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Redox Status as a Health Indicator of Economically Important Fish from the Northern Shelf of the Bulgarian Black Sea

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Abstract: Commercial fishing in the Bulgarian Black Sea includes small fish species whose well-being needs close monitoring. An emerging concept that defines fish metabolic status and health condition is their oxidative stress (OS) level. The present study aimed to assess the redox status of five economically important fish species (*Sprattus sprattus* Linnaeus, 1758, *Trachurus mediterraneus* Steindachner, 1868, *Mullus barbatus* Linnaeus, 1758, *Merlangius merlangus* Linnaeus, 1758, *Neogobius melanostomus* Pallas, 1814) sampled in summer from the northern Bulgarian Black Sea areas. The results showed that fish of the same species had different responses to marine environmental stress depending on the sampling locations. The measured OS indicators suggested that the marine environment in the regions of Shkorpilovtsi and Batova was more favourable, compared to Kamchia and Kavarna, for maintaining the redox balance and health of the studied fish. Interspecific comparison to the other investigated fish species indicated that the whiting (*M. merlangus*) had a higher antioxidant defence, indicating mobilisation of the mechanisms of adaptation to the environmental conditions, while in sprat, the investigator team recorded depletion of energy stores to adapt to stressors and a resilience reduction. Thus, monitoring the OS biomarkers could be a powerful tool to evaluate fish's metabolic and general health status.

Key words: Black Sea, Bulgaria, fish, morphometry, redox status, oxidative stress

Introduction

The Black Sea is characterised by a relatively low fish species diversity, which can be attributed to its unique features. These include a closed sea with a large freshwater inflow and a small outflow, as well as a lack of oxygen in over 80% of its water volume and the presence of a high hydrogen sulfide content (Bekova 2020). Specifically, the northern Bulgarian Black Sea sector is subject to increased environmental pressure mainly due to pollution from the inflow of the great European rivers – Danube, Dnieper, Southern Bug, and Dniester.

About 130 fish species are known in the Black Sea, of which only 10 are of significant economic importance in Bulgaria. These are mainly small fish species: sprat, anchovy, horse mackerel, and whiting (Bekova 2020). The largest share of the catch consists of sprats (about 80%), while the others are less than 5% (IARA 2021). An assessment of the status of exploited fish species in 2018 using accepted indicators found no species in "Good Status". Four species have not been assessed (spiny shark, sea fox, bream, and bluefish), and the remaining species caught are declared in "Poor" condition (sprat, whiting, horse mackerel, mullet, and turbot

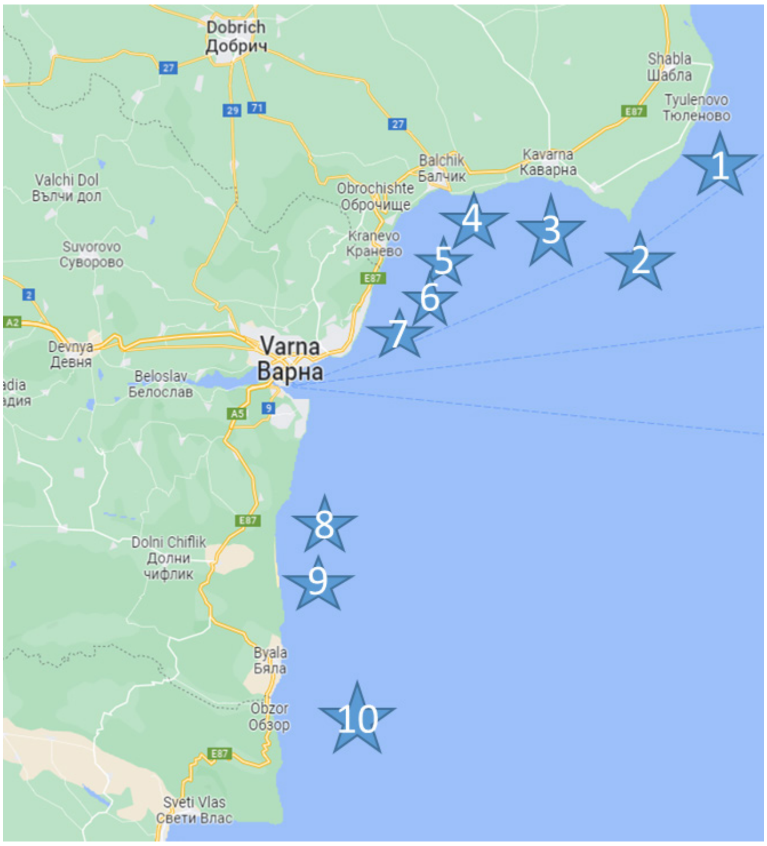

Fig. 1. Distribution of sampling localities in the northern Bulgarian Black Sea region: 1-Tulenovo; 2-Kaliakra; 3-Kavarna; 4-Albena; 5-Batova; 6-Kranevo; 7-Kabakum; 8-Kamchia; 9-Shkorpilovtsi; 10-Obzor.

