

Article

Microplastic Bioaccumulation and Oxidative Stress in Key Species of the Bulgarian Black Sea: Ecosystem Risk Early Warning

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Abstract

Plastic pollution in marine environments poses a new global threat. Microplastics (MPs) can bioaccumulate in marine organisms, leading to oxidative stress (OS). This study investigates MP accumulation and associated OS responses in six invertebrate species (Bivalvia, Gastropoda, and Malacostraca) and three key fish species of the Bulgarian Black Sea ecosystems. The target hydrobionts were collected from nine representative coastal habitats of the northern and southern aquatory. MPs were quantified microscopically, and OS biomarkers (lipid peroxidation, glutathione, and antioxidant enzymes) were analyzed spectrometrically in fish liver and gills and invertebrate soft tissues (STs). The specific OS (SOS) index was calculated as a composite indicator of the ecological impact, incl. MP effects. The results revealed species-specific MP bioaccumulation, with the highest concentrations in *Palaemon adspersus*, Rathke (1837) (0.99 ± 1.09 particles/g ST) and the least abundance in *Bittium reticulatum* (da Costa, 1778) (0.0033 ± 0.0025 particles/g ST). In *Sprattus sprattus* (Linnaeus, 1758), the highest accumulation of MPs was present (2.01 ± 2.56 particles/g muscle). The correlation analyses demonstrated a significant association between MP counts and catalase activity in all examined species. The SOS index varied among species, reflecting different stress responses, and this indicated that OS levels were linked to ecological conditions of the habitat and the species-specific antioxidant defense potential to overcome multiple stressors. These findings confirmed the importance of environmental conditions, including MP pollution and the evolutionarily developed capacity of marine organisms to tolerate and adapt to environmental stress. This study emphasizes the need for novel approaches in monitoring MPs and OS to better assess potential ecological risks.

Keywords: Black Sea; ecological risk; fish; marine invertebrates; microplastics; oxidative stress



Academic Editor: Tania Martellini

Received: 30 May 2025

Revised: 3 July 2025

Accepted: 24 July 2025

Published: 6 August 2025

Citation: Alexandrova, A.; Mihova, S.; Tsvetanova, E.; Andreeva, M.; Pramatarov, G.; Petrov, G.; Chipev, N.; Doncheva, V.; Stefanova, K.; Grandova, M.; et al. Microplastic Bioaccumulation and Oxidative Stress in Key Species of the Bulgarian Black Sea: Ecosystem Risk Early Warning. *Microplastics* **2025**, *4*, 50. <https://doi.org/10.3390/microplastics4030050>

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1. Introduction

The global production of synthetic polymers has been increasing rapidly, making plastic pollution one of the most critical environmental issues of the 21st century. In 2025, this output is estimated to reach approximately 450 million metric tons, an over 20-fold increase compared to six decades ago [1], and this is projected to rise to 590 million metric tons by 2050. Only a small amount of the plastic is recycled, and a large part is released into the environment as pollutants. Approximately 10% of annual production enters marine ecosystems [2]. Every hour, between 4 and 75 plastic objects enter the Black Sea only through river inflow [3] and account for 60–80% of marine litter [4–6].

Contamination of the marine environment with microplastics (MPs; plastics <5 mm) is now identified as an issue of global concern and documented extensively in seawater, marine sediments and also in marine biota [7,8]. The extent of MP pollution is notably high worldwide, and the Black Sea is no exception [9]. Studies on MPs in the Black Sea (including the Bulgarian part) have focused mainly on the sources of pollution [10–13] and their presence in sediments [14–20], seawater [15,18,21–26], and also in some species of invertebrates and vertebrates [27–35], including bivalves and fish caught along the Bulgarian coast [36–40].

Initially considered bioinert, MPs were thought to pass through the digestive systems of animals without being absorbed or causing harm. Marine organisms can be exposed to direct ingestion of MPs, indirect ingestion via consumption of contaminated prey, or through respiration. Regardless of the pathway, the intake of MPs can lead to adverse physical, chemical, and biological consequences [41–44]. Physical retention of MPs in digestive tracts [45], chemical leaching of plastic additives [46], and biofilm colonization [47] have been reported to cause toxic effects in hydrobionts. These impacts are often studied in controlled laboratory experiments using various endpoints such as growth rate [48,49], fecundity [50], and mortality [51]. An increasing body of evidence indicates that MPs can cross biological membranes, enter cells and tissues, and disrupt physiological functions [52–56]. The ability of MPs to penetrate cellular barriers largely depends not only on particle size but also on shape, surface properties, and the type of tissue or organism involved [57]. Smaller MPs can more easily pass through the inner layer of the intestinal epithelium into the circulatory system and translocate in tissues [58,59]. Larger particles may be internalized via phagocytosis (by immune cells) or endocytosis in some specialized cell types [60].

The negative effects of MPs at the cellular level include the induction of oxidative stress (OS) in exposed organisms [48]. Indeed, OS is a common biological response to various exogenous and endogenous factors. This condition is a result of an imbalance that favors pro-oxidant processes, during which reactive oxygen species (ROS) are generated in excess over the organism's antioxidant defenses. Severe oxidative stress can contribute to the development of various pathological conditions, as it has the potential to oxidatively modify proteins, lipids, and nucleic acids, ultimately leading to irreversible cellular damage and even cell death. In contrast, moderate and regulated levels of ROS may activate adaptive cellular signaling pathways [61].

Experimental studies on OS induced by MPs have been reported for various marine organisms, including both invertebrate species (representatives of Annelida, Arthropoda, Cnidaria, Mollusca, and Decapoda [40,62–64]) and vertebrates—predominantly fish [65–68]. These effects are associated with both MPs' direct action through the release of toxic monomers of plastics and the transfer of metals, persistent organic pollutants [56,59,69], and pathogenic microorganisms [70], which can be adsorbed onto their surface. These indicate that MPs can serve as vectors for other contaminants. As a general end-effect, directed laboratory studies have shown changes in the feeding, growth,

reproduction, and survival of different aquatic species after exposure to MPs [71,72]. In natural conditions, such effects pose significant ecological risks to marine organisms' health and ecosystems.

