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Research Article

Effects of anthropogenic and environmental stressors on the current status of red mullet (*Mullus barbatus* L., 1758) populations inhabiting the Bulgarian Black Sea waters

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Abstract

The red mullet (*Mullus barbatus* Linnaeus, 1758) is a keynote species for the Bulgarian Black Sea ecosystem and fisheries; nevertheless, existing knowledge on population status is very scarce. The present study was intended to assess the health status and adaptive potential of *M. barbatus* populations inhabiting the Bulgarian waters of the Black Sea. Our findings revealed that populations of *M. barbatus* are exposed to a variety of anthropogenic and environmental stressors. The species' status was assessed using representative genetic, morphological, biochemical and chemical biomarkers from specimens obtained in the research area's northern and southern regions. Based on mtDNA markers, genetic analysis revealed low haplotype and nucleotide diversity, typically observed in overexploited or "threatened" populations. Examining the morphology of the specimens revealed no discernible pattern of differentiation. Except for aluminium and chrome, metal and PAH concentrations in fish were below the regulatory thresholds. The specimens from the southern region ingested more microplastics than those from the northern region. The majority of specimens collected from the southern region also exhibited elevated levels of oxidative stress and decreased antioxidant defence, which can be interpreted as an early indication that they had reached the limits of their adaptive potential. Further research on the composite effects of the stressogenic environment on the Black Sea biota are critically needed, as well as the introduction of new indicators and thresholds at molecular and cellular levels for adequate monitoring of both the ecological state of the marine environment and its biota.

Key words: Black Sea, fish morphology, genetic diversity, metal accumulation, microplastics, oxidative stress, red mullet

Introduction

Progressively, marine ecosystems are exposed to multiple stressors of both natural and anthropogenic origin, which can have direct and indirect impacts with interdependent and complex effects on the marine environment and

biota. Human impacts on marine ecosystems include physical disturbance of the marine environment, biota and habitats; inputs of nutrients, litter and toxic substances; overexploitation of marine resources; introduction of waterborne pathogens and alien species; and increased use of coastal and seabed resources, which are overlaid on the effects of changing natural conditions (Bailly et al. 2015; Bernal et al. 2017).

As a result of environmental pressure, marine fish species are subjected to a wide variety of stressors that impair their optimal physiological functioning and adaptive capacity, which are heavily dependent on the ecological state and fluctuations of their immediate environment (Farag et al. 2009; Birnie-Gauvin et al. 2017). Multiple toxic contaminants can accumulate in the marine environment and biomagnify along the food chain (Streit 1998; Feng et al. 2020; Tenji et al. 2020; Butnariu 2022). The response of marine fish species to environmental stress can be polymorphic, depending on habitat, species sensitivity and vulnerability and other intrinsic variables, as well as the magnitude, duration and intensity of exposure to particular stressor/s. In addition, substantial evidence has recently accumulated suggesting that alterations at the molecular and cellular levels of biological organisation can have large effects at higher hierarchical levels by distressing the survival, growth and reproduction of organisms (Steinberg 2012; Petitjean et al. 2019; Fu et al. 2020).

The Black Sea has been documented as one of the seas heavily impacted by human activities, such as pollution and discharges from land-based sources on the territories of the central and eastern European countries along the river Danube (Zaitsev and Mamaev 2008). In the 1980s, the Black Sea ecosystem suffered substantial structural and functional changes as a result of multiple anthropogenic and natural influences (Ivanov and Beverton 1985; Daskalov 2002; Pauly and Watson 2005; Keskin et al. 2017). Eutrophication, overfishing and the introduction of alien species were referred to as the primary causes of the observed ecosystem disruption and severe loss of biodiversity (Prodanov et al. 1997; Zaitsev et al. 1997; Caddy 2008). Recently, the bioaccumulation of microplastics (MPs) has emerged as a new hazard, as it has been demonstrated to have harmful impacts on marine biota. In the Black Sea, MPs were registered in sediments (Săvucă et al. 2017; Cincinelli et al. 2021; Pojar et al. 2021a), seawater (Öztekin et al. 2017; Aytan et al. 2020a, 2020b; Berov and Klayn 2020; Pojar et al. 2021b; Bat and Öztekin 2022; Terzi et al. 2022) and marine organisms: zooplankton (Aytan et al. 2016, 2020a, 2020b), bivalves (Gedik and Gozler 2022) and fish (Atamanalp et al. 2021; Aytan et al. 2021; Eryaşar et al. 2022).

It has been proven that tracking explicit biomarkers in wild populations is an indispensable method for evaluating the harmful effects of an unstable environment. According to this perspective, changes in genetic diversity and genetic structure reflect the "health" of an ecosystem (Belfiore and Anderson 1998; Medina et al. 2007), which is defined as a comprehensive, multiscale, dynamic, hierarchical assessment of system resilience (Ehrenfeld 1992).

Various biomarkers, including oxidative stress' (OS) ones, are currently being used to study and monitor the marine environment and marine ecosystems. (van der Oost et al. 1996; Samet and Wages 2018). The stress-response approach is currently gaining attention in studies of the environment's multiple stressogenic effects on Black Sea biota and ecosystem functionality (Silkina et al. 2014; Vinagre et al. 2014; Yakimov et al. 2018; Bozcaarmutlu et al. 2020;

Alexandrova et al. 2021, 2022a, b). In addition, changes in cellular pro- and anti-oxidant balance can be utilised to assess the general response of all aerobic organisms to multiple stressors. Numerous studies have shown that, in fish, various factors of the aquatic environment can affect oxidative processes in cells (Lushchak and Bagnyukova 2006; Stoliar and Lushchak 2012; Vinagre et al. 2012; Chowdhury and Saikia 2020; Gopi et al. 2021). These effects, can intensify the production of reactive oxygen species (ROS) (pro-oxidant processes) and/or reduce the cellular antioxidant defence (antioxidant enzymes and non-enzymatic antioxidants). The imbalance in favour of oxidative processes is referred to as oxidative stress (OS). OS has been recognised as a promising molecular biomarker tool for determining the extent to which the environment has stressogenic effects on living organisms (Van Straalen 2003; Steinberg 2012; Hook et al. 2014; Samet and Wages 2018). Lower levels of biological organisation are more vulnerable to stressogenic environments; thus, alterations at these levels can be used as early warning indicators for changes that will eventually occur at the population, community and ecosystem levels.

Benthic and piscivorous marine species have been reported to be amongst the most vulnerable to the effects of pollutants (Tenji et al. 2020) and, hence, appropriate indicators of the state of the marine environment (Goksøyr et al. 1996; van der Oost R et al. 1996).