(Panayotova et al. 2018). However, as set up by EU directives, the traditional approach to assessing fish conditions does not include specific markers of fish health and functional state.

Currently, oxidative stress (OS) has the potential to be a tool to assess fish health status and metabolism. Oxidative stress consists of a disruption of the balance between the processes that generate reactive oxygen species (ROS) in the cells and the ability of the antioxidant system to neutralise them. The main sources of ROS are the electron transport chains in the mitochondria and the activity of some enzymes. The antioxidant system consists of enzymatic and non-enzymatic components that synchronously and consistently strive to prevent the formation of ROS, neutralise the generated ROS, or eliminate the consequences of their action (Ighodaro & Akinloye 2018). As a result, OS is considered to reflect the real metabolic status of animals and thus determines the extent to which their phenotype can sufficiently meet their metabolic/vital requirements and the effects of their living environment. The dynamics in the balance between the pro- and antioxidant processes is an expression of the organism's response to multiple endo- and exogenous factors of their living environment, containing various environmental stressors. It has recently been broadly accepted that the changes caused by OS at the cellular level subsequently affect the higher hierarchical levels of the organisation of living matter. This was recently defined as "stress ecology" (Steinberg 2012). Thus, OS biomarkers can serve as an early signal for the possible changes in the marine ecosystem and, hence, for assessing aquatic environmental health.

The present work aimed to assess the redox status and tolerance to environmental stress of several economically important fish from the northern region of the Bulgarian Black Sea coast (from Cape Kaliakra to Cape Emine) by using OS indicators. Knowing the stress status and health of the fish populations can help reveal the ecological and/or physiological causes of differences in growth, survival, and recovery among different species populations.

Materials and Methods

Sampling

Fish were randomly sampled from monitoring trawl catches from several localities (Fig. 1) of the northern Bulgarian Black Sea part (from Cape

Table 1. Sampling localities with geographical coordinates, depth of trawling, and surface water temperature.

№	Locality	Species	Start		End		Depth	Temperature
			N	E	N	E	[m]	[°C]
1	Tulenovo	*M. merlangus;* *N. melanostomus*	43.521269	28.728255	43.481384	28.717535	64	23.2
2	Kaliakra	*N. melanostomus*	43.358366	28.427258	43.365368	28.404088	16	22.6
3	Kavarna	*S. sprattus;* *T. mediterraneus;* *M. barbatus;* *N. melanostomus*	43.374105	28.391058	43.381897	28.358268	15	24.8
4	Albena	*N. melanostomus*	43.360965	28.166638	43.346865	28.155626	18	22.2
5	Batova	*T. mediterraneus;* *M. barbatus;* *N. melanostomus*	43.378802	28.157542	43.343525	28.142787	18	24.8
6	Kranevo	*S. sprattus;* *N. melanostomus*	43.342950	28.152812	43.326618	28.140454	16	23.2
7	Kabakum	*T. mediterraneus*	43.277175	28.095699	43.257614	28.064045	20	25.6
8	Kamchia	*M. merlangus;* *M. barbatus;* *N. melanostomus*	43.011383	27.999294	42.984703	27.974008	21	25.2
9	Shkorpilovtsi	*S. sprattus;* *M. merlangus*	42.976214	27.966946	42.952405	27.940966	27	25.6
10	Obzor	*M. merlangus*	42.829226	27.920851	42.823984	27.912522	22	25.2

Kaliakra to Cape Emine), using pelagic Midwater otter trawl (7x7 mm mesh size of the codend) in July 2021-2022 (Table 1). The fish species selected for this study were sprat (*Sprattus sprattus* Linnaeus, 1758), horse mackerel (*Trachurus trachurus* Linnaeus, 1758), whiting (*Merlangius merlangus* Linnaeus, 1758), red mullet (*Mullus barbatus* Linnaeus, 1758) and round goby (*Neogobius melanostomus* Pallas, 1814) (BSFishList 2020, FishBase ver. 2021). Fish samples were shock-frozen on board the research vessel for best preservation and subsequent transport to the laboratory (Secci & Parisi 2016). Ten specimens of each fish species from each trawl and year were used for the biochemical analysis. Specimens were selected with approximately the same morphometry (suggesting the same age) from the predominant size group of fish from the respective trawl. The obtained individual data on OS parameters of the specimens from the respective fish species from a given location over two consecutive years were combined into one joint group.

