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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 MPs accumulation and associated OS responses in six invertebrate species (Bivalvia, Gastropoda, Malacostraca) and three key fish species of the Bulgarian Black Sea ecosystems. Hydrobionts were collected from representative coastal habitats, MPs were quantified microscopically, and OS biomarkers (lipid peroxidation, glutathione, antioxidant enzymes) were analyzed spectrometrically in fish liver and gills, and invertebrate soft tissues. Results revealed species-specific MPs bioaccumulation, with *Palaemon adspersus*, Rathke (1837) and *Sprattus sprattus* (Linnaeus, 1758) having the highest concentrations per gram soft tissue and muscle, respectively. The alien Pacific oyster exhibited higher MPs loads than the native species. OS biomarkers varied markedly among species, reflecting different stress responses. Cluster analyses indicated that MPs were mainly grouped with catalase activity in all species. The specific OS (SOS) index showed that OS levels were linked to both habitat conditions and species-specific antioxidant defense. These findings confirmed the importance of environmental conditions, including MPs pollution, and evolutionarily developed adaptation capacity of organisms for tolerance and resilience to induced stress. The study emphasizes the need for monitoring MPs and OS to better assess the ecological risk.

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

1. Introduction

Plastic products are being produced at ever-increasing rates, making plastic pollution one of the most important contemporary environmental issues. Plastic is a very practical material that is long-lasting, resistant to degradation, inert, and easy to shape, with very low production costs. The annual global production of plastic materials is about 450 million tons in 2025, increasing more than 20-fold in the last 60 years [1]. The plastic production is expected to continue rising to approximately 590 million metric tons by 2050. Only a small amount of the plastics is recycled, and a large part is released into the environment as pollutants. About 10% of the plastic produced each year ends up in the oceans and seas [2]. A study reports that between 4 and 75 plastic objects enter the Black Sea every hour 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) has been identified as an issue of global concern [7] and documented extensively in seawater, marine sediments, and marine biota [8]. The pollution of the marine environment with MPs is particularly high worldwide, and the Black Sea is no exception [9]. Studies on MPs in the Black Sea (including the Bulgarian part) have focused on the sources of pollution [10–13], their presence in sediments [14–20] and seawater [15,18,21–26], as well as in different species of invertebrates and vertebrates [27–35], including bivalves and fish caught along the Bulgarian coast [36–40].

Initially, it was believed that MPs are bioinert, passing through the digestive system of animals without being absorbed and without affecting the organisms. Marine organisms can be exposed to direct ingestion of MPs or indirect ingestion via prey items, or also through respiration. Irrespective of the pathway, intake of MPs by marine organisms can result in adverse physical and chemical impacts [41–43].

Currently, there is increasing evidence that MPs can overcome biological membranes and, entering cells and tissues, can have various negative influences on the organism [44–48]. Physical retention of MPs in digestive tracts [49] and chemical leaching of plastic additives into tissues [50] have been reported to have toxic effects. These impacts are often investigated during controlled laboratory exposures using a variety of endpoints such as growth rate [51,52], fecundity [53], and mortality [54].

In wild-caught organisms, however, causality between MPs exposure pathways, accumulation, and observed effects is often difficult to ascertain due to the multitude of stressors present in the marine environment [55,56]. The ecological risks of plastic contamination can be defined as the likelihood of adverse health effects and their ecological consequences occurring as a result of the organisms' exposure to MPs [57,58]. The small size and variable buoyancy make MPs readily available for uptake by a wide range of organisms across different trophic levels and feeding strategies [59] and thus of particular concern as a pollutant affecting not only organisms but also ecosystems. Indeed, the uptake of MPs has been increasingly confirmed in wild populations of marine organisms across all trophic levels [4]. Hence, understanding endpoints such as MPs bioaccumulation and biomagnification can assist in improving our knowledge of the potential ecological effects associated with exposure pathways in the marine environment [56,60–63].

The mechanism of MPs' damage may be at least partly due to the induction of oxidative stress (OS) in organisms after their exposure [48]. Consequently, OS has the potential to be a reliable tool to assess the health and metabolic status of marine organisms, being an expression of the unified response of organisms to the multiple effects of the endo- and exogenous factors of their living environment. Nowadays, it is broadly accepted that OS-induced changes at the cellular level can subsequently impact higher hierarchical levels of biological organization - a phenomenon that underpins the theoretical framework of "stress ecology" [64,65].

Experimental studies on OS induced by MPs have been reported for various marine organisms, both invertebrate species (representatives of Annelida, Arthropoda, Cnidaria, Mollusca, Decapoda [40,66–68] and vertebrates – predominantly fish [69–72]. These effects are associated both with MPs direct action by the release of toxic monomers of plastics, but also with the transfer of metals, persistent organic pollutants [48,73,74], and pathogenic microorganisms [75], which can be adsorbed on their surface. As a general end-effect, laboratory studies have shown changes in feeding, growth, reproduction, and survival of aquatic species after exposure to MPs [76,77]. However, limited published evidence still exists for MPs' trophic transfer, biomagnification, and associated additives in marine food webs and the effects on the health of organisms and ecosystems.

The present study aimed to assess MPs transfer, accumulation, and effects in key species from Bulgarian Black Sea habitats and associated OS responses, with their adaptation capacity for tolerance and resilience to induced stress.

2. Materials and Methods

2.1. Species and Sampling Areas

The species for the study were collected from characteristic habitats of the Bulgarian Black Sea coastal areas, both in the northern (larger Varna Bay) and southern (larger Burgas Bay) regions (Figure 1). The invertebrates and fish species selected for this study are key ecosystem components. The invertebrates were *Mytilus galloprovincialis* Lamarck, 1819; *Rapana venosa* (Valenciennes, 1846), *Bittium reticulatum* (da Costa, 1778); *Palaemon adspersus* Rathke, 1837. The Pacific oyster, *Magallana gigas* (Thunberg, 1793), is a recent intruder in the Black Sea, and individuals of the species were also collected for comparative purposes. The fish samples of *Mullus barbatus* Linnaeus, 1758 and *Sprattus sprattus* (Linnaeus, 1758) were collected during the monitoring pelagic trawl survey of fish stocks, *Mesogobius batrachocephalus* (Pallas, 1814) individuals were collected using a fishing net cage trap, and the invertebrate species were collected using the diving method.