The red mullet is one of the most important species for the Black Sea's fisheries and marine ecosystem; nevertheless, little is known about their populations along the Bulgarian Black Sea coast. *M. barbatus*, as a benthivorous fish, has the potential to serve as a sentinel and bioindicator species for detecting the Black Sea ecosystem's stressogenic and genotoxic potential. Consequently, a multi-biomarker approach was employed to assess the current status of the *M. barbatus* populations as a multifaceted response to environmental stresses, with the incorporation of genetic and OS markers as an intelligible metric for ecological stress. Other physiological markers, such as morphological variation and physiological state, were found to be useful towards determining the health status of *M. barbatus* and, as a result, were also studied within the scope of this research.

The goal of this study was to provide an initial evaluation of the status of *M. barbatus* populations as a key species with significant economic value in the Bulgarian Black Sea waters, as well as its adaptive capacity, using representative biomarkers.

Material and Methods

Study area and sampling

Species sampling was carried out as part of a multispecies survey from 5 July to 15 July 2021. A random selection of 36 to 38 sampling sites was surveyed in the study area covering the coastal and shelf waters at depths 15 to 100 m in front of the Bulgarian Black Sea coast and the samples under consideration in this study were conditionally taken as representative for the "north region" – the area in front of Kavarna (sampling site 34) and the "south region" – the area in front of Sv. Vlas (sampling site 24) in coastal waters at depths 15–19 m (Fig. 1).

Figure 1. Sampling sites along the Bulgarian Black Sea coast.

The marine environment at both sites is known to be under substantial anthropogenic pressure. The eutrophication and organic content of the marine environment at the two sites were quantified using the pollution index PI (ranging from 0 to 1), in line with Guidance Document No. 23 on eutrophication of the overall strategy for WFD implementation (Guidance, WCE 2009). Furthermore, for the sampling regions, the time series of annual mean sea surface temperature (2003–2021) and salinity (1993–2022) were acquired using NASA OBPG (NASA OBPG 2020) and Copernicus Marine Service (Lima et al. 2020; Ciliberti et al. 2021). Breakpoints indicating trends in temperature and salinity regimes in the studied regions were detected using segmented regression analysis. The analyses and graphical representations were carried out using the statistics and programming software R 4.0.5 (R Core Team 2021), packages 'ggplot2' (Wickham et al. 2016) and 'segmented' (Muggeo 2008).

Assessment of heavy metals accumulation

A total of 25 fish specimens (15 sampled at the Sv. Vlas site and 10 sampled at the Kavarna site) were deep-frozen immediately after capture. The fish size range was consistent with Descriptor 8 "Pollutants in the Marine Environment" of the Monitoring Programme. The main elements were analysed on whole fish (pollutant priority substances: cadmium – Cd, mercury – Hg, lead – Pb, nickel – Ni, polyaromatic hydrocarbon – benzo (a) pyrene and specific pollutants: arsenic – As, chromium – Cr, aluminium – Al, iron – Fe, copper – Cu, manganese – Mn and zinc – Zn) were carried out on whole fish. The water content and free fats were measured in each sample, with the aim of subsequent normalisation of the concentration of pollutants. The analysis of the samples was carried out by an accredited laboratory (SGS Bulgaria Ltd., https://www.sgs.bg/en) using standard methods (atomic absorption and gas chromatography). The assessment of the state of the biota in terms of pollutants was based on the methodology described in Guidance Document No. 32 on Biota Monitoring (Guidance Document No. 32 2013). As a first step in the matrices, results that were below the limit of determination were replaced by ½ limit of quantification (LOQ). Metal concentrations in fish were then standardised by weight, whereas organic contaminants were normalised by fat content. The values obtained after normalisation by dry weight were compared to the maximum permissible limits for seafood (Commission Regulation (EC) No 1881/2006 of 19 December 2006), as enacted in Bulgarian national law (Ordinance No 5/9.02.2015 2015).

Fish morphology and biometric analyses

The biometric analysis includes the measurement and evaluation of 22 morphometric and four meristic features on 77 specimens (33 from Sv. Vlas and 40 from Kavarna). A Vernier caliper was used to measure the features with a precision of 0.1 mm. The methodology used by Mahmoud et al. (2016) to differentiate the morphology of *M. surmuletus* was applied to the morphometric measurements and meristic counts of *M. barbatus* (see Suppl. material 1: fig. S1).

To test statistical significance and validate the results, several statistical methods were used: parametric tests to verify the normal distribution of length-frequency data (LFD), which was used in linear regression models and non-parametric tests to identify statistically significant similarities in and between the samples (Analysis of Similarities – ANOSIM). The length-weight relationship (LWR) and ratios, such as standard length (SL), total length (TL), head length (HL), body depth/height (BD/BH) and BD/BH – SL (Fakunmoju et al. 2014) were modelled and their parameters were analysed.

All parametric and non-parametric statistical tests, modelling and computations were carried out using the MATLAB programming environment (THE MATH WORKS, INC. MATLAB version 2020a), the vegan package (Oksanen et al. 2019) and R version 4.0.5 (R Core Team 2021).

Molecular genetic analyses

Tissue samples were obtained from the dorsal fins of 79 specimens (39 from Kavarna and 40 from Sv. Vlas) and preserved in 96% ethanol at 4 °C for DNA

analysis. The genomic DNA was isolated using the DNeasy Blood & Tissue Kit (QIAGEN) and the target DNA was amplified with mitochondrial primers – cytochrome c oxidase subunit I (COI) and cytochrome b (Cyt b). The polymerase chain reaction (PCR) using mitochondrial primers (COI) and (Cyt b) was carried out in a reaction volume of 50 µl containing 2 µl of each primer, 25 µl of the Mastermix (MyTaqTM HS Mix, Bioline Reagent Ltd.) and 2 µl of the target DNA. The mitochondrial cytochrome c oxidase subunit I (COI) was amplified using universal primers, according to Ivanova et al. (2007) and for Cyt b using universal primers, according to Keskin and Can (2009). The conditions, conducive for PCR amplification for COI and Cytb included the following parameters: for COI 95 °C for 1 min, 95 °C for 15 s, 52 °C for 25 s, 72 °C for 1 min (35 cycles) and 72 °C for 1 min; for Cyt b: 95 °C for 2 min, 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s (35 cycles) and 72 °C for 10 min. PCR product quality control was performed by electrophoresis on 2% agarose gel. The mtDNA sequencing was performed by Macrogen Europe B.V. The mtDNA sequence data was further analysed by applying MEGA7 (Kumar et al. 2018). The number of haplotypes, haplotype connectivity and TCS Networks were constructed by means of Popart (Clement et al. 2002). The haplotype (H) and nucleotide (π) diversity were estimated with DnaSP 5.10.01 (Librado and Rozas 2009).