Condition factor

Fulton's condition factor (K) was computed according to the formula: $K = 100*TW/TL^3$, 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 (Nash et al. 2006). To assess the condition factor, 30 random specimens of each fish species and trawl were used.

Tissue preparation

Ten specimens of approximately the same size as each fish species from each trawl were thawed and sized in the laboratory. Following available protocols, they were dissected, and their liver and gills were extracted (Stoyanova et al. 2020a,b). The organs were homogenised in 0.1 M potassium phosphate buffer, pH 7.4 and centrifuged at 3000 g for 10 min to obtain a post-nuclear fraction to determine lipid peroxidation and glutathione levels. A portion of the post-nuclear fraction was re-centrifuged at 12 000 g for 20 min at 4°C to obtain a post-mitochondrial supernatant to measure the antioxidant enzyme activities.

Measurement of oxidative stress biomarkers

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

According to Lowry et al. (1951), protein concentration was measured and calculated from a

Table 2. Morphometric characteristics of the studied fish from different localities and calculated Fulton's condition factor (K).

Locality	Weight	Length	K
	[g]	[cm]	
Sprattus sprattus			
Kavarna	2.77±0.44	7.40±0.14	0.68±0.08
Kranevo	2.72±0.16	7.40±0.16	0.67±0.07
Shkorpilovtsi	4.20±0.42*	8.75±0.35*	0.63±0.01
Trachurus mediterraneus			
Kavarna	14.68±2.87	12.55±0.68	0.74±0.05
Batova	33.6±1.55*	15.5±1.00*	0.90±0.10
Kabakum	13.47±2.99	12.05±1.00	0.77±0.10
Merlangius merlangus			
Tulenovo	63.95±36.84*	21.00±4.24*	0.65±0.01
Kamchia	21.20±6.04	13.50±0.84	0.84±0.07
Shkorpilovtsi	15.00±0.85	14.00±1.41	0.57±0.20
Obzor	25.63±4.34	15.17±0.88	0.73±0.03
Mullus barbatus			
Kavarna	18.12±5.67	11.48±1.09	1.17±0.10
Batova	18.30±2.58	11.50±1.00	1.20±0.10
Kamchia	21.73±2.56	12.30±0.34	1.16±0.06
Neogobius melanostomus			
Tulenovo	33.20±2.69*	13.90±1.56	1.30±0.53
Kaliakra	32.40±2.42*	13.00±1.30	1.47±0.47
Kavarna	26.40±4.81	13.07±0.81	1.18±0.06
Albena	19.43±7.01	11.42±0.92	1.26±0.21
Batova	34.10±6.65*	13.00±0.01	1.55±0.30
Kranevo	23.38±3.46	11.63±0.48	1.48±0.10
Kamchia	7.01±1.08*	8.38±0.26*	1.18±0.08

*statistical significance of differences at $p<0.05$

standard curve obtained using bovine serum albumin as a standard.

Statistical analyses

The significance of comparing the OS biomarkers' raw data among species and localities was determined using the t-test, and cluster analyses studied the similarities in their interrelation. The calculations were carried out using the STATISTICA 10 package (StatSoft Inc., USA, 2010).

Results

In the present study, fish of the same species differed in size depending on the catch area. Significantly larger specimens of the sprat were caught near Shkorpilovtsi, of the horse mackerel – near Batova, of the whiting – near Tyulenovo, and of the round goby – near Batova, Tyulenovo and Kaliakra (Table 2). However, the Fulton condition factor (K) was not significantly different between fish of the respective species from the other locations. However, among fish species, the calculated Fulton condition factor of the pelagic fish – sprat, horse mackerel, and the demersal whiting was lower than that of the benthic fish – red mullet and round goby.

The level of OS in fish of the same species indicated a different response to marine environmental stressors depending on the sampling location (Table 3). The sprats from Kavarna and Kranevo were characterised by higher OS levels compared to sprats from Shkorpilovtsi, as indicated by the statistically higher levels of lipid peroxidation (LPO), depletion of the non-enzymatic antioxidant glutathione (GSH)

Table 3. Oxidative stress biomarkers in A) liver and B) gills of the studied fish species from the trawling localities.