All sampled individuals from the studied species were shock frozen for best preservation [78] and transported in dry ice to the laboratory. In the laboratory, they were stored at -20°C until the microplastic analyses and at -80°C until the biochemical analyses were performed.
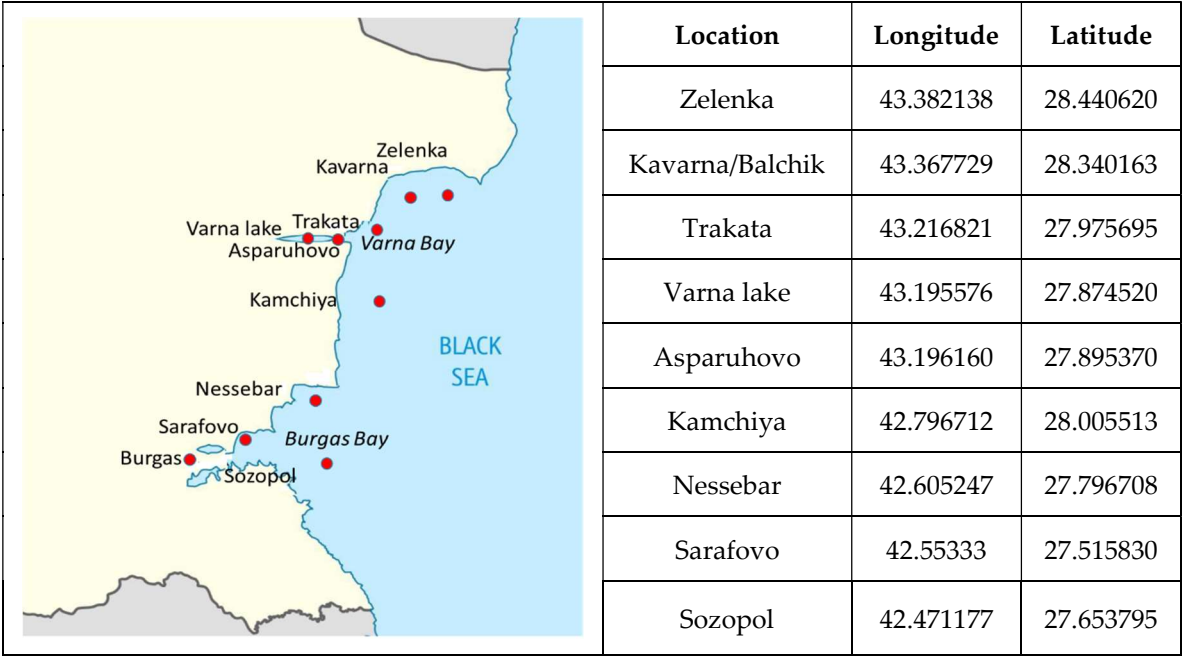

Location	Longitude	Latitude
Zelenka	43.382138	28.440620
Kavarna/Balchik	43.367729	28.340163
Trakata	43.216821	27.975695
Varna lake	43.195576	27.874520
Asparuhovo	43.196160	27.895370
Kamchiya	42.796712	28.005513
Nessebar	42.605247	27.796708
Sarafovo	42.55333	27.515830
Sozopol	42.471177	27.653795

Figure 1. Sampling areas.

In the analysis of MPs bioaccumulation, available data [38] from our data set for the characteristic species, lagoon cockle (*Cerastoderma glaucum* (Bruguière, 1789)) and soft-shell clam (*Mya arenaria* Linnaeus, 1758) from Varna Lake were also included.

2.2. Morphometry

On the day of analysis, the sampled individuals were thawed at room temperature, and the total length (L) and weight (W) of all sampled individuals were measured in centimeters and grams, respectively. In the fish, Fulton's condition factor (K) was computed according to the formula: $K = 100*W/L^3$, where W is the total body wet weight in grams and L is the total length in cm; the factor 100 is used to bring K close to a value of one [79].

2.3. Determination of Microplastics in Species Samples

To prevent plastic contamination in the laboratory, all surfaces were cleaned with ethanol (EtOH); all liquids were filtered; glass and metal consumables were rinsed thoroughly with filtered water before use and covered with metal foil. Procedural controls for liquids and air were performed and considered in the results of the samples. The filters are inspected and pre-cleaned under a

microscope to remove any impurities before analysis. Cotton laboratory clothes with distinguishable colors, nitrile gloves, and cuffs were used. The air in the laboratory was pre-cleansed to minimize self-contamination with an Oberon–520 air purifier and a HEPA filter.

In fish, the number of plastic particles in the gastrointestinal tract (GIT) and muscle tissue was counted and used as an indicator for MPs bioaccumulation and also for possible further transfer to humans. Before tissue digestion, the individuals were rinsed well with a filtered solution of 1:1 EtOH:dH$_2$O, the fish GIT and muscle tissues were carefully separated and the weight was recorded. The chitinous shell and head of the *P. adspersus* individuals were carefully cut. The surface of the *M. galloprovincialis* and *R. venosa* shells was first scraped to clean them of overgrown/attached species, and the soft tissues were carefully separated from the shells. The *B. reticulatum* shells were carefully broken with curved metal tweezers, and the soft tissue delicately separated.

The tissues were rinsed well with a filtered solution of 1:1 EtOH:dH$_2$O and then digested with filtered 10% potassium hydroxide at 40°C for 72 h in glass containers covered with metal foil. Thereafter, the resulting samples were filtered on glass microfiber filters with a pore size of 2.7 μm and a diameter of 47 mm (Filtratech, FV24A0047) with a stainless steel Rocker MultiVac 300 vacuum system, using a filtered solution of 1:1 EtOH:dH$_2$O. Before filtering, the *R. venosa* samples were additionally treated with 15-45 ml of 30% hydrogen peroxide (H$_2$O$_2$) for several minutes.

On a ZEISS Stemi 508 stereomicroscope, 435264-9200 (2.0x) objective with ZEISS ZEN core 3.6 software, protected with transparent polymethylmethacrylate plates, the microplastics were determined by type, color, and size directly from the glass Petri dishes, which are opened only to check the structure of the detected particles.