Oxidative stress assessment

Tissue preparation

M. barbatus specimens of the same length were analysed (supposedly to have individuals of the same age). The 26 samples were immediately shock-frozen and transported to the laboratory for optimal preservation (12 from the Sv. Vlas region and 14 from the Kavarna region) (Secci and Parisi 2016). On the day of analysis, the samples were dissected and their livers and gills were extracted and homogenised in 0.1 M potassium phosphate buffer (pH 7.4). After centrifuging homogenates at 3000 g for 10 minutes, the post-nuclear fraction was used to measure lipid peroxidation (LPO) and glutathione (GSH) contents. Antioxidant enzyme activities were measured using the supernatant obtained from re-centrifuging a portion of the post-nuclear fraction at 12,000 g for 20 minutes at 4 °C.

Measurement of oxidative stress biomarkers

Oxidative stress biomarkers were assayed using kits, purchased by Sigma-Aldrich Co. LLC, USA: MDA assay kit (Cat. No: MAK085) for determination of lipid peroxidation; Glutathione Assay Kit (Cat. No: CS0260), Superoxide dismutase (SOD) determination kit (Catalogue No: 19160), Catalase Assay Kit (Catalogue No: CAT100), Glutathione Peroxidase Cellular Activity Assay Kit (Cat. No CGP1), Glutathione-S-transferase (GST) Assay Kit (Cat. No CS0410) and Acetylcholinesterase Activity Assay Kit (Cat. No MAK119). The assays were performed strictly following the manufacturer's instructions. Protein concentration was measured according to Lowry et al. (1951) and calculated from a standard curve, obtained using bovine serum albumin as a standard.

This study also employed a version of a previously introduced Specific Oxidative Stress (SOS) index (Yakimov et al. 2018). The SOS index was com-

puted as the difference between the pro-oxidative (PrO) score and the antioxidant (AO) score of the OS markers measured in *M. barbatus* specimens. The pro-oxidative (PrO) score (index) is the mean Z-score of the pro-oxidant markers (LPO) measured (PrO = mean) (zLPO). The antioxidant (AO) score (index) is the average of the anti-oxidant Z-scores (in this case GSH, SOD, CAT, GPX and GST) measured (AO = average (zGSH + zSOD + zCAT + zGPX + zGST)). In order to calculate the Specific Oxidative Stress (SOS) index, the AO score was subtracted from the PrO score (SOS = PrO – AO). Close to zero values for each of the three indices represent the mean of the difference (i.e. the balance). Negative and positive values indicate deviation from the mean (above or below).

Determination of ingested microplastics in *M. barbatus*

Each sample (40 collected at Sv. Vlas and 39 collected at Kavarna) was wrapped in aluminium foil and frozen at -20 °C immediately after identification on-board. Lusher, Dehaut and Karami's (Dehaut et al. 2016; Karami et al. 2017; Lusher et al. 2020) methodology was utilised to extract and evaluate microplastics in biota. A reduced-cross-contamination methodology was applied during the analysis and liquid and air exposure controls were taken into account in the result processing. The samples were defrosted at room temperature and carefully rinsed in the laboratory. The gastrointestinal tract (GIT) was separated and weighed. The sampled stomach and intestines were digested with filtered 10% potassium hydroxide at 40 °C for 48–72 hours (until ready) in glass containers covered with metal foil. Subsequently, they were filtered with glass micro-fibre filters with a pore size of 1.2 µm and a diameter of 47 mm (Whatman glass micro-fibre filters, Grade GF/C) with a vacuum system, Rocker MultiVac 300, made of stainless steel. Using an Olympus SZ30 stereomicroscope with a 110AL2X WD 38 lens, the type, colour and size of the identified microplastics were determined directly from the glass Petri dishes, which were opened solely for the purpose of identifying the particle structure.

Results

Sampling sites' environmental characteristics and pressures

In general, the PI in the sampling locations was low to moderate and most pronounced in the south (Table 1, Fig. 2A). Both sampling sites are located in close proximity to or within regions with high physical disturbance pressure from fishing (swept area ratio (SAR) ≥ 2) (Todorova et al. 2021). During the sampling period, the fishing intensity was estimated to be 0.5 h/km^2 at the southern sampling site (Sv.Vlas) and 100+ h/km^2 at the northern sampling site (Kavarna) (Fig. 2B), despite the fact that cargo traffic intensity (Fig. 2C) was found to be low at both sampling sites in 2021.

Segmented regression analyses identified two breakpoints for both variables (temperature and salinity) during the studied period, the most recent of which occurred in the southern region in 2010 for SST and 2014 for SSS and in the northern region in 2011 and 2014, respectively (Table 2 and Suppl. material 1: fig. S2A–D), indicating a nearly decade-long increase in annual mean surface temperature and salinity.