A)

Liver	**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
Sprattus sprattus							
Kavarna	19.02	71.23	35.63	3.88	4.21	35.56	80.60
	±2.20	±7.30	±2.89	±0.36	±0.75	±10.01	±11.30
Kranevo	17.92	72.94	31.50	6.48	4.16	30.93	101.00
	±5.79	±17.94	±3.39	±1.73	0.78	±7.70	±17.29
Shkorpilovtsi	2.73	565.99	14.02	12.47	8.18	72.37	120.25
	±0.06	±79.87	±0.98	±1.70	±0.28	±7.92	±12.45
Trachurus mediterraneus							
Kavarna	1.60	575.32	21.03	14.30	20.46	57.14	37.85
	±0.46	±237.04	±11.46	±2.21	±3.49	±11.64	±12.99
Batova	0.48	744.78	28.17	13.52	22.59	30.81	43.86
	±0.08	±44.78	±2.17	±1.52	±2.5	±0.81	±3.86
Kabakum	0.64	582.36	26.99	9.02	20.64	27.27	81.34
	±0.12	±208.80	±21.47	±4.38	±12.37	±8.12	±12.94
Merlangius merlangus							
Tulenovo	2.15	734.25	27.61	17.90	11.07	17.80	67.0
	±0.01	±58.85	±3.96	±4.02	±1.39	±10.64	±0.41
Kamchia	22.01	1789.22	42.75	17.47	15.41	221.38	125.25
	±0.15	±404.87	±6.97	±2.81	±18.62	±54.74	±39.65
Shkorpilovtsi	0.60	1065.60	29.75	24.40	15.95	64.85	135.75
	±0.00	±147.88	±3.54	±1.23	±2.29	±7.14	±25.25
Obzor	11.93	489.42	65.25	21.26	9.63	266.07	115.98
	±7.69	±192.33	±7.54	±4.05	±2.71	±84.42	±55.99
Mullus barbatus							
Kavarna	0.62	413.22	25.69	4.84	7.03	76.50	70.46
	±0.26	±129.14	±5.83	±2.16	±8.87	±28.67	±21.73
Batova	0.47	693.7	53.76	27.74	4.59	78.99	64.03
	±0.05	±75.75	±7.25	±1.55	±0.75	±10.25	±9.75
Kamchia	1.28	393.30	6.78	5.92	5.70	203.44	107.78
	±0.49	±52.06	±1.00	±2.66	±1.83	±64.18	±44.06
Neogobius melanostomus							
Tulenovo	13.11	379.37	20.57	12.08	14.58	70.69	21.62
	±1.85	±139.89	±13.88	±6.18	±1.02	±55.02	±10.66
Kaliakra	1.11	361.66	6.78	3.92	12.58	15.69	11.02
	±0.25	±25.75	±0.87	±0.55	±2.25	±3.15	±1.75
Kavarna	6.85	821.46	8.61	15.19	14.83	258.21	65.17
	±7.47	±177.65	±5.82	±1.10	±4.19	±89.95	±8.66
Albena	4.89	393.10	32.05	10.48	31.92	225.37	23.19
	±2.57	±113.69	±3.20	±3.38	±7.69	±25.21	±29.71
Batova	1.30	790.63	4.45	22.96	24.75	59.96	16.92
	±1.04	±173.54	±1.33	±2.70	±5.81	±34.47	±3.02
Kranevo	11.67	370.60	34.37	10.88	9.77	162.88	18.15
	±9.85	±117.12	±7.21	±3.95	±3.84	±22.28	±1.64
Kamchia	1.40	300.76	47.66	6.12	29.94	141.81	58.99
	±0.34	±50.39	±12.96	±1.12	±4.20	±43.41	±4.33

Table 3. Oxidative stress biomarkers in A) liver and B) gills of the studied fish species from the trawling localities.