2.4. Tissue Preparation for Biochemical Analyses

Ten specimens of each species were used for the biochemical analyses. Before the analyses, the studied specimens were thawed and dissected. The soft tissues of invertebrates and fish gills and livers were extracted, and each tissue or organ was homogenized with cold 100 mM potassium phosphate (K-PO$_4$) buffer, pH 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 re-centrifuged at 12 000 rpm for 20 min at 4°C to obtain a post-mitochondrial supernatant in which were assayed the antioxidant enzyme activities: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), as well as acetylcholinesterase (AChE).

2.5. Biochemical Analyses

The protein concentrations of the tissue post-nuclear and post-mitochondrial fractions were measured according to Lowry et al. [80] using bovine serum albumin for generating the standard curve. The OS parameters were measured using kits, purchased from Sigma-Aldrich Co. LLC (USA): Lipid Peroxidation (MDA) Assay Kit MAK085, Glutathione Assay Kit CS0260, Superoxide dismutase Determination Kit 19160, Catalase Assay Kit CAT100, Glutathione Peroxidase Cellular Activity Assay CGP1, Glutathione Reductase Assay Kit GRSA, and Glutathione-S-Transferase Assay Kit, CS0410.

The AChE activity was measured using the method described by Ellman et al. [81], which is based on the production of thiocholine. The reaction mixture consisted of 100 mM K-PO$_4$ buffer at pH 8.0, 0.045 M acetylthiocholine iodide, 0.008 M 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), and an appropriate amount of tissue homogenate from either the invertebrates or fish liver and gills. Upon hydrolysis of acetylthiocholine by AChE, the resulting thiocholine reacted with DTNB to produce a yellow compound, 5-thio-2-nitrobenzoic acid. The absorbance of this product was measured at 412 nm, and AChE activity was expressed in units per milligram of protein (U/mg protein).

The Specific Oxidative Stress (SOS) index was calculated as the difference between the pro-oxidative (PrO) score and the antioxidant (AO) score (SOS = PrO – AO), based on oxidative stress markers measured in the studied specimens [82]. The PrO score represents the mean Z-score of the pro-oxidant marker LPO (PrO = mean (zLPO)), while the AO score is the average of the Z-scores of

the antioxidant markers GSH, SOD, CAT, GPX, and GST (AO = mean (zGSH, zSOD, zCAT, zGPX, zGR, zGST)). The SOS values near zero indicate a balanced oxidative state.

2.6. Statistical Analysis

Statistical analyses followed Mosaheb et al. [83]. The significance of differences of means between groups was determined using Student's t-statistic. The correlations between studied variables were assessed using the Spearman rank coefficient, and cluster analysis was applied to organize the observed data into meaningful structures of dependent variables. The calculations were carried out using the STATISTICA 10 package (StatSoft Inc., USA). For detecting the presence of significant thresholds of change in MPs accumulation in species individuals, the Sequential Three-step Analysis of Regime Shifts (STARS 6.3) was used [84–86].

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, crustaceans) and vertebrates (fish) that belong to different trophic levels and are key species for different habitats.

The studied invertebrate species were significantly different in size and weight (Table 1). The analyses showed that the percentage of occurrence (%FO) of MPs in the soft tissues of the different invertebrates studied 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 of the key invertebrate species studied differed significantly. The estimation of the number of MPs per gram of soft tissue 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). Practically the alien species *M. gigas* had the highest accumulation of MPs.

There was no significant correlation present between the accumulated particles of MPs and the individual length and weight of the invertebrate species. The dependence of size on the accumulation of MPs was analyzed by correlation analyses of MPs content in the soft tissues and the size of the individuals of the different invertebrate species.

Table 1. Morphological characteristics of the studied invertebrate species (mean±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
M. galloprovincialis	13.62[b,c,d,e] ±3.30	6.04[c] ±0.42	4.04[b,c,d,e] ±0.95	63.33	1.13[e] ±1.11	0.320 ±0.31
R. venosa	37.87[a,c,d,e] ±6.31	5.78[c] ±0.35	11.87[a,c,e] ±2.33	46.67	0.60[e] ±0.83	0.050 ±0.07
B. reticulatum	0.051[a,b,d,e] ±0.010	1.09[a,b,d] ±0.098	0.01[a,b,e] ±0.03	83.33	0.30[e] ±0.19	0.0033 ±0.0025
P. adspersus	1.54[a,b,c,e] ±0.66	5.86[c] ±1.02	-	70.00	1.37[e] ±1.27	0.990 ±1.09
M. gigas	136.21[a,b,c,,d] ±19.72	11.8 ±0.76	22.2[a,b,c,d] ±4.54	66.67	100.5 ±87.36	4.530 ±11.08

6 of 22

W=Total weight; L=Total length; ST=Soft tissues; FO=Frequency of occurrence. Significant difference from: [a] - *M. galloprovincialis*; [b] - *R. venosa*; [c] - *B. reticulatum*; [d] - *P. adspersus*; [e] – *M. gigas*.

The studied fish significantly differed in their size and weight. The biggest individuals were those of *M. batrahocephalus* 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. batrachocephalus* and *M. barbatus* (Table 2).

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

	W	L	GIT weight	MPs			
Species	gr	cm	gr	%FO in GIT	%FO in muscle	particles/ GIT	particles/ g muscle
M. batrachocephalus	90.5[b,c] ±24.37	21.32[b,c] ±1.83	7.57[b,c] ±4.18	53.3[c]	26.7[c]	0.80 ±1.08	0.24[c] ±0.44
M. barbatus	21.64[a,c] ±6.68	12.55[a] ±1.11	1.21[a,c] ±0.34	46.7	33.4	0.93 ±1.16	0.27[c] ±0.48
S. sprattus	4.19[a,b] ±0.80	9.12[a] ±0.47	0.17[a,b] ±0.07	33.4[a]	46.5[a]	0.40 ±0.63	2.01[a,b] ±2.56

W=Total weight; L=Total length; GIT=Gastrointestinal tract; FO=Frequency of occurrence. Significant difference from: [a] - *M. batrachocephalus*; [b] - *M. barbatus*; [c] - *S. sprattus*.