In the marine environment, establishing a causal relationship between MP exposure, accumulation, and their specific biological effects remains challenging due to the presence of multiple interacting environmental stressors [73,74]. Oxidative stress, as a common physiological response in organisms, may serve as a sensitive and reliable indicator for assessing the health, metabolic status, and physiological resilience of marine species. It reflects an integrated response to both internal and external environmental influences, among which MPs play a significant role. It is now widely recognized that OS-induced cellular alterations can propagate to affect higher levels of biological organization, which constitutes the essence of ecological risk—a concept fundamental to the framework of “stress ecology” [75–77].

Given their small size and variable buoyancy, MPs are readily available for uptake by a wide range of organisms across various trophic levels and feeding strategies [78]. As such, they are of particular concern as pollutants capable of affecting not only individual organisms but also entire populations and ecosystems [77]. Therefore, understanding the extent of MP bioaccumulation and the biological reactions of key hydrobionts is essential for improving/advancing our knowledge of their potential ecological impacts in marine environments [74,79–83].

This study aimed to assess MP accumulation in key species from representative habitats along the Bulgarian Black Sea coast and to evaluate the potential health impacts on these organisms by measuring OS levels. A set of reliable OS biomarkers was used to investigate the biological response to MP exposure and to analyze their correlation with MP burden. To further evaluate the ecological implications, a composite Specific Oxidative Stress (SOS) index will be used to provide insight into the potential ecological risk posed by MPs as part of multiple environmental stressors, as well as the organisms' adaptive capacity and stress tolerance.

The results and findings of this study are expected to assert the role of MP accumulation in compromising the health of marine biota and ecosystems. In this respect, this study can present novel indicators and approaches for monitoring and the development of methods to cope with global change issues.

2. Materials and Methods

2.1. Species and Sampling Areas

Specimens for this study were collected from characteristic habitats along the Bulgarian Black Sea coast, encompassing both the northern region (Greater Varna Bay) and the southern region (Greater Burgas Bay) (Figure 1).

The selected invertebrate and fish species represent key components of the local marine ecosystem. The invertebrates included *Mytilus galloprovincialis* Lamarck, 1819, *Rapana venosa* (Valenciennes, 1846), *Bittium reticulatum* (da Costa, 1778), and *Palaemon adspersus* Rathke, 1837. Fish species comprised *Mullus barbatus* Linnaeus, 1758, *Sprattus sprattus* (Linnaeus, 1758), and *Mesogobius batrachocephalus* (Pallas, 1814). Fish samples of *M. barbatus* and *S. sprattus* were obtained during pelagic trawl surveys for fish stock monitoring, and individuals of *M. batrachocephalus* were collected using cage traps. Invertebrates were gathered through scuba diving. All individuals were immediately shock-frozen to ensure optimal preservation [84] and transported to the laboratory in dry ice. Upon arrival, samples were stored at $-20\text{ }^{\circ}\text{C}$ for MP analysis and at $-80\text{ }^{\circ}\text{C}$ for biochemical assays.

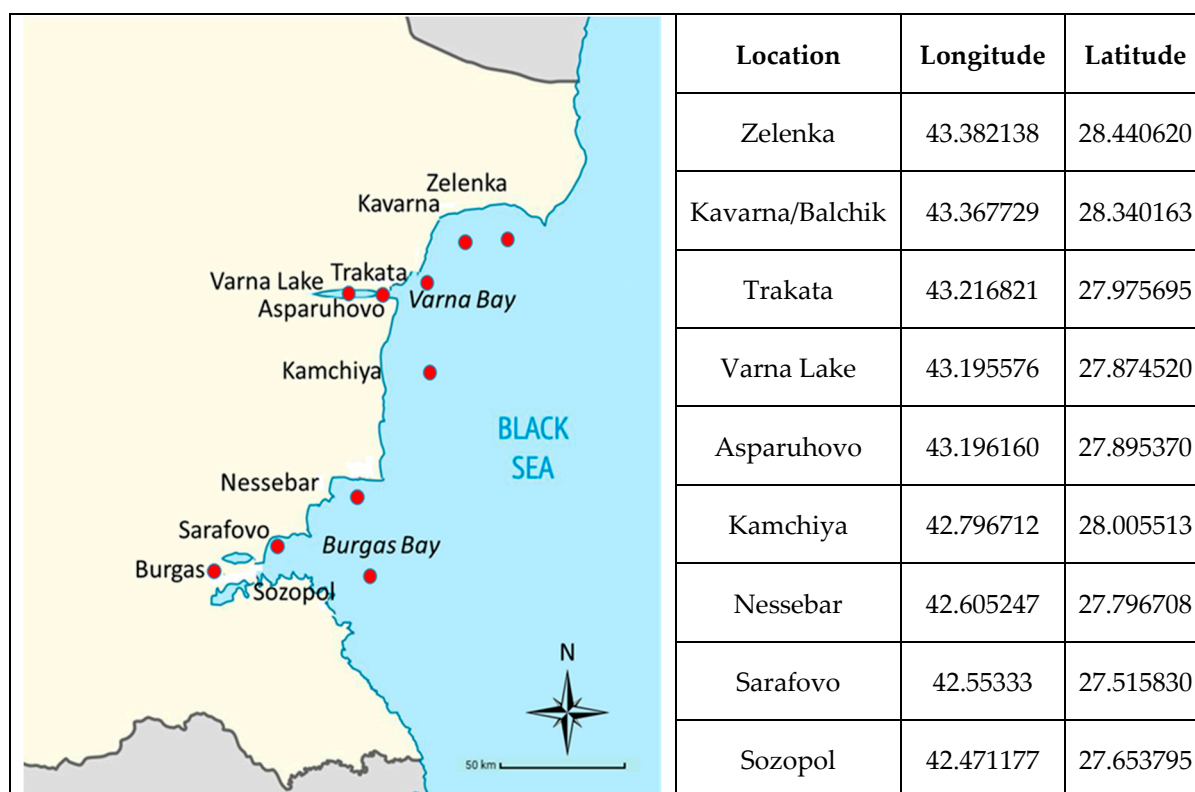


Figure 1. Sampling areas and sites.

2.2. Morphometry

On the day of analysis, the sampled individuals were thawed at room temperature, and the total length (L) and weight (W) were measured in centimeters and grams, respectively.

2.3. Determination of Microplastics in Species Samples

2.3.1. Quality Control

To minimize plastic contamination during laboratory procedures, all work surfaces were cleaned with ethanol (EtOH), and all liquids were pre-filtered. Glass and metal consumables were thoroughly rinsed with filtered water before use and covered with aluminum foil to prevent airborne contamination. To avoid cross-contamination between samples, all tools and glassware were rinsed three times with a 1:1 solution of filtered EtOH and deionized water (dH₂O) between each sample.