B)

gills	**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**
Sprattus sprattus							
Kavarna	20.59	81.39	8.71	1.79	4.29	7.76	153.98
	±1.72	±12.25	±1.46	±0.25	±1.09	±0.56	±37.70
Kranevo	21.29	70.87	11.66	3.12	5.08	8.63	215.54
	±0.45	±7.59	±1.06	±0.42	±1.40	±1.53	±18.91
Shkorpilovtsi	6.70	763.91	35.33	17.56	8.73	51.34	225.00
	±0.95	±102.12	±19.20	±0.31	±0.88	±7.00	±20.2
Trachurus mediterraneus							
Kavarna	21.88	509.85	12.95	0.40	24.43	44.01	167.46
	±0.71	±104.35	±5.84	±0.08	±8.55	±6.13	±25.04
Batova	2.51	928.31	58.82	0.51	6.56	31.30	52.23
	±0.18	±24.78	±1.17	±0.05	±0.5	±0.31	±1.86
Kabakum	4.87	540.85	15.98	0.74	14.82	22.95	209.99
	±0.61	±180.80	±6.08	±0.30	±3.15	±5.02	±26.68
Merlangius merlangus							
Tulenovo	3.09	905.92	5.41	1.83	3.57	12.76	7.84
	±1.24	±68.34	±2.17	±1.47	±0.96	±3.49	±2.07
Kamchia	19.00	1568.02	10.37	1.28	54.11	34.66	62.48
	±2.55	±169.40	±3.55	±0.78	±6.23	±3.81	±12.53
Shkorpilovtsi	1.34	1673.19	7.25	2.24	1.46	14.61	75.75
	±0.37	±444.75	±1.25	±0.34	±0.15	±1.27	±5.25
Obzor	13.57	577.05	33.84	1.62	5.26	83.68	29.91
	±5.01	±142.49	±5.90	±0.40	±2.24	±35.30	±2.59
Mullus barbatus							
Kavarna	11.20	449.09	23.62	1.55	12.32	37.16	300.43
	±9.20	±122.76	±8.66	±0.42	±2.48	±9.74	±115.73
Batova	1.46	382.11	57.87	1.06	15.88	22.15	123.30
	±0.10	±55.75	±6.75	±0.75	±1.25	±3.50	±15.75
Kamchia	3.31	315.91	3.20	1.02	9.54	68.32	300.07
	±1.44	±43.80	±0.70	±0.17	±3.76	±10.47	±36.52
Neogobius melanostomus							
Tulenovo	1.84	495.35	21.22	0.55	26.12	15.07	41.07
	±0.81	±65.06	±12.42	±0.06	±3.17	±8.90	±10.30
Kaliakra	3.73	337.01	1.91	0.68	7.22	33.13	8.79
	±0.45	±45.45	±0.25	±0.07	±0.75	±2.25	±1.75
Kavarna	1.92	414.73	11.19	0.85	15.38	83.97	58.44
	±0.10	±15.25	±3.64	±0.36	±4.94	±28.61	±32.33
Albena	5.52	255.39	43.98	0.66	7.77	263.75	29.56
	±4.85	±48.48	±32.39	±0.22	±2.78	±27.05	±3.49
Batova	4.68	590.73	31.50	1.73	12.21	30.21	8.01
	±0.50	±22.42	±2.60	±0.10	±9.77	±8.23	±0.85
Kranevo	1.96	132.83	23.08	0.10	8.01	43.93	25.56
	±0.90	±20.63	±2.83	±0.04	±3.50	±9.51	±5.43
Kamchia	4.24	260.13	49.91	0.67	7.96	337.23	47.80
	±2.99	±76.34	±19.78	±0.30	±3.08	±68.05	±13.23

Table 4. Mean values of OS biomarkers of studied fish species.

Liver	***S. sprattus***	***T. mediterraneus***	***M. barbatus***	***M. merlangus***	***N. melanostomus***
LPO	13.93	0.97	0.72	13.07	5.62
GSH	155.33	594.56	402.04	1079.47	450.48
SOD	26.96	24.93	21.84	47.67	29.10
CAT	6.04	11.35	6.02	19.81	10.94
GPx	5.98	20.75	6.25	64.80	27.42
GST	37.34	38.45	99.33	193.13	162.76
AChE	80.71	62.12	74.27	104.34	34.55
F	0.66	0.80	1.12	0.74	1.35

Gills	***S. sprattus***	***T. mediterraneus***	***M. barbatus***	***M. merlangus***	***N. melanostomus***
LPO	23.72	10.84	8.39	12.77	2.68
GSH	194.26	564.80	393.89	1097.37	307.41
SOD	13.83	18.77	19.88	19.72	33.19
CAT	4.98	0.59	1.33	1.60	0.67
GPx	4.62	17.56	11.28	24.32	10.68
GST	15.29	31.36	40.98	47.80	173.22
AChE	164.23	180.18	275.22	40.72	35.36
F	0.66	0.80	1.12	0.74	1.35

in the liver and gills, and activation of superoxide dismutase (SOD) in the liver. For these fish, low glutathione-S-transferase (GST) and glutathione peroxidase (GPx) activity was also reported in the liver and gills, possibly due to low levels of GSH, which is a co-substrate in the reactions. In the gills of fish from Shkorpilovtsi, the activity of all enzymes was high.