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

The transfer, bioaccumulation and the possible biomagnification of MPs in ecosystem food chains (and webs) and the ecological consequences are still not well understood. Due to the high variance of means in our study for the detecting of significant thresholds and shifts in MPs content in the studied species individuals, the Sequential Three-step Analysis of Regime Shifts (STARS 6.3) [84–86] was used. In this analysis, our previous data on MPs accumulation in individuals of *C. glaucum* and *M. arenaria* from the same regions (Varna Lake) [38] were also included. The results are graphically presented in Figure 2.
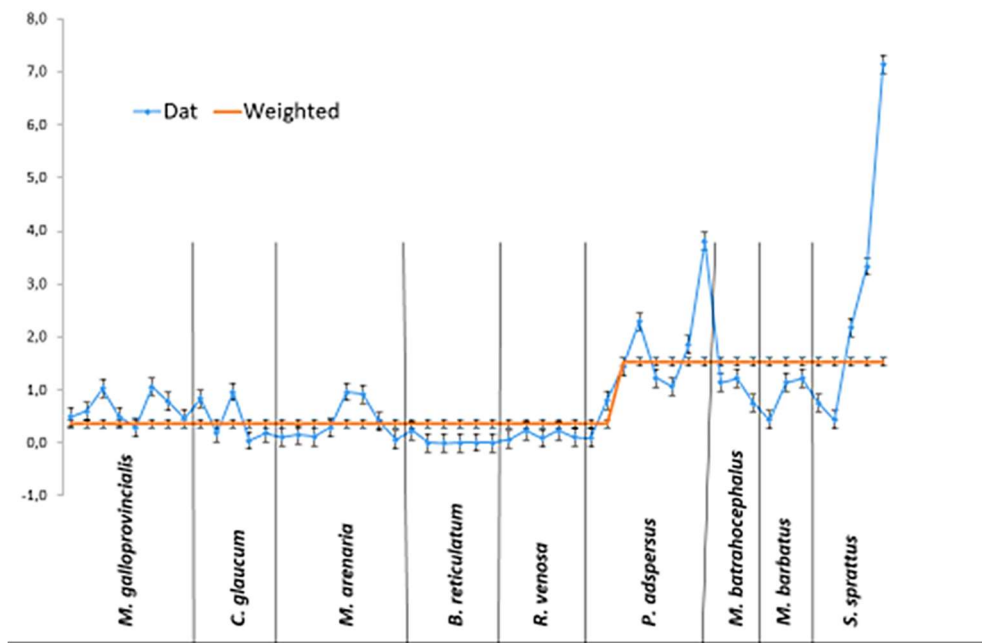
Figure 2. Thresholds of significant shifts in MPs accumulation in the studied species individuals.

The graphic clearly indicated the presence of a significant increase in the MPs accumulation in the individuals of *P. adspersus* and the fish species studied. These results suggested the presence of differences in MPs accumulation in species from different habitats and different trophic levels.

3.2. Oxidative Stress Levels in the Studied Species

Under natural conditions, it is almost impossible to differentiate the specific ecological effects of MPs, and in this study, the stress-response effects 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 measured OS indicators varied 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 – were recorded, which indicate the presence of higher OS. The values of GST and AChE were also low. The lowest LPO and the highest values of GSH, SOD, CAT, GR, and GST were measured in *R. venosa*, which indicated the presence of low OS level, together with a relatively low accumulation of MPs per gram tissue (0.05±0.06 particles/g). Compared to native invertebrate species, the alien Pacific oyster showed a relatively high LPO, accompanied, however, by markedly higher values of GSH, SOD, and GR; as well as high CAT and GPx activities.

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

Species	LPO nM/mg protein	GSH ng/mg protein	SOD U/mg protein	CAT U/mg protein	GPX U/mg protein	GR U/mg protein	GST U/mg protein	AChE U/mg protein
M. galloprovincialis	3.69[b,c,d] ±0.33	93.39[c] ±9.75	21.72 [b,d] ±1.05	0.63[a,b,c,d] ±0.07	8.92[c] ±0.43	12.78[b,d,e] ±1.23	13.86[a,b,c,d] ±2.00	33.72[b,c,d] ±1.60

R. venosa	0.37[a,c,d] ±0.04	118.5 ±69.60	32.81[a,c,d] ±2.86	1.30[a,b,d] ±0.23	8.48[c] ±1.57	20.40[a,b,d] ±5.17	323.62[a,b,c,d,e] ±10.72	181.56[a,d,e] ±22.67
B. reticulatum	1.51[a,b,d] ±0.03	130.9[a,d] ±2.74	23.56[b,d] ±0.48	1.01[a,c,d] ±0.12	17.92[a,b,d] ±1.20	12.12[d,e] ±1.27	86.70[a,b,c,e] ±1.73	293.52[a,d,e] ±16.31
P. adspersus	6.32[a,b,c] ±1.13	84.24[c] ±4.69	5.31[a,b,c] ±0.55	0.12[a,b,c,e] ±0.01	8.03[c] ±0.99	1.48[a,b,c,d,e] ±0.15	34.65[a,b,c] ±4.00	47.51[a,b,c] ±5.70
M. gigas	5.38[b,c] ±1.22	266.55[a,b,c,d] ±62.79	74.60[a,b,c,d] ±7.82	0.72[d] ±0.09	14.90[a,b,d] ±1.56	23.81[a,c,d] ±1.61	33.10[a,b,c] ±3.44	40.00[b,c] ±6.45

Significant difference from: [a] - M. galloprovincialis; [b] - R. venosa; [c] - B. reticulatum; [d] - P. adspersus; [e] – M. gigas.

The lowest concentration of MPs per gram tissue was recorded in *B. reticulatum*, where also a relatively low level of LPO, high concentration of GSH, and high activity of the antioxidant enzymes were present, together with the highest AChE values. In the tissues of *M. galloprovincialis*, where average MPs values were present, relatively high LPO and low levels of GSH and antioxidant enzyme activities were found. In these mussels, there were statistically significantly lower GST and AChE activities compared to the other invertebrate species studied (Table 3).