Procedural blanks for both air and liquid phases were included and accounted for in the results. All filters were pre-cleaned and examined under a microscope to ensure the absence of impurities before use. Laboratory personnel wore cotton clothing in distinguishable colors, nitrile gloves, and protective cuffs. Airborne contamination was further minimized using an Oberon-520 air purifier equipped with a HEPA filter.

2.3.2. Sample Processing

A total of 210 specimens were analyzed for MPs, including 75 *Mullus barbatus*, 15 *Mesogobius batrachocephalus*, 15 *Sprattus sprattus*, 30 *Mytilus galloprovincialis*, 15 *Rapana venosa*, 30 *Bittium reticulatum*, and 30 *Palaemon adspersus*.

Prior to tissue digestion, all individuals were thoroughly rinsed with a filtered 1:1 solution of EtOH and dH₂O to remove any externally adhering plastic [85]. For fish species, the gastrointestinal tracts (GITs) were carefully extracted by making an incision from the anus along the ventral side to the upper esophagus, avoiding damage to adjacent internal organs. A portion of the dorsal muscle tissue was also dissected using sterile scalpels and

scissors and placed in glass Petri dishes. The wet weights of both the GIT and muscle tissues were recorded.

In *P. adspersus*, the chitinous exoskeleton and head were carefully removed. For *M. galloprovincialis* and *R. venosa*, external shells were first scraped to remove any epibionts, after which the soft tissues were separated from the shells. The delicate shells of *B. reticulatum* were carefully cracked using curved metal tweezers, and soft tissues were extracted.

2.3.3. Tissue Digestion

The tissues were digested with filtered 10% potassium hydroxide (KOH) at 40 °C for 72 h in glass containers covered with metal foil [85–88]. Thereafter, the resulting samples were filtered on glass microfiber filters (FV24A0047) with a pore size of 2.7 µm and a diameter of 47 mm (FiltrateCH (SAS), Saint Jean de Braye, France) with a stainless steel Rocker MultiVac 300 vacuum system (Rocker Scientific Co., Ltd., New Taipei City, Taiwan (R.O.C.)) using a filtered solution of 1:1 EtOH:dH₂O. Before filtering, the *R. venosa* samples were additionally treated with 15–45 mL of 30% hydrogen peroxide (H₂O₂) for several minutes.

2.3.4. Visual Inspection of Microplastics

Using a ZEISS Stemi 508 stereomicroscope (Carl Zeiss Microscopy GmbH, Jena, Germany) with 435264-9200 (2.0×) objective and ZEISS ZEN core 3.6 software, which was protected with transparent polymethylmethacrylate plates, the MPs were determined by type, color, and size directly from the glass Petri dishes, which were opened only to check the structure of the detected particles.

2.4. Biochemical Analyses

2.4.1. Tissue Preparation

Ten individuals from each species with confirmed MP accumulation were selected for biochemical analyses. Prior to testing, specimens were thawed and carefully dissected. The soft tissues of invertebrates and fish gills and livers were extracted, and each tissue or organ was homogenized with a cold 100 mM potassium phosphate (K-PO₄) buffer with a pH of 7.4. The homogenates were centrifuged at 3000 rpm for 10 min at 4 °C to obtain a post-nuclear fraction in which lipid peroxidation (LPO) and glutathione concentration (GSH) were measured. A part of the post-nuclear fraction was recentrifuged at 12,000 rpm for 20 min at 4 °C to obtain a post-mitochondrial supernatant, and the antioxidant enzyme activities were assayed: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST).

2.4.2. Oxidative Stress Assay

Protein concentrations in post-nuclear and post-mitochondrial tissue fractions were determined using the Lowry method [89], with bovine serum albumin used as the standard. Oxidative stress (OS) biomarkers were quantified using commercially available assay kits from Sigma-Aldrich Co. LLC (Saint Louis, MO, USA), including the following: Lipid Peroxidation (MDA) Assay Kit (MAK085), Glutathione Assay Kit (CS0260), Superoxide Dismutase Determination Kit (19160), Catalase Assay Kit (CAT100), Glutathione Peroxidase Cellular Activity Assay Kit (CGP1), Glutathione Reductase Assay Kit (GRSA), and Glutathione-S-Transferase Assay Kit (CS0410).

To assess the overall oxidative status of the organisms, a specific oxidative stress (SOS) index was calculated, defined as the difference between the pro-oxidative (PrO) and antioxidant (AO) scores (SOS = PrO – AO), based on the measured OS markers [90]. The PrO score was computed as the mean Z-score of the lipid peroxidation marker

(PrO = mean(zLPO)), while the AO score represented the average Z-scores of the antioxidant biomarkers: GSH, SOD, CAT, GPX, GR, and GST (AO = mean(zGSH, zSOD, zCAT, zGPX, zGR, zGST)). SOS values close to zero indicate a balanced redox state.

2.5. Statistical Analysis

Statistical analyses were carried out following Mosaheb et al. [91]. The significance of differences in means between groups was determined using Student's t-statistic. The correlations between the studied variables were assessed using the Pearson r-statistic. The calculations were carried out using the STATISTICA 10 package (StatSoft Inc., Tulsa, OK, USA). For detecting the presence of significant thresholds of change in MP accumulation in species individuals, the Sequential Three-Step Analysis of Regime Shifts (STARS 6.3) was used [92–94].

3. Results

3.1. Morphological Characteristics of the Studied Species and Accumulated Microplastic Particles in Them

The indicator species selected for this study were both invertebrates (bivalves, gastropods, and crustaceans) and vertebrates (fish) that have different lifestyles and that are key species for different habitats. For each specimen, all MPs were counted irrespective of their size, shape, or color.

The studied invertebrates were significantly different in size and weight (Table 1). The analyses showed that the percentage of occurrence (%FO) of MPs in their soft tissues varied between 47% and 83% (Table 1). The lowest percentage was in *R. venosa*, where MPs were recorded in approximately every second individual (46.67%), and the highest percentage was in *B. reticulatum* (83.3%). The accumulated MPs in the soft tissues also differed significantly. The estimation of the number of MPs per gram of soft tissue (ST) showed that they were most abundant in *P. adspersus* (0.99 ± 1.09 particles/g ST) and least abundant in *B. reticulatum* (0.0033 ± 0.0025 particles/g ST) (Table 1).