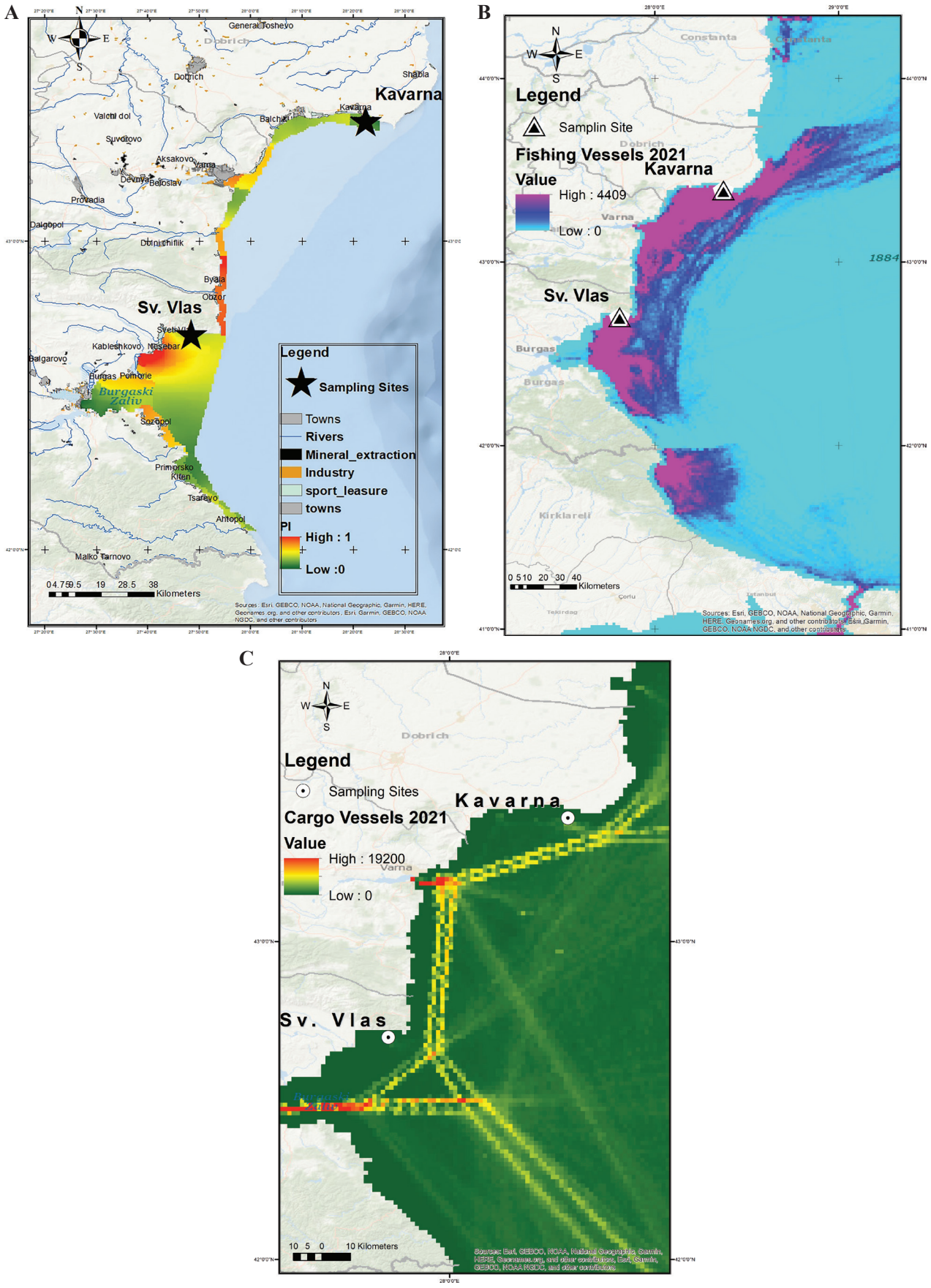

Figure 2. A sampling sites, overlain with PI (eutrophication + organic) **B** sampling sites overlain with fishing intensity layer (routes km^{-2}) in 2021 and **C** cargo ships traffic intensity layers in 2021 (EMODNet 2022).

Table 1. Sampling stations and anthropogenic impact data in the sampling period.

Date	Sampling station	Lon [DD]	Lat [DD]	Depth [m]	Region position	Pollution index (PI)*	Fishing density (h/km² per month in July 2021**	Fishing intensity in 2021 (routes/km²) **	Cargo traffic intensity in 2021 (routes/km²) **
13.07.21	Kavarna	43.388	28.329	15	northern	0.213	> 100+	1155	13
9.07. 21	Sv. Vlas	42.698	27.775	19	southern	0.288	0.5	450	0

*Report analysis and interpretation of data on the ecological state of marine waters – 2021,
** (EMODNet 2021).

Table 2. Breakpoint analysis of annual mean surface sea temperature (SST) and salinity (SSS) time series (2003–2021 for SST and 1993–2022 for SSS) in the sampling regions.

Sampling station	Variable	Time series data period	Estimated breakpoints (year)	Std. error and R^2	Data source
Kavarna	T_{mean} [°C]	2003–2021	2007 ↘ and **2010** ↗	R^2=0.59; *std.err*=0.48	1*
Kavarna	S_{mean} [PSU]	1993–2022	2008 ↘ and **2014** ↗	R^2=0.61; *std.err*=0.23	2*
Sv. Vlas	T_{mean} [°C]	2003–2021	2007 ↘ and **2011** ↗	R^2=0.49; *std.err*=0.53	1*
Sv. Vlas	S_{mean} [PSU]	1993–2022	2007 ↘ and **2014** ↗	R^2=0.55; *std.err*=0.26	2*

*1 (NASA OBPG 2020),
*2 (Lima et al. 2020; Ciliberti et al. 2021).

Heavy metal accumulation

The majority of the normalised concentrations of the studied elements were below the maximum allowable concentrations for seafood specified in the relevant regulatory documents (Guidance Document No. 32 2013) (Table 3). The aluminium concentration in fish from the Kavarna site (higher than 30 mg kg^{-1}) and the chromium concentration in fish from the Sv. Vlas site (higher than 0.3 mg/kg) both exceeded the toxicologically acceptable level in biota. The results for mercury were below its limit of determination (0.05 mg kg^{-1}) and, therefore, not taken into account.

Fish morphology

Analysis of morphometric characteristics revealed that specimens captured in the southern region had a lower allometric coefficient than those sampled in the northern region (Table 4, Suppl. material 1: fig. S3A, B); however, specimens from both regions exhibited negative allometric growth. The estimated Fulton condition coefficient for Sv. Vlas specimens was slightly higher than the value calculated for Kavarna specimens, with the most likely cause being the larger size class range represented in the length frequency data (LFD) collected for Kavarna specimens (Suppl. material 1: table S1). The BD-HL ratio varied significantly by sampling site and all other ratios displayed very close values, with a difference of 0.01–0.02 in favour of the samples collected at the Kavarna site, indicating greater variability in the morphometric characteristics of specimens within each size class.

According to ANOSIM results, the morphometric and meristic characteristics of male and female *M. barbatus* specimens did not differ statistically and the specimens collected from the two sites appeared to have a relatively similar biometric structure (Suppl. material 1: table S2).

Table 3. Normalised concentrations of metal elements in *M. barbatus*.

Element	As	Cd	Pb	Al	Fe	Sn	Co	Mn	Cu	Ni	Cr	Zn	Benzo (a) pyrene
Sampling station	μg kg^{-1}												
Sv. Vlas	1.17	0.028*	0.028	21.1	263	0.028*	0.028*	1.34	1.05	0.07	0.55	9.54	0.266
Kavarna	0.93	0.024*	0.066	61	525	0.024*	0.024*	1.64	0.83	0.19	0.32	10.2	0.169

* – below the detectable limit.

Table 4. Length-weight relationship (LWR) modelling results and TL-SL, BD-HL/SL, HL-SL/TL ratios and Fulton's condition factor, calculated for specimens taken at Sv. Vlas and Kavarna.