For the integrative analysis of the degree of OS present and the specific accumulation of MPs per gram of tissue in the invertebrates, the percentage ratio of the individual indicators in the species studied was measured and is presented in Figure 3.
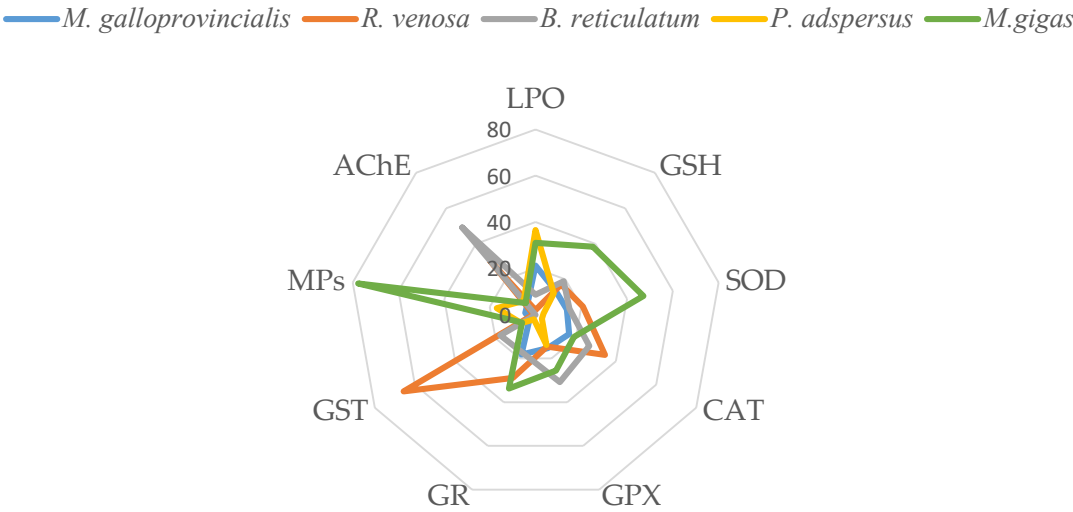

Figure 3. Percentage ratio of measured OS parameters and MPs accumulation in the tissues of the studied invertebrates.

The graphic clearly shows that the recently introduced foreign species *M. gigas* was characterized by the highest MPs accumulation and high LPO. But here, high levels of the anti-stress system activity were found (SOD, CAT, GSH). Similarly, *P. adspersus* was also characterized by high MPs accumulation together with high stress level (high LPO and the lowest GSH, SOD, CAT, GR and GST activities) compared to the other studied species. In *B. reticulatum* low MPs accumulation was present together with low OS level (lowest LPO, highest GSH and GPx activities, and relatively high SOD, CAT, GR and GST activities) compared to the other studied invertebrates.

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

Table 4a. Biometrics (W – Total weight; L – Total length; K – Fulton's condition index) and oxidative stress biomarkers (mean±SD) in fish liver.

	biometrics			oxidative stress biomarkers							
	W	L	K	LPO	GSH	SOD	CAT	GPx	GR	GST	AChE
Species	g	cm		nM/mg protein	ng/mg protein	U/mg protein	U/mg protein	U/mg protein	U/mg protein	U/mg protein	U/mg protein
M. batrachocephalus	73.0	19.5	0.98	7.45 [b]	32.9[b,c]	80.5[b,c]	2.02[b]	74.6[b,c]	-	225.9[b,c]	17.8[b,c]
	±3.62	±0.35	±0.02	±0.83	±4.30	±1.94	±0.16	±6.25	-	±11.78	±0.52
M. barbatus	9.60	9.44	1.04	0.93[a,c]	135.1[a]	86.1[c]	8.00[a,c]	27.5[a]	17.42	476.8[a,c]	50.1[a,c]
	±4.17	±1.40	±0.05	±0.19	±67.45	±14.89	±4.00	±9.15	±1.25	±16.65	±13.02
S. sprattus	2.94	7.70	0.61	10.3[a]	130.9[a]	19.97[a,b]	1.87[b]	32.2[a]	17.10	850.4[a,b]	32.9[a,b]
	±1.12	±0.87	±0.04	±3.84	±13.95	±2.64	±0.27	±10.78	±2.62	±10.05	±8.12

Significant difference from: [a] - *M. batrachocephalus*; [b] - *M. barbatus*; [c] - *S. sprattus*.

In the liver of *M. barbatus* (Table 4a), the lowest LPO levels were measured. This finding, together with the relatively high levels of antioxidant enzymes (including statistically significant 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, probably because GSH is a co-substrate in the enzymatic reaction. 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 also statistically higher compared to both *M. batrachocephalus* and *M. barbatus*, which indicates an increased level of detoxification processes (Table 4a).

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

Table 4b. Biometrics (W – Total weight; L – Total length; K – Fulton's condition index) and oxidative stress biomarkers (mean±SD) in fish gills.

	biometrics			oxidative stress biomarkers							
	W	L	K	LPO	GSH	SOD	CAT	GPx	GR	GST	AChE
Species	g	cm		nM/mg protein	ng/mg protein	U/mg protein	U/mg protein	U/mg protein	U/mg protein	U/mg protein	U/mg protein
	73.0	19.5	0.98	19.2	320.1[b,c]	19.3[c]	3.68[b,c]	24.8[b,c]	-	121.8[b,c]	187.2[b,c]

M. batrachocephalus	±3.62	±0.35	±0.02	±1.29	±33.50	±2.03	±0.58	±3.11	-	±3.16	±18.95
M. barbatus	9.60 ±4.17	9.44 ±1.40	1.04 ±0.05	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	57.3[a,c] ±16.2
S. sprattus	2.94 ±1.12	7.70 ±0.87	0.61 ±0.04	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	17.3[a,b] ±1.92

Significant difference from: [a] - *M. batrachocephalus*; [b] - *M. barbatus*; [c] - *S. sprattus.*