Table 1. Morphological characteristics of the studied invertebrate species (mean $\pm$ SD) and accumulated microplastic particles in soft tissues.

Species	W	L	ST Weight	MPs		
	gr	cm	gr	%FO in ST	Particles/ST	Particles/g ST
<i>M. galloprovincialis</i> (n = 30)	13.62 ^{b,c,d} ± 3.30	6.04 ^c ± 0.42	4.04 ^{b,c,d} ± 0.95	63.33	1.13 ± 1.11	0.320 ± 0.31
<i>R. venosa</i> (n = 15)	37.87 ^{a,c,d} ± 6.31	5.78 ^c ± 0.35	11.87 ^{a,c} ± 2.33	46.67	0.60 ± 0.83	0.050 ± 0.07
<i>B. reticulatum</i> (n = 30)	0.051 ^{a,b,d} ± 0.010	1.09 ^{a,b,d} ± 0.098	0.01 ^{a,b} ± 0.03	83.33	0.30 ± 0.19	0.0033 ± 0.0025
<i>P. adspersus</i> (n = 30)	1.54 ^{a,b,c} ± 0.66	5.86 ^c ± 1.02	-	70.00	1.37 ± 1.27	0.990 ± 1.09

W = Total weight; L = total length; ST = soft tissues; FO = frequency of occurrence; significant difference at $p < 0.05$ in the following: ^a—*M. galloprovincialis*; ^b—*R. venosa*; ^c—*B. reticulatum*; ^d—*P. adspersus*.

There was no significant correlation present between the accumulated MPs and the individual length and weight of the invertebrate species.

The studied fish differed significantly in size and weight. The biggest individuals were those of *M. batrachocephalus*, and the smallest were the individuals of *S. sprattus* (Table 2). The frequency of occurrence (%FO) of MPs in the GIT was significantly higher in *M. batrachocephalus* (53.3%) compared to *S. sprattus* (33.4%) (Table 2). Conversely, the %FO of MPs in the muscle tissue of *M. batrachocephalus* (26.7%) was significantly lower

compared to *S. sprattus* (46.5%). The number of particles per gram of muscle in *S. sprattus* was significantly higher compared to *M. batrachiocephalus* and *M. barbatus* (Table 2).

Table 2. Morphological characteristics of the studied fish and accumulated microplastic particles in the gastrointestinal tract and muscle (mean $\pm$ SD).

Species	W	L	GIT Weight		MPs		
	gr	cm	gr	%FO in GIT	%FO in Muscle	Particles/ GIT	Particles/g Muscle
<i>M. batrachiocephalus</i> (n = 15)	90.5 ^{b,c} ± 24.37	21.32 ^{b,c} ± 1.83	7.57 ^{b,c} ± 4.18	53.3	26.7	0.80 ± 1.08	0.24 ^c ± 0.44
<i>M. barbatus</i> (n = 75)	21.64 ^{a,c} ± 6.68	12.55 ^a ± 1.11	1.21 ^{a,c} ± 0.34	46.7	33.4	1.39 ± 1.37	0.27 ^c ± 0.48
<i>S. sprattus</i> (n = 15)	4.19 ^{a,b} ± 0.80	9.12 ^a ± 0.47	0.17 ^{a,b} ± 0.07	33.4	46.5	0.40 ± 0.63	2.01 ^{a,b} ± 2.56

W = Total weight; L = total length; ST = soft tissues; FO = frequency of occurrence; significant difference at $p < 0.05$ in the following: ^a—*M. batrachiocephalus*; ^b—*M. barbatus*; ^c—*S. sprattus*

The only species where a significant correlation was present between the number of MPs/g of muscle and the fish size was *S. sprattus*.

Due to the high variances in the number of MPs, comparisons of the means by conventional statistics in our study were not applicable. For the detection of significant differences and thresholds of shifts in MP accumulation in species individuals, the Sequential Three-Step Analysis of Regime Shifts (STARS 6.3) was applied [92–94]. The results are graphically presented in Figure 2.

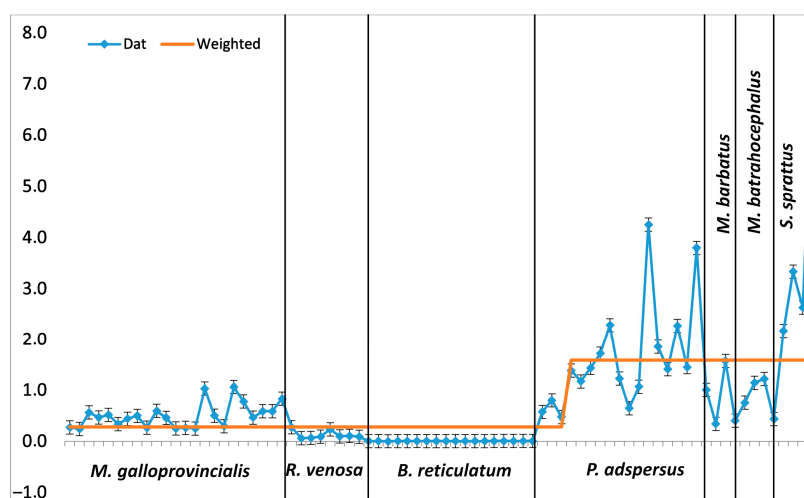


Figure 2. Thresholds of significant shifts in MP accumulation in the individuals of the studied species.

The figure clearly indicates the presence of significant differences in MP accumulation in the individuals of the studied species. In *P. adspersus* and fish species individuals, significantly higher MP accumulation was present, indicating differences in MP transfer.

3.2. Oxidative Stress Levels in the Studied Species and MP Effects

Under natural conditions, it is almost impossible to differentiate the specific ecological effects of MPs, and in this study, the stress-response effects obtained by measuring OS levels were used as the basic component of the stress ecology approach.

The measured values of the OS biomarkers in the soft tissue of the studied invertebrate species are shown in Table 3. The values of the OS markers varied significantly among the studied invertebrate species. In *P. adspersus*, against the background of the highest

content of MPs per gram of tissue, the highest LPO and the lowest values of antioxidants (non-enzymatic GSH and the enzymes SOD, CAT, GPx, and GR) and also low GST values were recorded, which indicate elevated OS levels. The lowest LPO and the highest values of GSH, SOD, CAT, GR, and GST were measured in *R. venosa*, which indicated lower OS levels, together with a relatively low accumulation of MPs per gram tissue (0.05 ± 0.06 particles/g).