The horse mackerel caught off Kavarna also showed higher levels of OS in the detoxification processes; contamination of the waters from this location with pro-oxidative xenobiotics can be assumed. The mackerels from Batova had low levels of OS, characterised by low levels of LPO and high concentrations of GSH in both examined organs (Table 3).

LPO was highest in whiting from Kamchia and lowest in those from Shkorpilovtsi, both in the liver and gills. In whiting from these areas, the concentration of GSH was high in the liver and gills, while in whiting from Obzor, the lowest concentration was found. The highest GST activity in fish from Obzor was measured in both organs. Similar trends were observed in red mullet fish from Kamchia, which had the highest LPO in the liver and lowest concentrations of GSH and activated GST in the liver and gills (Table 3).

In the round goby, the highest LPO in the liver was found in fish from Kranevo and the lowest in those from Batova. In the gobies from Batova, high GSH levels, catalase (CAT), and GPx activities, along with low GST activities were measured in both the liver and gills (Table 3), indicating that the gobies in the region of Batova tolerate the present environmental conditions successfully.

Based on the results obtained from measuring OS indicators for all types of fish, the environmental conditions near Shkorpilovtsi and Batova are the most favourable, and the waters near Kamchia and Kavarna are the least favourable.

The fish of the different species studied were sensitive to oxidative stress; however, there were trends distinguishing the individual types of sensitivity and response. Comparing pooled values (all fish of a given species, regardless of capture site) showed that whiting had the highest GSH levels in both the liver and gills, as well as the highest activities of all measured antioxidant enzymes in the liver and GPx in gills (Table 4). Against the background of the relatively high LPO of whiting compared to all the other fish species, this indicated the activation of the protective mechanisms mobilised to combat stressor pressures so that the organism maintained a higher level of sustainability. On the other hand, sprat was found to have the highest LPO (especially in the gills) and lowest antioxidant defence in contrast to all the other studied fish species, as indicated by the lowest concentrations of GSH and the lowest activities of glutathione-related enzymes (GPx and

GST) in both liver and gills, as well as the lowest CAT activity in liver and SOD activity in gills (Table 4). This means that in the sprat, a depletion of adaptive energy stores occurred, limiting the capacity to adapt to stressors' effects, which may reduce the population resilience capacity.

The cluster analysis (Fig. 2) confirmed dissimilarities in the responses to OS between the fish species in the liver and the gills. This can be expected as the stressor effects in the liver differed from those in the gills, which have direct contact with the marine environment. Whiting appeared to differ significantly from all the other fish species studied in the response to OS in the liver and gills.

Discussion

Fishing in the Black Sea is limited to the coastal zone due to the anoxic nature of the deeper waters. A significant problem for the coastal areas of the northern region of the Bulgarian Black Sea is eutrophication by anthropogenic nutrients and pollutants carried by the large European rivers (Danube, Dnieper, Dniester), which lead to significant variations in the chemical and biological regimes (Bekova 2020). This determines the precarious status of wild fish populations, which entails concerns about fish health.

Fulton's body condition factor (К) is a marker widely used in fisheries and fish biology studies of the effects of physical and biological fluctuations on fish condition (Le Cren 1951, Datta et al. 2013). The К-factor values measured by us clearly showed similarity between the red mullet and round goby on the one hand, which differed from that of the sprat, whiting, and horse mackerel on the other. The feeding mode and the habitat fish occupy probably determined the differences. In general, fish with elongated, torpedo-shaped bodies that chase their prey were shown to have a lower К-factor (Ragheb 2023). The red mullet and round goby in our study had К-factor above 1.0, indicating good habitat conditions, well tolerated by these species. For example, data show that low levels of eutrophication can be favourable and thus well tolerated by benthopelagic fish (Zymaroieva et al. 2023). The demersal goby (*N. melanostomus)* can tolerate exposure to low-oxygen water for several days (Skora et al. 1999).