The visualization of the ratios of OS parameters (means in liver and gills) and MPs accumulation of the studied fish are presented in Figure 4. It is evident from the data that *S. sprattus* exhibits the highest stress parameters values, as indicated by the highest LPO and the lowest/inhibited SOD and CAT activities, together with highest MPs accumulation in this species. In contrast, *M. barbatus* had the lowest MPs accumulation and stress, represented by the lowest LPO and the highest SOD and CAT activities compared to the other fish species studied.
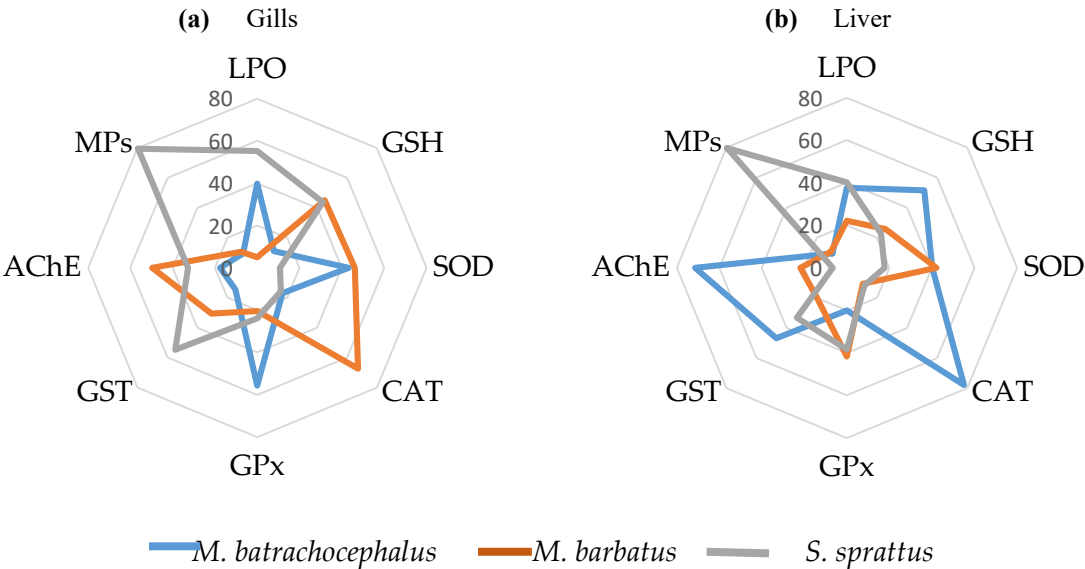

Figure 4. Percentage ratio of MPs accumulation (particles/g muscle) and the OS parameters in gills (**a**) and liver (**b**) of the studied fish species.

In order to identify the possible interactions of MPs and the measured OS parameters with potential ecological consequences, cluster analysis was applied. The results revealed the close grouping of MPs accumulation with OS indicators in the key invertebrate species studied (Figure 5). The data of *M. gigas* is intentionally not presented as this is not a key species but is a foreign intruder recently beginning to settle down in the Bulgarian Black Sea part.

In *M. galloprovicialis*, the cluster analysis results (Figure 5a) showed very close relationship between MPs accumulation and CAT activity, which were also closely related to LPO. More distant were the relationships with a group including the glutathione-dependent enzymes GPx and GR. Overall, MPs and OS indicators formed a relatively compact group with different relations with others, where the most distant was the relation with GSH. Similarly, a cluster suggesting close relations between MPs and CAT activity, along with that of GR, was also present in *P. adspersus* (Figure 5c). These clusters in turn were also closely related to LPO and more distantly to SOD and GPx. The activities of GST and AChE formed a separate cluster. In *P. adspersus*, as in *M. galloprovicialis*, the relation with GSH was the most distant (Figure 5c). In *R. venosa* and *B. reticulatum*, a cluster suggesting very close relation between the accumulation of MPs and LPO was present, and in *B. reticulatum*, CAT activity was also involved (Figure 5b,d). In *R. venosa*, a cluster of close relations was

present with the antioxidant enzymes (GPx, CAT, GR and SOD), all forming a common group. A separate cluster of GSH levels and AChE activity was formed, which was most distant from GST and the other studied OS parameters. In *B. reticulatum*, SOD and GPx activities were closely related to each other and formed a cluster also related to GR activity. A separate cluster was formed by GSH levels and GST activity, also related to MPs accumulation and the other OS indicators. In *B. reticulatum*, AChE activity had the most distant relationship with the other measured parameters, and also MPs (Figure 5d).
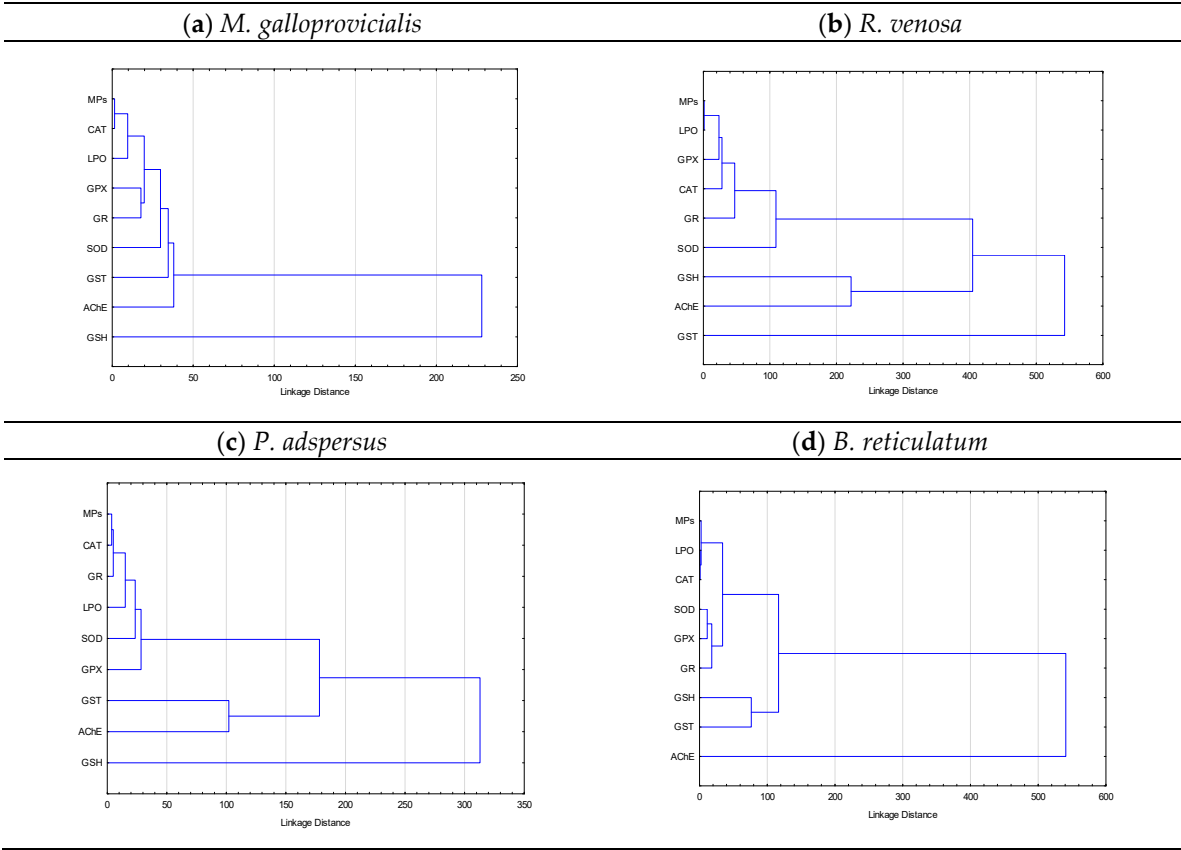