Table 3. Values of oxidative stress biomarkers in the soft tissue of the studied marine invertebrate species (mean $\pm$ SD).

Species	Oxidative Stress Biomarkers						
	LPO	GSH	SOD	CAT	GPx	GR	GST
	nM/mg Protein	ng/mg Protein	U/mg Protein	U/mg Protein	U/mg Protein	U/mg Protein	U/mg Protein
<i>M. galloprovincialis</i> (n = 10)	3.69 ^{b,c,d} ± 0.33	93.39 ^c ± 9.75	21.72 ^{b,d} ± 1.05	0.63 ^{b,c,d} ± 0.07	8.92 ^c ± 0.43	12.78 ^{b,d} ± 1.23	13.86 ^{b,c,d} ± 2.00
<i>R. venosa</i> (n = 10)	0.37 ^{a,c,d} ± 0.04	118.5 ± 69.60	32.81 ^{a,c,d} ± 2.86	1.30 ^{a,c,d} ± 0.23	8.48 ^c ± 1.57	20.4 ^{a,c,d} ± 5.17	323.62 ^{a,c,d} ± 10.72
<i>B. reticulatum</i> (n = 10)	1.51 ^{a,b,d} ± 0.03	130.9 ^{a,d} ± 2.74	23.56 ^{b,d} ± 0.48	1.01 ^{a,d} ± 0.12	17.92 ^{a,b,d} ± 1.20	12.12 ^d ± 1.27	86.70 ^{a,b,d} ± 1.73
<i>P. adspersus</i> (n = 10)	6.32 ^{a,b,c} ± 1.13	84.24 ^c ± 4.69	5.31 ^{a,b,c} ± 0.55	0.12 ^{a,b,c} ± 0.01	8.03 ^c ± 0.99	1.48 ^{a,b,c} ± 0.15	34.65 ^{a,b,c} ± 4.00

Significant difference at $p < 0.05$ in the following: ^a—*M. galloprovincialis*; ^b—*R. venosa*; ^c—*B. reticulatum*; ^d—*P. adspersus*.

From the invertebrate species studied, the lowest number of MPs per gram tissue was recorded in *B. reticulatum* (Table 1). Here, a relatively low level of LPO, high concentration of GSH, and high activity of the antioxidant enzymes were also present. In the tissues of *M. galloprovincialis*, where average MP values were present (Table 1), relatively high LPO and low levels of GSH and antioxidant enzyme activities were found. In *M. galloprovincialis*, there were also statistically significant lower GST activities compared to the other invertebrate species studied (Table 3). High MP accumulation together with high OS levels (high LPO and the lowest GSH, SOD, CAT, GR, and GST activities) were present in *P. adspersus* compared to the other invertebrate species and also compared to the fish species (Tables 1 and 3). In *B. reticulatum*, low MP accumulation (Table 1) was present together with low OS levels (lowest LPO, highest GSH and GPx activities, and relatively high SOD, CAT, GR, and GST activities) compared to the other studied invertebrates (Table 3).

In the fish species, OS biomarkers were examined in the liver and gills, as these organs are most susceptible to stress, in which it is possible for a larger amount of MPs to accumulate (Table 4).

Table 4. Values of oxidative stress biomarkers in liver and gills of the studied fish species (mean $\pm$ SD).

Species	Oxidative Stress Biomarkers						
	LPO	GSH	SOD	CAT	GPx	GR	GST
	nM/mg Protein	ng/mg Protein	U/mg Protein	U/mg Protein	U/mg Protein	U/mg Protein	U/mg Protein
<i>liver</i>							
<i>M. batrachocephalus</i> (n = 10)	7.45 ^b ± 0.83	32.9 ^{b,c} ± 4.30	80.5 ^{b,c} ± 1.94	2.02 ^b ± 0.16	74.6 ^{b,c} ± 6.25	- -	225.9 ^{b,c} ± 11.78
<i>M. barbatus</i> (n = 10)	0.93 ^{a,c} ± 0.19	135.1 ^a ± 67.45	86.1 ^c ± 14.89	8.00 ^{a,c} ± 4.00	27.5 ^a ± 9.15	17.42 ± 1.25	476.8 ^{a,c} ± 16.65
<i>S. sprattus</i> (n = 10)	10.3 ^a ± 3.84	130.9 ^a ± 13.95	19.97 ^{a,b} ± 2.64	1.87 ^b ± 0.27	32.2 ^a ± 10.78	17.10 ± 2.62	850.4 ^{a,b} ± 10.05

Table 4. Cont.

Species	Oxidative Stress Biomarkers						
	LPO	GSH	SOD	CAT	GPx	GR	GST
	nM/mg Protein	ng/mg Protein	U/mg Protein	U/mg Protein	U/mg Protein	U/mg Protein	U/mg Protein
<i>gills</i>							
<i>M. batrachocephalus</i> (n = 10)	19.2 ±1.29	320.1 ^{b,c} ±33.50	19.3 ^c ±2.03	3.68 ^{b,c} ±0.58	24.8 ^{b,c} ±3.11	- -	121.8 ^{b,c} ±3.16
<i>M. barbatus</i> (n = 10)	11.3 ±6.62	160.5 ^a ±62.66	20.2 ^c ±3.90	0.50 ^a ±0.20	51.7 ^a ±23.4	23.7 ±5.2	51.8 ^{a,c} ±5.54
<i>S. sprattus</i> (n = 10)	20.5 ±0.47	141.7 ^a ±11.59	8.49 ^{a,b} ±1.25	0.55 ^a ±0.12	47.6 ^a ±6.81	13.7 ±1.81	86.2 ^{a,b} ±6.08

Significant difference at $p < 0.05$ in the following: ^a—*M. batrachocephalus*; ^b—*M. barbatus*; ^c—*S. sprattus*

In the liver of *M. barbatus*, the lowest LPO levels were measured (Table 4). This finding, together with the relatively high levels of antioxidant enzymes (including statistically significantly higher CAT activity) compared to the other fish species studied, suggested low levels of OS. In *M. batrachocephalus* and *S. sprattus*, LPO levels were high. In *M. batrachocephalus*, a low concentration of GSH was measured, and the lowest GST activity was observed. On the other hand, the GPx activity of these fish individuals was significantly higher than that of *M. barbatus* and *S. sprattus*, which suggests a possible activation of the recovery processes of GSH from GSSG under OS conditions. In *S. sprattus*, the lowest SOD activity was recorded compared to the other studied fish. In *S. sprattus*, GST activity was statistically higher compared to both *M. batrachocephalus* and *M. barbatus*, which indicates an increased level of detoxification processes (Table 4).