	LWR	
Sampling site:	**Sv. Vlas**	**Kavarna**
No of samples	***n = 33***	***n = 40***
LWR model: $W_{LWR}(i) = q * L(i)^b$	$W_{LWR}=0.029 * L^{2.62}$	$W_{LWR}=0.016 * L^{2.685}$
R^2 ***(α=0.05)***	R^2=0.90	R^2=0.92
Fulton condition factor: $K=W/L^3 * 100$	*K=1.11*(*std. dev ± 0.129*)	*K=1.08* (*std. dev ± 0.131*)
Ratios		
TL/SL ratio	*TL/SL ratio=1.21*(*std. dev ± 0.069*)	*TL/SL ratio=1.22*(*std. dev ± 0.048*)
BD-HL ratio	*BD/HL ratio=0.55*(*std. dev ± 0.09*)	*BD/HL ratio=0.61* (*std. dev ± 0.11*)
BD-SL ratio	*BD/SL ratio=0.16*(*std. dev ± 0.04*)	BD/SL ratio=0.18 (*std. dev ± 0.05*)
HL-SL ratio	*HL/SL ratio=0.29* (*std. dev ± 0.04*)	*HL/SL ratio=0.3* (*std. dev ± 0.04*)
HL-TL ratio	*HL/TL ratio=0.24*(*std. dev ± 0.03*)	*HL/TL ratio=0.25* (*std. dev ± 0.04*)

Molecular genetic analyses

The obtained mitochondrial DNA sequences were used to determine the number of different haplotypes. A total of 16 haplotypes for COI (626 bp) and 13 haplotypes for Cyt b (298 bp) were identified (Table 5). The majority of identified COI haplotypes originated from one prevalent set of haplotypes (Hap 3) following a single nucleotide substitution (Fig. 3A). Five haplotypes were unique for Sv. Vlas and three for Kavarna populations, while for Cyt b, the observed unique haplotypes were three in Sv. Vlas and four in Kavarna (Table 5, Fig. 3A, B).

The analyses of COI showed high values of haplotype diversity (Hd > 0.5) only in the population of Sv. Vlas (0.520) and lower diversity in the region of Kavarna (0.396), as well as low values of nucleotide diversity (π < 0.5%), varying from 0.00077 (Kavarna) to 0.00245 (Sv. Vlas) (Table 5).

Analyses of Cyt b showed high values of haplotype diversity (Hd > 0.5), ranging from 0.533 (Sv. Vlas) to 0.613 (Kavarna) and low values of nucleotide diversity (π < 0.5%), ranging from 0.00199 (Sv. Vlas) to 0.00245 (Kavarna) (Table 5).

Oxidative stress assessment

Assessing the level of OS biomarkers in individual *M. barbatus* fish can provide a direct indication of their condition. The biomarker values demonstrated significant differences between the liver and gills of *M. barbatus*, the two organs that actively respond to environmental stresses. There were differences in the levels

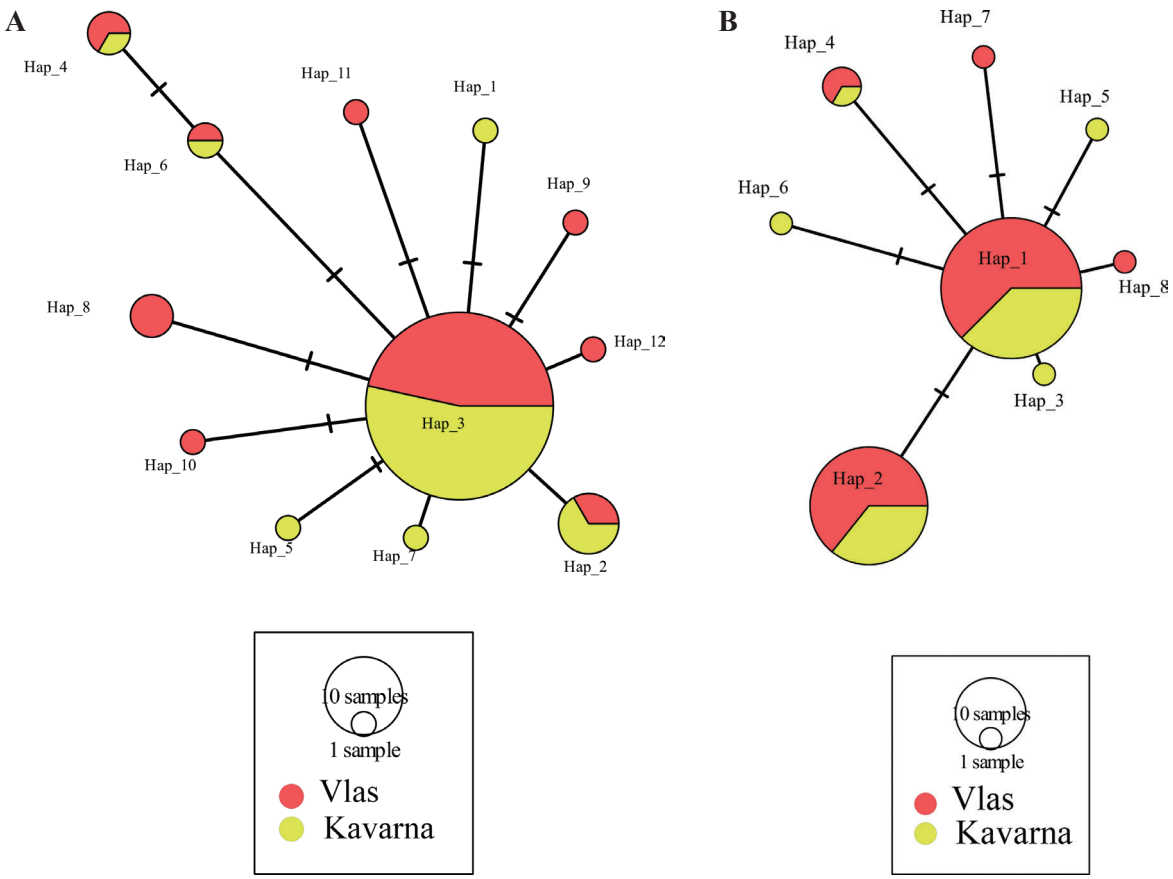

Figure 3. A haplotype network obtained from the TCS analysis, based on the distribution of COI haplotypes **B** haplotype network obtained from the TCS analysis, based on the distribution of Cyt b haplotypes*. *The size of the circles indicates the frequency of occurrence of each haplotype by the studied region. Small lines represent substitutions between haplotypes.

Table 5. Genetic diversity parameters of two sampling sites of *M. barbatus*, based on mtDNA sequence data.

	Sampling site	n	H	pHap	Hd	π	D	k	Fs
COI	**Sv. Vlas**	39	9	5	0.520 ± 0.096	0.00111 ± 0.00026	-1.81204*	0.696	-6.660
	Kavarna	40	7	3	0.396 ± 0.095	0.00077 ± 0.00022	-1.75657	0.482	-5.205
Cyt b	**Sv. Vlas**	40	6	3	0.55 6 ± 0.072	0.00212 ± 0.00036	-1.17640	0.63205	-2.732
	Kavarna	39	7	4	0.632 ± 0.048	0.00257 ± 0.00033	-1.23402	0.76653	-3.310

n – number of sequences; H – number of haplotypes; pHap – number of private haplotypes; H_d – haplotype diversity; PS – polymorphic sites; π – nucleotide diversity; D – Tajima's D value; *p < 0.05; Fs – Fu's Fs value.

of lipid peroxidation (LPO), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and acetylcholine esterase (AChE) in fish from both sampling sites, as well as superoxide dismutase (SOD) activity in fish from the Sv. Vlas sampling site (Table 6). Similar GSH concentrations were found in the liver and gills of fish from Kavarna and Sv. Vlas. In contrast, the LPO, CAT and GST activities in the liver and gills of fish from the Sv. Vlas sampling site were significantly greater than those of fish from the Kavarna sampling site (Table 6).