Figure 5. Results of cluster analysis of the oxidative stress biomarkers and microplastics accumulated in the invertebrate species: (**a**) *M. galloprovicialis*, (**b**) *R. venosa*, (**c**) *P. adspersus*, and (**d**) *B. reticulatum.*

The cluster analyses of the grouping relations of MPs accumulation and the OS indicators measured in the liver of the studied fish species are presented in Figure 6 (a, b). The results show that the estimated accumulation of MPs in the fish species is grouped with the main OS biomarkers, and this suggests the presence of interactions.

In the liver of all the fish species studied, the MPs belonged to a distinct group, including also CAT, LPO, or GR (Figure 6a). Additionally, in *S. sprattus* associated with this group was also SOD and in *M. barbatus* - GPx. The AChE in *M. batrahocephalus* was also related to these OS indicators and thus belonged to this group (Figure 6a). In the fish species *M. batrahocephalus* and *M. barbatus*, the liver GST activity was most distant from the clusters of OS biomarkers, suggesting that the activity of this enzyme seemed not directly related to the presence of MPs. In *S. sprattus*, liver GSH concentration and AChE activity formed a separate cluster, which was only distantly related to the accumulated MPs and the other OS biomarkers (Figure 6a).

In the gills of the studied fish species, the grouping and the possible relations of MPs with closely associated OS variables were similar to those in the liver (Figure 6b).
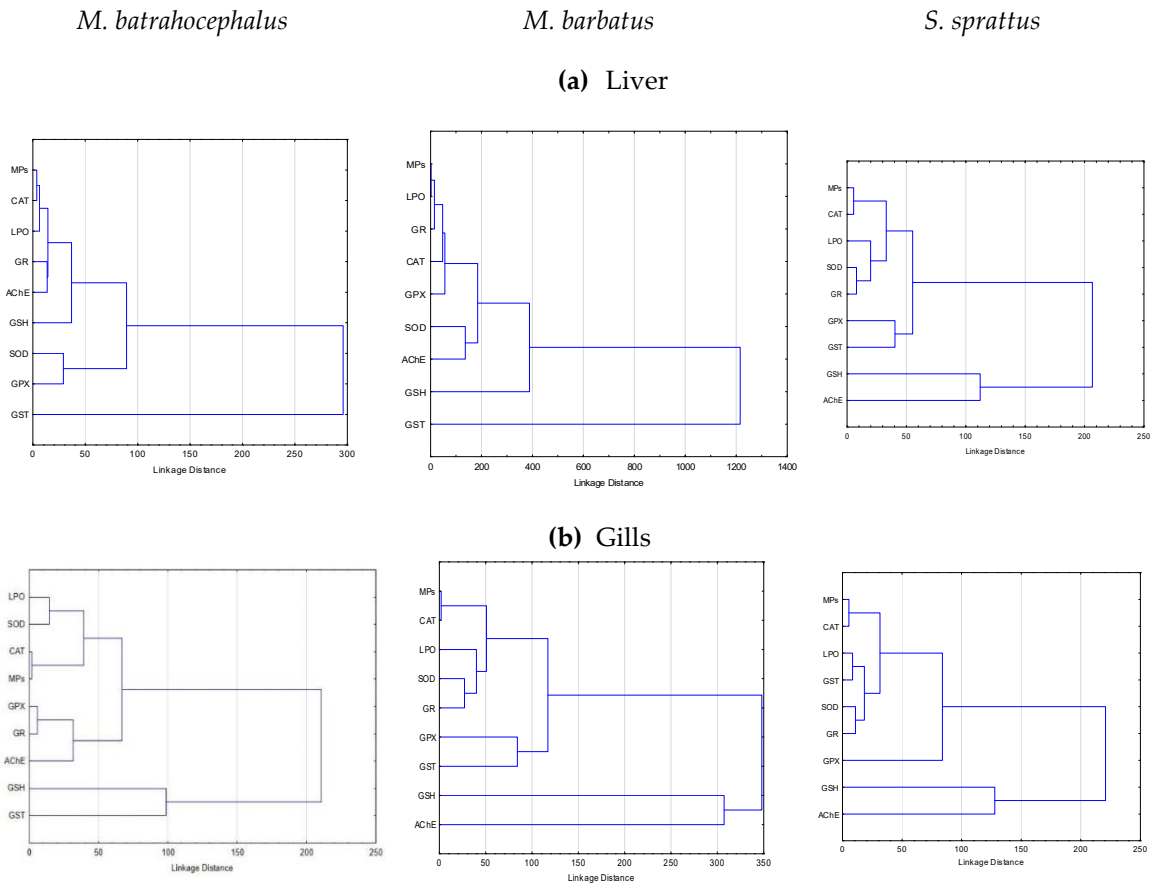
M. batrahocephalus   *M. barbatus*   *S. sprattus*

(a) Liver

(b) Gills

Figure 6. Results of cluster analysis with the meaningful structures of accumulated microplastics and oxidative stress biomarkers in fish (**a**) liver and (**b**) gills. .

