullus barbatus							
	0.64	**692**	**58.13**	**15.08**	**10.94**	**55.45**	**54.64**	**2.77**	**294**	**20.88**	**1.77**	**19.46**	**9.77**	**292.4**
S1	±0.12 *s^{3}G	±217 *G	±3.75 *s^{3G}	±2.12 *s^{3}G	±3.24 *s^{3}	±7.3 *s^{3}G	±12.0 *s^{3}G	±0.86 *s^{3}L	±51 *L	±5.18 *L	±0.33 *L	±5.07	±1.08 *s^{3}L	±7.60 *L
	0.53	**459**	**45.89**	**17.86**	**4.19**	**76.69**	**73.11**	**2.64**	**350**	**23.08**	**1.88**	**16.32**	**14.97**	**355.8**
S2	±0.05 *s^{3}G	±118	±8.27 *G	±3.65 *s^{3}G	±1.51 *s^{3}G	±10.4 *s^{3}G	±10.9 *s^{3}G	±0.19 *s^{3}L	±27 *s^{3}	±3.59 *L	±0.11 *L	±4.73 *L	±2.62 *s^{3}L	±75.3 *s^{3}L
	2.09	**383**	**23.71**	**4.07**	**24.57**	**102.3**	**30.24**	**10.26**	**218**	**12.17**	**2.55**	**17.74**	**68.00**	**168.8**
S3	±0.60 *$s^{2,3}$G	±75 G	±12.4 *s^{1}	±1.64 *$s^{1,2}$	±3.08 *$s^{1,2}$G	±4.72 *$s^{1,2}$G	±3.40 *$s^{1,2}$G	±1.25 *$s^{1,2}$L	±12 *s^{2L}	±7.16	±0.31	±0.07 *L	±1.91 *$s^{1,2}$L	±1.9 *s^{2}L
Site Mean	**1.09**	**511**	**42.58**	**12.34**	**13.23**	**78.15**	**52.66**	**5.22**	**287**	**18.71**	**2.07**	**17.84**	**30.91**	**272.4**
	±0.26 *G	±136 *G	±8.14 *G	±2.47 *G	±2.61	±7.49 *G	±8.79 *G	±0.77 *L	±30 *L	±5.31 *L	±0.25 *L	±3.29	±1.87 *L	±28.3 *L
							Trachurus mediterraneus							
	2.93	**335**	**14.80**	**1.30**	**46.51**	**79.55**	**51.92**	**21.90**	**393**	**7.64**	**1.04**	**31.49**	**34.84**	**214.4**
S1	±1.48 *L	±68	±3.08	±0.72	±16.3	±18.2 *s^{3}G	±20.3 *G	±0.39 *L	±21 *s^{3}	±5.16	±0.27	±9.08	±10.5 *L	±60.4 *L
	2.09	**249**	**14.37**	**0.60**	**36.09**	**59.07**	**44.05**	**22.32**	**368**	**7.57**	**1.16**	**27.36**	**35.68**	**199.6**
S2	±0.58 *L	±8	±7.73	±0.17 *s^{3}	±14.6	±11.0 *G	±13.5 *s^{3}G	±0.43 *L	±49	±6.38	±0.33	±8.80	±1.55 *L	±28.7 *L
	3.57	**227**	**9.64**	**1.73**	**34.37**	**48.43**	**66.00**	**24.41**	**246**	**4.79**	**1.31**	**28.48**	**39.17**	**203.3**
S3	±1.25 *L	±18	±0.86 *G	±0.20 *s^{2}	±5.42	±3.49 *s^{1}G	±3.68 *s^{2}G	±0.96 *L	±19 *s^{1}	±0.95 *L	±0.24	±3.12	±0.89 *L	±7.81 L
Site Mean	**2.86**	**270**	**12.94**	**1.21**	**38.99**	**62.35**	**53.99**	**22.8***	**336**	**6.67**	**1.17**	**29.11**	**36.6**	**205**
	±1.10 *L	±31	±3.89	±0.36	±12.1	±10.9	±12.5 *G	±0.59 *L	±30	±4.16	±0.28	±7.00	±4.31 *L	±32.3 *L
							Sprattus sprattus							
	16.27	**376**	**12.57**	**12.25**	**26.66**	**99.89**	**112.6**	**22.56**	**896**	**4.28**	**1.16**	**39.41**	**20.56**	**218.0**
S1	±8.22	±8.63 *G	±3.28 *G	±1.97 *G	±11.2 *s^{3}	±11.5 *s^{3}G	±59.1 *s^{2}G	±0.57	±343 *L	±1.18 *L	±0.44 *L	±17.8 *s^{3}	±5.16 *s^{3}L	±26.9 *L
	19.15	**413**	**13.27**	**13.27**	**31.90**	**72.44**	**35.91**	**22.05**	**1044**	**6.73**	**2.01**	**49.49**	**24.87**	**228.9**
S2	±4.15	±63 *G	±0.72 *G	±0.72+= *G	±11.6 *s^{3}	±6.73 *s^{3}G	±6.03 *s^{1}G	±0.80	±41 *L	±1.0 *L8	±0.58 *L	±23.6	±2.45 *s^{3}L	±28.0 *L
	14.11	**312**	**8.67**	**15.30**	**12.71**	**40.66**	**52.78**	**22.00**	**341**	**5.17**	**2.83**	**74.48**	**47.72**	**142.9**
S3	±2.78 *G	±100	±3.61	±1.23 *G	±0.26 *$s^{1,2}$G	±3.02 *$s^{1,2}$	±6.20 *G	±0.8 *L2	±49	±0.90	±0.06 *L	±6.74 *s^{1}L	±1.80 *$s^{1,2}$	±41.9 *L
Site Mean	**16.51**	**367**	**11.50**	**13.61**	**23.76**	**71.00**	**67.12**	**22.2**	**760**	**5.39**	**2.00**	**54.4**	**31.0**	**196**
	±5.05	±57 *G	±2.54 *G	±1.3 *G1	±7.73	±7.09 *G	±23.8 *G	±0.73	±145 *L	±1.05 *L	±0.36 *L	±16.1	±3.14 *L	±32.3 *L