In general, the majority of fish samples from the Sv. Vlas sampling site had elevated OS, as indicated by the high levels of LPO in the liver and the low levels of GSH; nonetheless, they also had elevated CAT and GST activities in the liver and gills.

Table 6. Biomarkers of OS (mean ± SD) in liver and gills and weight and length of *M. barbatus* from two different sampling sites (Kavarna and Sv.Vlas) off the Bulgarian Black Sea coast.

Weight [g]	Length [cm]	organ	Biomarker							Mean SOS
			LPO (lipid peroxidation)	GSH glutathione	SOD (superoxide dismutase)	CAT (catalase)	GPx (glutathione peroxidase)	GST (glutathione-S-transferase)	AChE (acetylcholine esterase)	
			nmoles/mg prot	ng/mg prot	U/mg prot					
Kavarna										
19.84 ± 2.15*	11.84 ± 0.41	**Liver**	0.57 ± 0.05†,*	413.22 ± 34.41*	25.69 ± 1.55*	4.84 ± 0.58†,*	3.45 ± 0.25	76.50 ± 7.64†,*	73.96 ± 5.45†	-0.659
		Gills	13.26 ± 1.43†	434.11 ± 33.63*	23.92 ± 2.16*	1.87 ± 0.32†,*	8.33 ± 0.83*	39.01 ± 3.00†,*	277.96 ± 24.59†	
Sv. Vlas										
16.43 ± 0.96*	11.36 ± 0.19	**Liver**	0.91 ± 0.04†,*	245.03 ± 16.05*	17.94 ± 1.51*	9.37 ± 0.82†,*	3.30 ± 0.34†	268.75 ± 24.63†,*	82.69 ± 7.72†	-0.103
		Gills	8.81 ± 2.26†	203.91 ± 13.49*	13.21 ± 0.69*	4.26 ± 0.24†,*	12.86 ± 0.85†,*	65.30 ± 3.04†,*	284.10 ± 26.09†	

†- significance of difference ($p < 0.05$) between the liver and gills of the fish from the same region,
* – significance of difference ($p < 0.05$) between sampling sites.

In this study, the Specific Oxidative Stress (SOS) index was utilised for an integrated assessment of the cellular oxidative process balance. The results of the SOS measurements revealed that only a few *M. barbatus* specimens from Kavarna exhibited activation of pro-oxidative processes and activation of their antioxidant system (Fig. 4). In contrast, the majority of *M. barbatus* specimens from the Sv. Vlas sampling site exhibited a high level of oxidative stress and, in some cases, the antioxidant system was even suppressed (Fig. 4). This strongly suggested that the marine environment of the southern Black Sea region (Sv. Vlas sampling site) was ultimately more stressful for *M. barbatus* than the marine environment of the northern region (Kavarna sampling site). The mean values of the SOS index for the two sampling locations also reflected these results (Table 6).

In an effort to evaluate the potential effects of the OS on the body condition of *M. barbatus* individuals, we intended to measure the correlation between OS markers and Fulton's K or the length and weight of the fish. However, in this study, we utilised only *M. barbatus* specimens of equal length, indicating that they were likely of the same age (Table 6). This practically limited the analyses of the possible relationships of the OS markers and body condition indicators; consequently, it was relevant in this case to analyse only the relationships between the OS markers and fish body weight. The interdependence between individual fish weight and measured OS markers in fish from the two sampling sites was investigated using correlation analyses (Pearson's r). No significant correlations were observed between fish weight and OS markers in the liver and gills of *M. barbatus* individuals from the Kavarna sampling site. On the contrary, significant correlations were discovered between weight and LPO in red mullet from the Sv. Vlas sampling site ($r = -0.812$; $p < 0.05$) and CAT ($r = -0.786$; $p < 0.05$) for liver and between fish weight and LPO ($r = -0.763$; $p < 0.05$) and AChE ($r = -0.794$; $p < 0.05$) for gills.

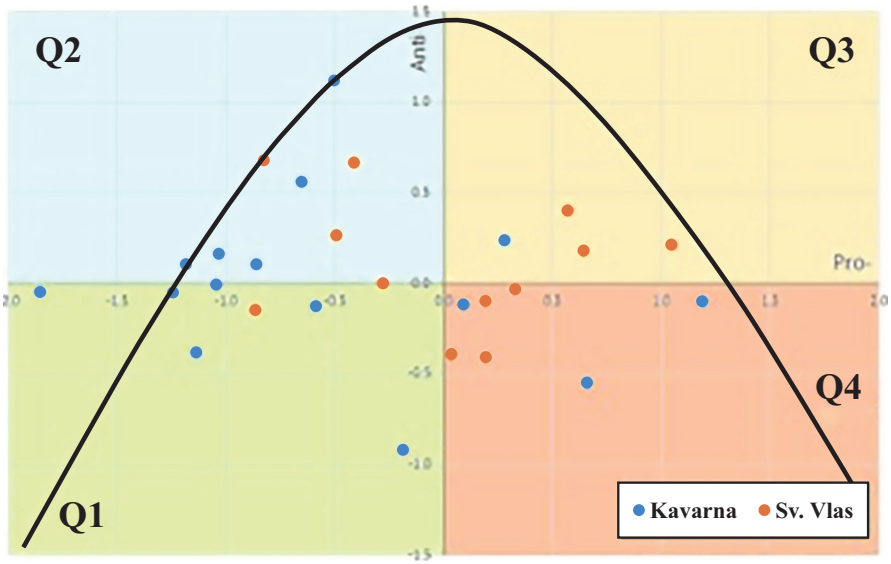

Figure 4. Distribution of the Specific Oxidative Stress (SOS) indices for *M. barbatus* specimens from the sampling sites Kavarna and Sv. Vlas in the space of the pro- and antioxidant indices axes (standardised data) divided into four quadrats (Q*) corresponding to the deviation of the balance of the pro- and antioxidant processes. * Q1 – low pro-oxidants, low antioxidants; Q2 – low pro-oxidants, high antioxidants; Q3 – high pro-oxidants, high antioxidants; Q4 – high pro-oxidants, suppressed antioxidants.