In the gills, there were no statistically significant differences in the LPO levels among the fish species (Table 4). High antioxidant defense was present in *M. batrachocephalus*, including significantly higher concentrations of GSH and activities of CAT and GST compared to those in *M. barbatus* and *S. sprattus*. Low levels of GSH, together with the lowest activity of SOD and GR, were measured in *S. sprattus* compared to the other fish species studied.

In order to identify the possible interactions of MPs and the measured OS parameters with potential ecological consequences, correlation analysis was applied. The results revealed the presence of some significant correlations between accumulated MPs and the values of the OS biomarkers in the invertebrate species (Table 5).

Table 5. Correlation coefficients of OS biomarkers and MP number in the invertebrate species (red—significance $p < 0.05$).

Species	Mean MPs/g ST	LPO	GSH	SOD	CAT	GPx	GR	GST
		Correlation Coefficients						
<i>M. galloprovincialis</i>	0.320	−0.514	0.132	0.288	0.621	0.272	−0.329	−0.448
<i>R. venosa</i>	0.050	0.592	−0.161	−0.533	0.072	0.456	0.147	0.358
<i>P. adspersus</i>	0.990	0.014	−0.177	0.531	0.748	0.067	−0.632	−0.357
<i>B. reticulatum</i>	0.0033	−0.646	0.177	−0.546	0.563	−0.544	0.546	0.273

The correlation coefficients showed that, in *M. galloprovincialis*, *P. adspersus*, and *B. reticulatum*, a statistically significant correlation between CAT and MPs was present. In *B. reticulatum* and *R. venosa*, LPO was significantly correlated with MP numbers. In *P. adspersus*, a significant correlation was also present between GR and MPs (Table 5).

In the fish species studied, correlations between OS markers and MPs in the liver and gills were measured, and significant correlations were present (Table 6).

Table 6. Correlation coefficients of OS biomarkers and MP number in the fish species liver and gills (red—significance $p < 0.05$).

Species	Mean MPs/g Muscle	LPO	GSH	SOD	CAT	GPx	GR	GST
		Correlation Coefficients (Liver)						
<i>M. barbatus</i>	0.24	0.751	−0.290	0.158	0.631	−0.481	−0.656	−0.253
<i>M. batrachocephalus</i>	0.27	0.679	−0.441	0.507	0.766	0.419	−0.506	−0.466
<i>S. sprattus</i>	2.01	0.501	0.325	0.424	−0.739	0.101	−0.113	−0.342
Species	Mean MPs/g Muscle	Correlation Coefficients (gills)						
		LPO	GSH	SOD	CAT	GPx	GR	GST
<i>M. barbatus</i>	0.24	0.559	−0.131	−0.484	0.631	−0.348	−0.528	0.126
<i>M. batrachocephalus</i>	0.27	0.528	−0.545	0.879	0.789	0.447	0.594	0.468
<i>S. sprattus</i>	2.01	−0.456	0.274	−0.186	−0.674	0.229	−0.023	−0.481

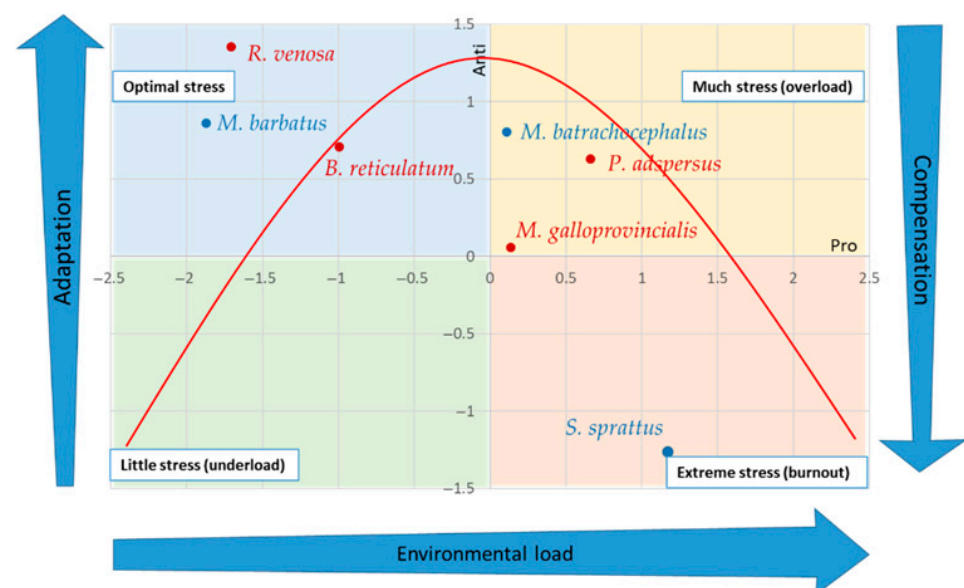
In the liver of all fish species studied, significant correlations were found between CAT and MPs (Table 6). In *M. barbatus* and *M. batrachocephalus*, significantly high correlation coefficients were measured for LPO and MP contents. In *M. barbatus*, the significant correlation of GR with the present MPs was also present.

In the gills of all three fish species studied, there was a significant correlation between CAT and the number of accumulated MPs (Table 6). From the other OS markers, only SOD had significant high correlations with the MPs present in *M. batrachocephalus*. In *M. barbatus*, the correlation coefficient of LPO with MPs was also relatively high, although it was not statistically significant.

To assess the effects of multiple environmental stressors (incl. MP accumulation) on the studied species, the composite indicator SOS index was calculated after data normalization to overcome the differences in the magnitude between the different biomarker values (Table 7). The results of the SOS analysis are visualized in Figure 3.

Table 7. Calculated values of the SOS index of the studied species.