Marine organisms' state depends on the conditions of their environment from which they draw resources for their life processes. Monitoring the condition of coastal ecosystems has traditionally used measurements of eutrophication and pollutant concentrations in sediment and water. Such chemical monitoring alone cannot be objective enough because the environmental factors form complex relationships, and their interactions can modulate the effects compared to the stand-alone effect of an individual factor (Steinberg 2012, Heys et al. 2016; Banaee et al. 2019). A more objective assessment of the environment's multifactorial stressogenicity and the organisms' tolerance to stress can be obtained by analysing suitable markers of biological effects, also known as stress ecology (Van der Oost et al. 2003, Hook et al. 2014). With the development of stress ecology, it is accepted that lower levels of biological organisation (molecular and cellular) are more sensitive to the stressful conditions of the marine environment. Changes in them allow them to be used as early warning markers for changes in populations and communities and to introduce measures to deal with stress (Lemos 2021). Several studies have shown that environmental factors of both anthropogenic and natural origin can induce oxidative stress in organisms. Thus, OS markers are becoming widely used in the research and monitoring of the marine environment and marine ecosystems.

Our results showed that fish of the same species have different OS levels depending on the sampling location. The regions of Kamchia and Kavarna appear to be more stressful to most fish species. In the fish species from these locations, significantly more intense pro-oxidant reactions and decreased non-enzymatic antioxidants (GSH) were found, especially in the liver. The role of nonenzymatic antioxidants in the defence system is important because of their abundance in the tissues and their ability to be mobilised as soon as oxidative stress intensifies (Gostyukhina & Andreenko 2015). Increases in pro-oxidant processes accompanied by a decrease in GSH concentration have been reported as a result of exposure of fish to a large variety of stressors such as metal pollution, persistent organic pollutants, bacterial toxins, climate change, hypoxia etc. (Bozcaarmutlu et al. 2008). Specifically, the area of Kavarna is known to be more affected by pollution carried in by the waters of the Danube (Doncheva et al. 2020, Chițescu et al. 2021). The Kamchia River estuary region is assumed to affect marine water because of the river's inflow of pollutants of anthropogenic origin. As a result of river runoff, the nearshore zone is characterised by low transparency and high nutrient concentrations (Kalinov et al. 2019; Doncheva et al. 2019, 2020). This leads to eutrophication of the area with inherent hypoxia, which is directly related to organisms'