Analyses of microplastics in *M. barbatus* gastrointestinal tract

A total of 74 plastic particles were found in GIT of 26 specimens, 15 sampled at Sv. Vlas and 11 sampled at the Kavarna site (33% of all examined, 16 out of 44 females and 10 out of 35 males), pointing to the fact that 60.8% of MPs were found in female specimens. The average number of plastic particles per fish was estimated to 0.94 ± 1.81 particles individual^{-1} for all individuals and respectively 2.85 ± 2.15 particles individual^{-1} only for those who have ingested plastics. The number of particles documented varied from 0 to 6 per specimen in Sv. Vlas and 0 to 10 per specimen in Kavarna (Fig. 5). The types of plastics detected were predominantly fibres (75.7%) and fragments (24.3%) (Suppl. material 1: fig. S4). The most common plastic colour distinguished for fibres was grey (36.5%), followed by blue (29.7%) and for fragments green (21.6%) (Suppl. material 1: fig. S5).

The vast majority (98.4%) of the ingested particles were MPs in the size class < 5 mm. The small MPs size class (1 µm – 1mm) represented 73.4% of all plastics found, of which the share of the 1–500 µm size class was estimated to be 48.4% of the total number of small particles, with an average length of 255.7 µm and the 501–1000 µm size class was represented with a 25% share, with an estimated average length of 778.4 µm. Large microplastics (1–5 mm) accounted for 25% of all plastics reported, with an average length of 2034.2 µm. Mesoplastics (> 5 mm) were represented by only 1.6% of all plastics registered in the study. Particles with a length above 25 mm (macroplastics) were not detected.

A correlation between the total length and weight of the sampled fish, the weight of their GIT and the number of MPs was not established (verified by Spearman Rank Correlation, rho ~ 0.2).

Additionally, the results showed that the specimens sampled at Sv. Vlas had a higher number of ingested MPs (1.125 ± 1.842 particles individual^{-1}),

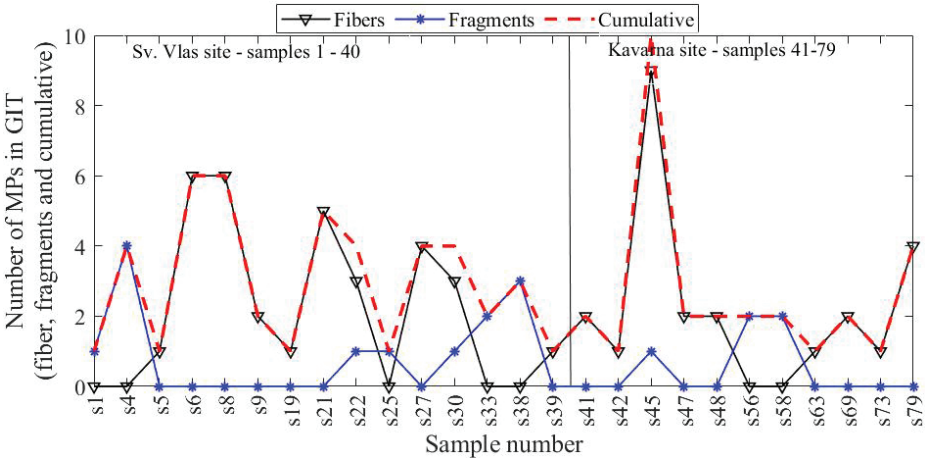

Figure 5. Number of microplastics found in GIT of *M. barbatus*, in the specimens sampled at Sv. Vlas and Kavarna sites.

compared to those sampled at Kavarna (0.743 ± 1.787 particles individual^{-1}), despite the fact that the LFD (Sv. Vlas TL_{mean} = 11.767 ± 0.798; Kavarna TL_{mean} = 12.377 ± 1.859) showed that only smaller size classes and age groups were represented in the Sv. Vlas sample.

Discussion and conclusions

Historically, physico-chemical analyses of environmental parameters formed the backbone of the risk assessment of pollution and alterations to the Black Sea ecosystem. Recently, multi-biomarker approaches have been developed (Beliaeff and Burgeot 2002; Yancheva et al. 2018) to assess the effects of exposure to contaminants and the responses of marine biota to environmental stress. Biochemical and physiological indicators are presently an essential tool for ecological research (Steinberg 2012). The general response of marine organisms to environmental pressures (abiotic, biotic and anthropogenic) is the activation of oxidative processes in their cells (Birnie-Gauvin et al. 2017) and the latter were also utilised in the assessment of the condition of the studied *M. barbatus* populations.

Depending on the spatial dispersal of the species, the findings of this study indicate that Black Sea marine environment pressures can have diverse and multidimensional effects on *M. barbatus* populations. The alterations and variations in the balance of oxidative processes in *M. barbatus* are a polymorphic response to the stressogenic effects of the environment. Specimens from the southern Bulgarian Black Sea coast had elevated OS levels, whereas those from the northern had low genetic diversity. Based on PI data and other stressors, such as the accumulation of heavy metals and MPs in biota, the southern region was evidently more polluted and its stressogenic effects from an OS perspective were more pronounced. In the northern region, fishing was clearly identified as a major threat and its effects on the genetic diversity of the *M. barbatus* population were more distinct.

Genetic diversity is one of the major determinants of the "health" and resilience of fish populations. Reduced genetic diversity may result in decreased population viability and small effective population size, despite the possibility of a high abundance or biomass and an increased probability of extinction (Hauser et al. 2002; Kenchington 2003; Martinez et al. 2018; Prunier et al.

2020). Our study revealed the absence of genetic structure amongst the analysed populations, indicating the existence of a single stock of the studied species. In addition, the low haplotype and nucleotide COI diversity of the *M. barbatus* population in the northern region (Kavarna) was correlated with the higher values of commercial catches reported in the northern Black Sea during the period 2018–2020 (Suppl. material 1: table S3). The effects of overfishing have been linked to a decline in genetic diversity, which may result in the loss of fish species' capacity to adapt to future environmental changes (Zhang et al. 2020; Petit-Marty et al. 2021). Moreover, fishing is typically a highly selective activity, so it has the potential to alter the population structure by favouring slow-growing, early-maturing specimens (Kenchington 2003). Practically, fishing pressure may have significant genetic effects on fish stocks without clearly pronounced contributions to the near-extinction of fish stocks. These findings indicate the need for further investigation of *M. barbatus* populations inhabiting Bulgarian Black Sea waters and implementation of conservation plans and measures.