	<i>M. batrachocephalus</i>	<i>M. barbatus</i>	<i>S. sprattus</i>	<i>M. galloprovincialis</i>	<i>R. venosa</i>	<i>B. reticulatum</i>	<i>P. adspersus</i>
ProO	0.109	−1.872	1.410	0.136	−1.709	−0.994	0.663
AO	0.804	0.863	−1.335	0.057	1.3541	0.708	0.631
SOS	0.694	−2.736	2.745	0.079	−3.063	−1.702	0.032

**Figure 3.** Distribution of the studied species in four quadrants of the normalization distribution of the specific oxidative stress (SOS) index.

Three of the studied species (*B. reticulatum*, *R. venosa*, and *M. barbatus*) were characterized by low prooxidant reactions (negative values for Pro), probably due to activated antioxidant defense (positive values for AO); therefore, the calculated SOS index is < 0 (Table 7; Figure 3). The two invertebrate species (*M. galloprovincialis* and *P. adspersus*) and the fish species *M. batrachocephalus* exhibited positive pro-oxidant reactions (positive Pro values), along with positive AO values (Figure 3). The only species showing a significantly elevated OS was *S. sprattus* (Table 7; Figure 3). Individuals from this species exhibited suppressed antioxidant responses (negative values for AO) and high pro-oxidant activity (positive values for Pro). Notably, the pronounced OS in *S. sprattus* (Table 4) coincided with the high levels of accumulated MPs.

4. Discussion

The results of the present study showed that MPs were present in all the species studied, and hence, they were present in the organisms' habitats. The high percentage of MPs (%FO) in the soft tissues of *B. reticulatum* (83.33%) and *P. aspersus* (70%) individuals can be related exclusively to the significant amount of plastics in their living environment. Both species inhabit shallow sandy areas in algal and eelgrass beds, and algae play a significant role in the retention of MPs and their removal from the water column [95–98]. MP retention is a result of their adhesion to algae or embedment/encrustation within the epibiont matrix of algae, thereby preventing MPs from migrating with water currents [98]. It was found that vegetated marine sites had much higher MPs than non-vegetated sites and that filamentous species contain more MPs due to their entanglement [99]. On the other hand, the significantly higher content of MPs in the tissues of *P. aspersus* compared to *B. reticulatum* was probably due also to species-specific factors, including size, food preferences, metabolism, etc. Furthermore, *P. aspersus* are omnivorous invertebrates and, as such, they consume organisms that are likely to be highly loaded with MPs in their kelp habitat. Although *B. reticulatum* also inhabits kelp habitats, it is significantly smaller and thus consumes a much smaller amount of food, and it is also a microalgae herbivore unlike *P. aspersus* [100].

Concerning fish species, the relatively higher percentage of occurrence of MPs in the GIT of *M. batrachocephalus* and *M. barbatus* (about 50%) compared to *S. sprattus* was probably due to their different lifestyles. The accumulation of MPs was reported to be higher in demersal fish than in pelagic fish species [101]. Both *M. batrachocephalus* and *M. barbatus* are demersal and less mobile fish, while *S. sprattus* is a pelagic–neritic species [95]. In the present study, a significant increase in the ingested (in GIT) number of MPs related to fish lifestyle and size was found, and the accumulation of MPs was higher in demersal fish than in pelagic fish species [102]. Most likely, their feeding habits lead to the difference in MP bioaccumulation. Demersal fish are more stationary than pelagic fish and feed mostly on the prey species present on the sea floor. Specifically, *M. batrachocephalus* feeds predominantly on fish [103], *M. barbatus* feeds on small benthic crustaceans, worms, and mollusks [104], and *S. sprattus* feeds on planktonic crustaceans. Furthermore, a study reported that the mean abundance of MPs in herbivorous fish species was almost two times lower than the abundance of MPs in carnivores and omnivores [105], and Rasta et al. [106] detected more MP particles in omnivorous than in carnivorous fish. Additionally, the established value in this study, which is almost 10 times more MPs per g of muscle in *S. sprattus* compared to the demersal fishes (*M. batrachocephalus* and *M. barbatus*), may depend at least to some extent on the type of muscle. Demersal fish typically have white muscle tissue, whereas pelagic species have a great proportion of red muscle fibers, related to the demands of sustained swimming. Due to its rich vascularization and higher metabolic activity, red muscle tissue may have a higher likelihood of MPs being transported via the

bloodstream. This can explain the observed significant differences in MP accumulation in *S. sprattus*. Significant differences in MP bioaccumulation were generally found between fish organs, being higher in the gills and the liver [107,108]. Gills are major hotspots for MP accumulation compared to other organs since they are used to filter water for respiration, and MPs can be trapped there [108].

Recently, efforts to determine the ecological impacts of MP pollutants have increased because of the rising plastic contamination in the marine environment. Due to the presence of multiple pollutants in the marine environment, it is almost impossible to assess the specific effects of every separate pollutant, including MPs, on organism health [73,74]. Recent data show that MPs can contribute to increasing OS in marine organisms by generating excess reactive oxygen species (ROS) directly produced by the plastic particles themselves [64,109,110]. MPs can release toxic chemicals such as phthalates, bisphenol A, and polycyclic aromatic hydrocarbons, which can induce ROS production [111]. Another mechanism is through the adsorbed pollutants onto the MPs' surface, acting as a sink for metal elements, organic compounds, and pesticides [109,112–114]. All of these pollutants can lead to ROS formation through various chemical reactions. Adsorption of transition metals such as iron or copper could produce hydroxyl radicals via the Fenton reaction [109,113]. The entirety of OS damage may not solely represent structural damage to the cells, but it may, in fact, alter metabolic functions irrespective of the pathways of intake. Bioaccumulation of MPs in marine organisms can result in OS and adverse impacts on organism health [41–43,73,74]. Herein, significant correlations were established between MP accumulation and OS biomarkers. The ultimate goal for the use of biomarkers is to have a predictive tool that may give a mechanistic overview of the impact on a given organism. More importantly, different OS biomarkers may provide insight about the potential impacts on an organism's health and fitness, as well as the cascading effects on the sustainability of populations, communities, and ecosystems.

In this study, quite different ratios of OS parameters (Tables 3 and 4), along with different MP accumulation in the studied species (Tables 1 and 2), were present. In *P. adspersus*, high MP accumulation was found together with high OS levels. In *B. reticulatum*, low MP accumulation was present, and OS was low compared to the other studied invertebrates. In the studied fish species, the ratios of OS parameters (means in liver and gills) and MP accumulation showed differences, but the highest OS in *S. sprattus* was accompanied by the highest MP accumulation. Here, GST activity was also high, indicating an increased level of detoxification processes, as it is a major phase II detoxification enzyme, detoxifying many endogenous compounds and breaking down xenobiotic substrates through conjugation of reduced glutathione with various substrates [115].