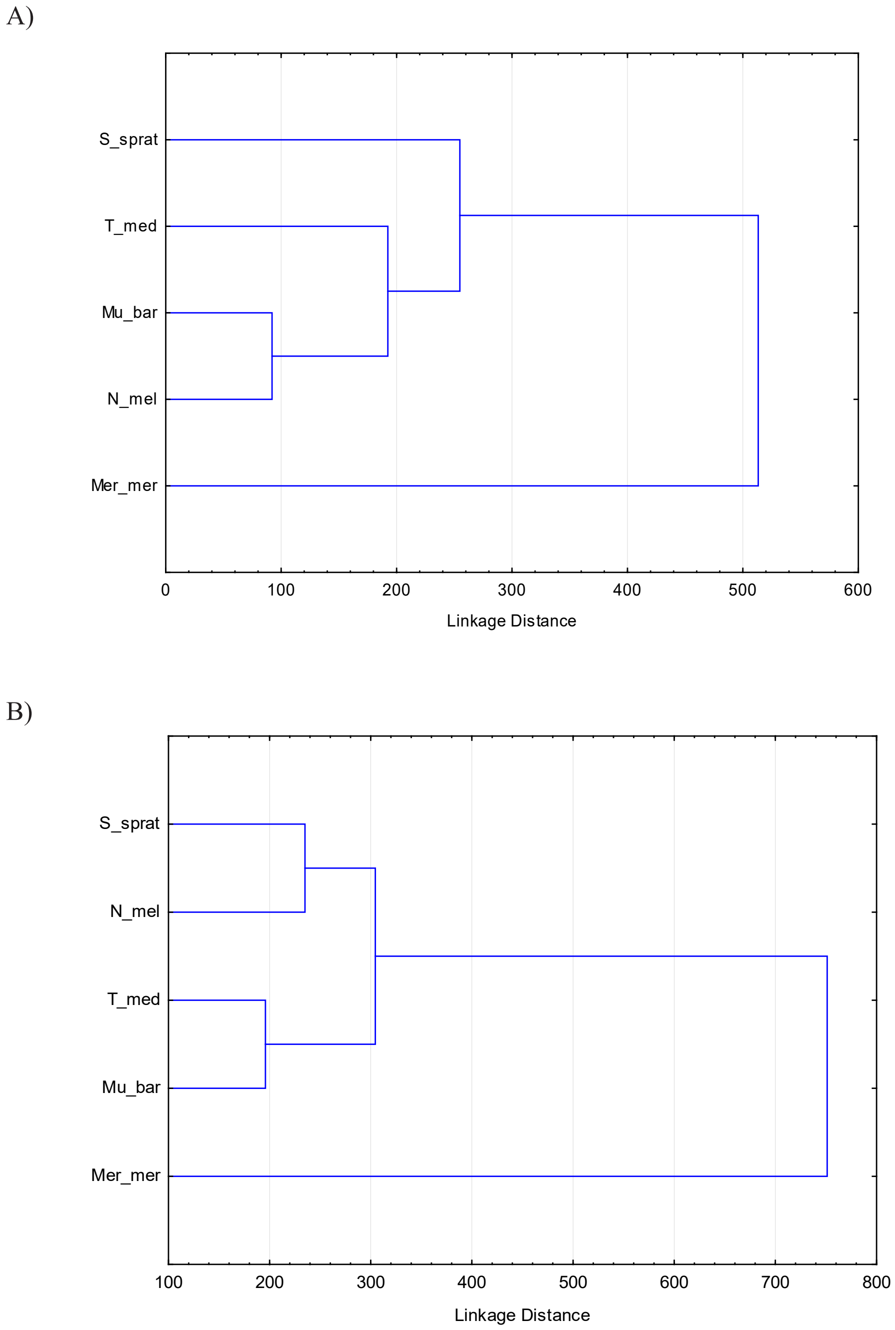

Fig. 2. Cluster analysis of Euclidean distances between different spicies of fish in relation to OS biomarker arrangement similarities in A) liver and B) gills.

metabolic processes and energy supply (Ding et al. 2020). Hypoxia also influences the OS status of marine organisms (Parrilla-Taylor & Zenteno-Savín 2011). It has been reported that hypoxia causes overproduction of ROS by disturbing the electron transport chain (Luo et al. 2020), followed by an increase of the antioxidant potential to counteract the OS development (Hermes-Lima & Zenteno-Savín 2002, Lushchak & Bagnyukova 2007, Wang et al. 2021). In addition, the data we obtained showed intraspecies variations in the OS reaction of the fish studied, as clearly demonstrated in the whiting, where the highest levels of antioxidant defence were established. The OS biomarkers could be affected by factors such as fish habitat, trophic level, feeding behaviour, and nutritional factors (Martinez-Alvarez et al. 2005, Rudneva et al. 2010, Rudneva & Kuz'minova 2011). Our data showed that whiting differed from the other species studied in its OS bioindicator value ratios and, thus, in its ability to tolerate environmental conditions. In comparison to other fish species, the results for the analysed whiting indicated relatively low K factor (< 1), high LPO, and very high GSH level and GPx activity in both liver and gill, as well as the highest values of antioxidants in the liver (Table 4). Whiting is classified as a supra-benthic, less mobile predator, feeding on molluscs, crustaceans, worms, and small fish accumulating xenobiotics (Rudneva et al. 2010). It has been suggested that most sluggish fish from the benthic and supra-benthic groups usually live in a more contaminated environment because most pollutants precipitate in low water layers and sediments. In addition, the benthic invertebrates, serving as food for such fish, might accumulate the xenobiotics from the bottom and transfer them via trophic nets, magnifying the toxic effect. Based on these characteristics, the activated antioxidant defence system in whiting from the studied sites seems to be the expected result. Much data indicates higher enzymatic activity in fish from polluted localities (Hamed et al. 2003, Bozcaamutlu et al. 2008, Rudneva et al. 2010, Kumar et al. 2019). On the other hand, the sprat in this study was in a poor physical condition (lowest Fulton's condition factor) along with a high OS level (high LPO and low antioxidant defence). The poor condition of the sprats was also noted during annual monitoring (Panayotova et al. 2018, Bekova 2020).

In conclusion, the investigated fish species demonstrated differences in their adaptation capacity and resilience in response to the multistressor impacts of the marine environment. This suggests that OS biomarkers can provide an objective assessment of their metabolic and general health condition, which provides the basis for uncovering the ecological or physiological reasons for differences in growth, survival, or recovery between populations of a fish species on a smaller spatial scale. Hence, monitoring the stress-response status can be a powerful tool to evaluate the tolerance of fish to changing marine environmental conditions.

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