The analysis of morphometric features revealed that specimens captured in the southern region had a lower allometric coefficient and specimens from both regions exhibited negative allometric growth. The condition of the fish specimens, as estimated by the Fulton condition coefficient (K), was comparable at both sites, although the mean value of K for the samples captured in the Sv. Vlas region was slightly higher than the value calculated for the samples captured at the Kavarna site. The larger size class range represented in the length frequency data (LFD) collected for the specimens sampled at the Kavarna site may account for this difference. The BD-HL ratio varied significantly between both sampling sites, whereas all other ratios exhibited very similar values. The absence of morphological variation between the sexes of *M. barbatus* was previously reported for Mullidae species (Mahe et al. 2014). Our data on OS biomarkers allowed us to study the interdependence only between individual fish weight and measured OS markers at the two sampling sites. In *M. barbatus* individuals from the Kavarna sampling site, no significant correlations were found between fish weight and OS markers both in the liver and in the gills. On the contrary, in the specimens sampled at Sv. Vlas, significant negative correlations were found between fish weight and LPO and CAT for liver and between fish weight and LPO and AChE for gills. Nevertheless, the observed BD-HL ratio variation between the specimens sampled in the two regions need further investigation to allow generalisation, as species occupying different substrate types are more likely to develop a diverse diet composition resulting in consequent morphological variation (Mahe et al. 2014). The latter may add valuable knowledge specifically in terms of the *M. barbatus* ability to adapt; nonetheless, given the outcomes of the genetic analyses, it is more likely that the observed differences are due to phenotypic plasticity than to genetic variation.

Our study provided the first data on the types and quantities of MPs ingested by *M. barbatus* in the Black Sea waters of Bulgaria. The particles found in the GIT of the sampled fish were more than twice as numerous as those found in the same species by Aytan et al. (2021) and Eryaşar et al. (2022) in the Turkish Black Sea waters and nearly comparable to Atamanalp's (Atamanalp et al. 2021) findings (Suppl. material 1: fig. S6). In all of the latter case studies, including our own, fibres were the most prevalent morphological form, followed by fragments (Eryaşar et al. 2022; our study) and microsheets/films (Aytan et

al. 2021). The MPs particles discovered in the GIT of *M. barbatus* in our study were more abundant in Sv.Vlas samples, which corresponded with the higher OS in fish specimens from this location. The possible effects of MPs bioaccumulation in marine biota are still being investigated. Nonetheless, some case studies have reported the induction of OS by MPs in the tissues of both invertebrate (Hu and Palić 2020; Costa et al. 2022) and vertebrate – primarily fish (Alomar et al. 2017; Espinosa et al. 2019; Kim et al. 2021). In this regard, our findings suggest that the higher number of accumulated MPs may have also contributed to the increased OS observed in *M. barbatus* specimens from Bulgaria's southern Black Sea coast (Sv. Vlas).

The toxic effects of various heavy metals and PAHs may be one of the numerous potential causes of OS induction in fish. Chromium concentrations were found to be higher in the tissues of Sv. Vlas specimens in our study. Particularly, trivalent and hexavalent chromium forms are involved in redox cycling (Stohs and Bagchi 1995). In a comparison of the effects of hexavalent and trivalent chromium ions on goldfish, it was discovered that both ions induce oxidative stress (Kubrak et al. 2010). The exposure of Chinook salmon *Oncorhynchus tshawytscha* (Farag et al. 2006) and *Channa punctatus* (Awasthi et al. 2018) to hexavalent chromium resulted in oxidative DNA damage and elevated LPO in the tissues; and that of *Labeo rohita* resulted in significantly elevated activity of SOD, CAT and GR in the liver, gills, muscles and brain (Kumari et al. 2014); that of *Oncorhynchus mykiss* led to increased brain SOD and GR (Li et al. 2011). As a conclusion, the specific effects of heavy metals accumulation in *M. barbatus* tissues in the Black Sea on their health status and physiological state need to be further studied.

Water temperature and salinity are proven to be basic abiotic factors that govern species' spatial distribution and developmental stages, having multiple effects on species' physiology and can consequently also affect OS induction in marine organisms. In our study, breakpoint analysis of mean annual surface sea temperature and salinity time series revealed shifts towards increase of the mean annual temperatures and salinity in the last decade in both of the studied regions. In general, the rise in seawater temperature can cause higher production of ROS in marine organisms. It has been recognised that higher water temperatures can trigger intracellular ROS production and metal-induced cell death to a greater extent (Park et al. 2020). A rise in the seawater temperature is also associated with elevated metabolic rates, higher oxygen consumption and respiratory chain flux and, thereby, increased ROS production (Halliwell and Gutteridge 2015). The OS response, on the other hand, is related to the optimal temperature for the species. It is at its lowest at the optimal temperatures and increases outside the upper and lower thermal optimum limits (Vinagre et al. 2014). Adaptation to temperature changes with a reduction in OS has also been previously reported (Vinagre et al. 2014). Thus, the anticipated increases in temperature due to climate change are very likely to induce higher OS and trigger adaptive mechanisms in marine biota. Moreover, as the various environmental stressors interact with one another, the strength of their effects on OS cannot be attributed to a single stressor (Min et al. 2014; Fadhlaoui and Couture 2016). Consequently, further research is needed to explain the relative proportion of species' synergetic responses to multiple stressors and their interactive effects that remain unaccounted for.

In conclusion, the present study demonstrated evidently that *M. barbatus* populations along the Bulgarian Black Sea coast are exposed to a variety of

stressors that differ by region and habitat's ecological conditions. Our study displayed that the species can tolerate environmental and anthropogenic variations to some extent; however, some of the studied specimens from the southern region exhibited high oxidative stress and suppressed antioxidant defence, while those from the northern region exhibited low genetic diversity. The latter can serve as an early indicator that the studied population may be approaching its adaptive capacity limits. In order to effectively monitor the ecological condition of the marine environment, it is recommended that research into the multiple stressor effects of the Black Sea environment on the biota must be intensified. This includes the development of new indicators and the determination of thresholds, also at the molecular and cellular levels.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

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Author contributions

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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Supplementary material 1

Supplementary data

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Data type: figures and tables (.docx file)

Explanation note: Schematics of measurements taken for biometric analysis on the body of *M. barbatus* (Mullidae species – Mahmoud et al., 2016). Breakpoint analyses (segmented regression models fit) of SST and in a) southern region in the area of Sv. Vlas – sampling site 24 and b) northern region in the area of Kavarna – sampling site 34 and SSs c) in the region of sampling site Sv. Vlas and d) in the region of sampling site Kavarna. Graphic representation of LWR models vs. measured weight-length values per sampling site (a) – Sv. Vlas, b) – Kavarna). First row: large fragments identified in the GIT of two female specimens sampled in Kavarna (first row, first image) and Sv. Vlas (first row, second image); Second row: fibers identified in the GIT of *M. barbatus*. Percentage share of fragments and fibers identified in *M. barbatus* GIT by color. Number of particles found in GIT of M. Barbatus per case study in the Black Sea region. Summary statistics of morphometric features measurements of sampled specimens per sampling site. Analysis of Similarities (ANOSIM) results of M. barbatus morphometric and meristic features in (by sex) and between the samples. EAFA Official statistics for M. barbatus landings reported in the period 2018-2020.

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