The OS and response processes are also specific for different organs and have different effects. The gills and liver are most susceptible to OS. The liver is a major metabolic and detoxifying organ of the body, and during these processes, ROS can be generated as byproducts [116]. The liver also plays a crucial role in immune responses by activating immune cells upon encountering pathogens or toxins [117]. This activation can trigger inflammation and the subsequent excess production of ROS. Disruption of the redox balance causes OS and contributes to cellular damage, which affects liver function modulation. The results obtained proved that, in the gills of the studied fish species, a correlation of MPs with closely associated OS variables was present. The gills, being in direct contact with the aquatic environment, serve as a primary target for pollutants. They are richly vascularized and possess a large surface area, facilitating gas exchange and filtration processes [118]. Research on contamination in marine fishes found that MPs were present in the gills, dorsal muscle, and gastrointestinal tract. MP-contaminated fish had increased LPO levels in the brain, dorsal muscle, and gills [119].

The correlation analyses performed (Tables 5 and 6) confirmed the presence of relations between MP content and the measured OS indicators. In most of the studied species, a closer relation between MP accumulation and CAT activity was observed. Thus, our findings support the suggestion that CAT can be used as a model enzyme to assess the biological effects of MPs [120]. Catalase is an essential enzyme that specifically decomposes H_2O_2 , preventing the formation of the most damaging agent—the hydroxyl radicals. A study of the effects of polyvinyl chloride MPs on hepatic antioxidant enzymes showed a time-dependent decrease in the activities of SOD, GPx, and CAT and an increase in LPO levels [121]. The results also suggested that MPs directly interact with CAT. Therefore, MPs themselves or through toxic substances or pathogens carried on their surface are really significant pro-oxidants and, upon prolonged exposure, can lead to depletion or inhibition of antioxidants in organisms [120,121].

Recent review articles and reports have identified current research gaps [122,123] concerning indicators and assessment of ecological risks related to MPs. The specific ecological risks of plastic contamination can be defined as the likelihood of adverse health effects on different marine organisms and their consequences on populations, communities, and ecosystems in the sense of stress ecology. The changes associated with OS at the cellular level can subsequently affect higher hierarchical levels, which is defined as “stress ecology” [75–77]. In the present research, we used the previously introduced SOS index as a general composite risk indicator to measure the complex effects of multiple environmental stressors, including predominantly MPs, on the studied species (Table 7; Figure 3). The species *B. reticulatum*, *R. venosa*, and *M. barbatus* showed low levels of OS. Their Pro and AO index values showed that the environmental pressure by multiple factors (incl. MPs) was controlled successfully by the antioxidants, and the stress could be defined as optimal. In other species (*M. galloprovincialis*, *P. adspersus*, and *M. batrachcephalus*), although antioxidant defense was still active, it was not sufficient to prevent OS development. Therefore, pro-oxidant activity dominated, resulting in an SOS index greater than zero (positive). However, in these cases, the SOS values were close to zero, suggesting moderate OS levels. A really high OS was present only in the studied *S. sprattus*. Likely, the intense environmental pressure led to depletion of non-enzymatic and inhibition of enzymatic antioxidants, i.e., the anti-stress system could not effectively compensate for the oxidative challenge. In this species, the highest MP accumulation was also present. Hence, the SOS index proved that it can serve as an integral indicator of the OS effects on organism health and also indicate ecological risk for the higher levels of biological organization. However, the SOS index cannot differentiate the specific factor/s causing OS changes, which also applies to MP accumulation. The concrete effects of different MPs can be studied in controlled laboratory experiments.

5. Conclusions

Much remains unknown about the consequences of MP pollution, and further in-depth studies are essential to accurately assess its ecological and human health risks. The specific oxidative stress (SOS) index demonstrated that OS levels are influenced by both habitat conditions and species-specific antioxidant responses. The findings of this study underscore the critical role of the marine environment, including MP contamination, in triggering oxidative stress, as well as the importance of the evolutionarily developed physiological resilience and adaptive capacity of marine organisms to environmental stressors. These results emphasize the urgent need for targeted monitoring of MPs and their accumulation in marine organisms to better evaluate ecological risks. Additional research is strongly recommended to elucidate the broader impacts of MPs at the ecosystem level.

Author Contributions: Conceptualization, N.C. and A.A.; methodology, A.A. and S.M.; software, N.C.; formal analysis, A.A., S.M., E.T., M.A., G.P. (Georgi Pramatarov), G.P. (Georgi Petrov), V.D., K.S., M.G., H.S., E.H., D.D. and V.R.; investigation, A.A., S.M., E.T., M.A., G.P. (Georgi Pramatarov) and G.P. (Georgi Petrov); resources, V.R., D.D., P.I. and A.A.; data curation, N.C.; writing—original draft preparation, N.C. and A.A.; writing—review and editing, N.C., A.A., D.D. and V.R.; visualization, A.A.; supervision, N.C. and A.A.; project administration, P.I. and A.A.; funding acquisition, P.I., N.C. and A.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Science Fund, Sofia, Bulgaria: grant № KII-06-H61/10.

Institutional Review Board Statement: No laboratory experimental animals were involved in this study and approval from Local Ethics Committee was not required. Species sampling was conducted under the authorization of the Executive Agency for Fisheries and Aquaculture, Ministry of Agriculture and Food, Bulgaria, following the permit for capture of fish and other aquatic organisms for scientific research purposes No. 32/21.06.2023 and No. 36/24.07.2024 under contract No. D-156 dated 13 March 2023.

Informed Consent Statement: Not applicable.

Data Availability Statement: Researchers wishing to access the data used in this study can make a request to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

AO	Antioxidant score;
CAT	Catalase;
FO	Frequency of occurrence;
GIT	Gastrointestinal tract;
GPx	Glutathione peroxidase;
GR	Glutathione reductase;
GSH	Glutathione;
GST	Glutathione-S-transferase;
L	Total length;
LPO	Lipid peroxidation;
MPs	Microplastics;
OS	Oxidative stress;
PrO	Pro-oxidative score;
SOD	Superoxide dismutase;
SOS	Specific oxidative stress index;
ST	Soft tissues;
W	Total weight.

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