*LPO was expressed as nmoles MDA/mg protein; GSH was expressed as ng/mg protein, all enzymes were expressed as U/mg protei

Nessebar Bay demonstrated significantly lower levels of GSH, higher activities of CAT, GPx and GST, along with significantly lower AChE activities in the gills, compared to those from Maslen Nos and Staro Oryahovo, thus indicating clear OS signs. No differences in the values of the studied OS biomarkers between sprat from Maslen Nos and Staro Oryahovo were found, indicating a relatively similar level of OS in the fish from these localities. There were no significant differences in the levels of the studied OS indicators in horse mackerel from the three studied localities.

In order to identify significant similarities (dissimilarities) between the values of the measured OS biomarkers MDS was applied (Fig. 1). The MDS clearly demonstrated the presence of differences in the OS level and the enzyme defense activation between the fish species studied (Fig. 1A). The red mullet differed significantly from the other studied species (separated at the right side of the two dimensional plane). The round goby was the other species with specific and different OS reaction (clearly separated from the other species in the two dimensional plane). Both the red mullet and the round goby have similar benthic live style and hence should have been subjected to similar environmental pressures resulting in OS. The level of OS in horse mackerel and sprat appeared more similar to each other than to the other two species (Fig. 1A).

The activity of the pro/antioxidative processes in the studied fish species showed some indicative similarities (and dissimilarities) (Fig. 1B). The different degree of activation of the fish antioxidant defense system, as a response to multiple environmental stressors, showed two lines of defense (including ancillary factors). The first one reflected the activation of the first line of defense by antioxidant enzymes, indicated by the activity of CAT and SOD (grouped together in the left part of the two dimensional plane). The second one was reflected by the grouping (although loose) of GSH, GPx and AChE at the right side of the factor plane, thus indicating the activation also of the detoxification defense system of the fish species as additional response to toxic pollutants.

As a general measure of fish condition and health, we used Fulton's condition factor (K) (Fig. 2). The results strongly indicated the presence of differences of the K

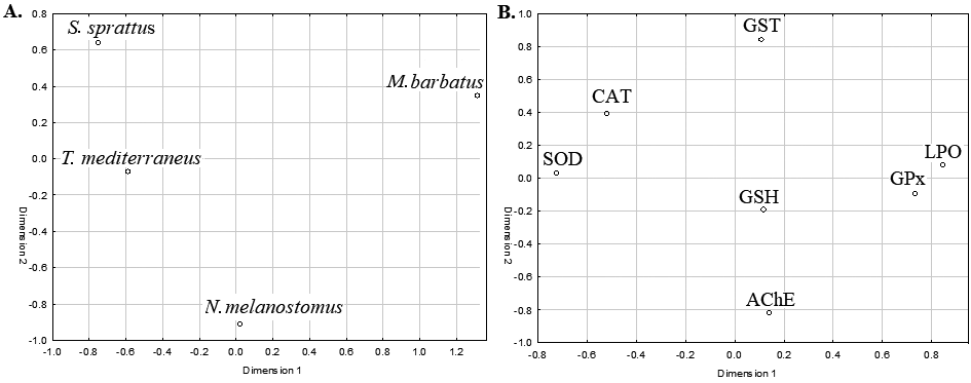

Figure 1. Multidimentional scaling of similarity (dissimilarity) between the studied fish species in their level of OS (**A**) and the induced activity of the pro/antioxidant processes (**B**).

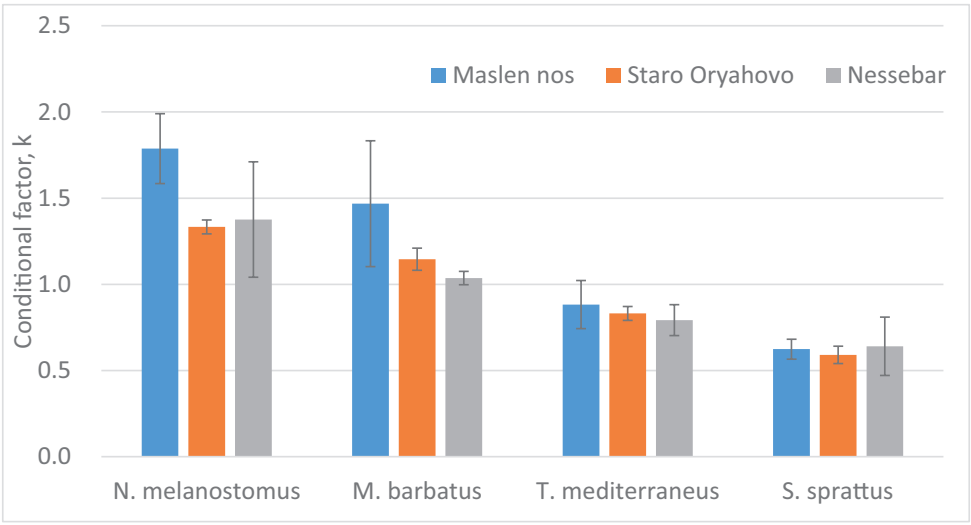

Figure 2. Fulton's condition factor (K) of studied fish species from the sampled localities of the Black Sea coastal zone (Mean±SD).

factor among fish species and also among the trawling localities. As a whole, the values of K for round goby and red mullet were significantly higher (also higher than 1) that those of the other species for all localities. The highest K factor values were present in round goby and red mullet from Maslen Nos. The K factor of sprat and horse mackerel was significantly lower (also less than 1) for all trawling localities studied.

Discussion

Changes in the balance of cell oxidative processes are a basic reaction of marine organisms to the impact of environmental pressure. The increasing contamination of the sea with metals, polycyclic aromatic carbohydrates and eutrophication leads to increase of LPO and alters the activity of antioxidant protection in the tissues of marine fish and other organisms (Regoli and Giuliani 2014).

In this study, we reported preliminary data on the OS level in the liver and gills of four commercially important fish species (sprat, horse mackerel, round goby and red mullet) from the Bulgarian Black Sea region. The first overall finding of our study was that in the gills and liver of fish different oxidative changes were present, most probably due to their unique composition and metabolic profile. Secondly, the studied fish species differed in the level of their OS and antioxidant systems activation, which depended on their life style and habitat (food preferences and habits, mobility and hence metabolism levels). The round goby and red mullet are classified as slow-swimming and carnivorous, while horse mackerel and sprat are fast-swimming and plankton feeders (Rudneva et al. 2010). The more active species compared to low-mobile forms

require higher oxygen consumption and hence, have a higher metabolic rate which determines higher ROS production and OS, and induction of different levels of antioxidant protection (Martinez-Alvarez et al. 2005; Filho 2007; Rudneva et al. 2010). In line with these findings were our results indicating significantly higher levels of LPO and GST in the gills of sprat and horse mackerel, i.e. the fish species with higher mobility. On the other hand, the benthic and suprabenthic, more sluggish, fish species (as round goby and red mullet), are assumed to live in more contaminated marine environments, because many pollutants accumulate in low water layers and bottom sediments. In support of this assumption, our data showed significantly higher OS levels in round goby and red mullet individuals from Nessebar bay (a bustling tourist center), compared to the individuals from Maslen Nos (protected area under the European ecological network NATURA). Furthermore, the extremely high GST values established, especially in the liver of the studied fish species from Nessebar Bay, indicated the presence of activated detoxification metabolism of xenobiotics, most probably entering the fish body from the sediment. This was confirmed by the performed MDS which showed activation of detoxification processes and decreased neurotransmission capacity (indicated by AChE activity). Our results corresponded with the observations of Rudneva et al. (2010), who also demonstrated higher activity of GST in the liver of slower swimming and sluggish fish species. It can be concluded that the studied by us round goby and red mullet appeared to be more vulnerable to OS induced by multiple marine environmental stressors than the horse mackerel and sprat. However, their antioxidant defense systems allowed them to tolerate and adapt to the changing environmental conditions of their habitats.

Our findings on the specific pro/antioxidative processes in the studied fish species were confirmed by values of the Fulton's condition factor (K). This biometric tool is used to indicate the general health and wellbeing of the fishes (Datta et al. 2013), as well as the quality of the marine environment. The value of K reflects the interactions between feeding conditions, parasitic infections and physiological nutritional/energy/reserves and hence, OS levels cannot alone determine the K factor variations. In our study we found that the round goby and red mullet from all three localities were in good condition according to K (K>1), whereas the condition of the sprat and horse mackerel from all studied localities was not so good (K<1). These findings corresponded, as a whole, to the estimated OS levels in these fish species.

Conclusion

It can be concluded that round goby and red mullet are more vulnerable to OS induced by marine environmental factors than the horse mackerel and sprat. However, their antioxidant defense system allows them to tolerate and adapt to the environmental conditions of their habitats. Therefore, it could be assumed that demersal fish species are more convenient for monitoring the state of the marine environment and the risk for impairing the fitness of fish. Obviously, further studies are needed for more comprehensive assessment of OS in fish of economic importance in the Bulgarian part of the Black Sea.

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