In the gills of the three fish species studied, the MPs' presence formed a distinct and very compact cluster with CAT, thus clearly indicating the presence of functional relations. To this compact main group in the fish studied there were also other clusters of specific OS indicators including LPO, SOD, GR which were also more or less close to the MPs-CAT group.

To study the cumulative effect on OS levels, the values of the SOS index in the studied species were calculated (Table 5a,b).

Table 5a. Calculated values of the SOS index of the studied species.

	M. batrachocephalus	*M. barbatus*	*S. sprattus*	*M. galloprovincialis*	*R. venosa*	*B. reticulatum*	*P. adspersus*	*M. gigas*
PrO	0.109	-1.872	1.410	-0.132	-1.118	-0.736	0.149	0.561
AO	0.804	0.863	-1.335	-0.891	-0.681	0.357	-0.457	0.627
SOS	**-0.694**	**-2.736**	**2.745**	**0.758**	**-0.437**	**-1.093**	**0.606**	**-0.066**

Table 5b. Classification of the stress level of the studied species according to the SOS index.

Very low stress (<-2)	Low stress (-1 to -2)	Stress actively compensated (-1 to +1)	Moderate stress (+1 to +2)	High stress (+2<)
	B. reticulatum	*R. venosa* *M. galloprovincialis* *M. gigas* *P. adspersus*	-	
M. barbatus		*M. batrachocephalus*	-	*S. sprattus*

Two of the studied species (*B. reticulatum* and *M. barbatus*) showed low levels of SOS (Table 5b). Most of the invertebrate species (*R. venosa, M. galloprovincialis, M. gigas, P. adspersus*) and the fish species *M. batrachocephalus* were in stress balance (SOS near zero), as the OS was actively compensated by the anti-stress cell system. The only species having a significantly high OS that the anti-stress system could not effectively compensate was *S. sprattus* (Table 5b). It is indicative that in the studied *S. sprattus* individuals, this coincides with the high level of accumulated MPs.

4. Discussion

The small size of MPs and their variable buoyancy make them easily accessible for uptake by organisms inhabiting different habitats and with different nutritional characteristics, indicating the "natural" trophic transfer of MPs in marine biota with implications for the broader food web, including humans [56,61].

The present study showed that MPs were present in most of the individuals of the species studied. The high percentage of MPs (%FO) in the soft tissues of *B. reticulatum* (83.33 %) and *P. aspersus* (70%) can be related to the number of plastics present in their habitats. Both species inhabit shallow sandy areas in algal and eelgrass beds, often in brackish water [87–89], and algae play a significant role in the retention of MPs and their removal from the water column [90]. The MPs' retention is achieved by their adhesion to algae or embedment/encrustation within the epibiont matrix of algae, thereby preventing MPs from migrating with water currents [90]. It was found that vegetated sites had much higher MPs than non-vegetated sites and that filamentous species contain more MPs due to the entanglement [91]. 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 much smaller amount of food, and is also a microalgae herbivore unlike *P. aspersus* [92].

Concerning the fish species, the relatively higher percentage of occurrence (%FO) of MPs in the GIT of *M. batrachocephalus* and *M. barbatus* (about 50%) compared to *S. sprattus* was probably also due to their lifestyle. Both *M. batrachocephalus* and *M. barbatus* are demersal and less mobile fish, while *S. sprattus* is pelagic-neritic species [93]. Similar to other studies [94,95], in the present study there was a significant increase of ingested (in GIT) number of MPs related to fish lifestyle and size. The accumulation of MPs was found to be higher in demersal fish species than in the pelagic species [96]. It was assumed that their feeding habits most likely influenced the difference in MPs accumulation. Demersal fish are more stationary than pelagic fish and mostly feed on the prey species present on the sea floor. Specifically, *M. batrachocephalus* feeds mainly on fish [97], *M. barbatus* feeds on small benthic crustaceans, worms, and mollusks [98], and *S. sprattus* feeds on planktonic crustaceans. Furthermore, a study has reported the mean abundance of MPs in herbivorous fish species to be almost two times smaller than the abundance of MPs in carnivores and omnivores [99], and Rasta et al. [100] detected more MPs particles in omnivorous than in carnivorous fish. The established in this study almost 10 times more MPs per g muscle in *S. sprattus,* compared to the demersal fishes (*M. batrachocephalus* and *M. barbatus*), probably depends at least to some extent on the type of muscle. Demersal fish often have white meat, but pelagic fish typically have red flesh, indicative of the powerful swimming muscles they require. Tissues with high blood flow may have a higher likelihood of MPs being transported to them via the bloodstream. It has been found that different tissues and organs of fish have significant differences in MPs accumulation (concentrations of 0.84 ± 0.45 items/g in the digestive tract, 2.56 ± 0.73 items/g in the gills, and 0.3 ± 1.72 items/g in the muscle tissue) [95]. The organs of fish where MPs accumulation is generally higher include the gills and liver. Gills are significant hotspots for MPs accumulation compared to other organs, since the gills are used to filter water for respiration, and MPs can be trapped there [101].

The transfer and accumulation of MPs in species belonging to different levels of food chains (and webs) remains still not well studied. The applied STARS analysis (graphic in Figure 2) showed that in the individuals of _P. adspersus_ and in the individuals of the fish species studied significantly higher numbers of MPs were present but this could hardly be interpreted as some kind of MPs trophic transfer from lower to higher trophic levels which is in agreement with other studies [56].

Irrespective of the pathways, intake of MPs can result in adverse physical and chemical impacts on organisms [41–43]. Due to the presence of multiple stressors in the marine environment it is almost impossible to assess specific effects of MPs on organism health [55,56]. In order to reveal the ecological effects of MPs in line with the stress ecology approach in this study, the effects of OS and the organism response were assessed.

Recent data show that MPs can contribute to OS in marine organisms [68,102,103] by generating excess reactive oxygen species (ROS) directly produced by the plastic particles themselves. MPs can release toxic chemicals such as phthalates, bisphenol A, and polycyclic aromatic hydrocarbons, which can induce ROS production [104]. Another mechanism is through the adsorbed pollutants onto the MPs' surface, acting as a sink for heavy metals, organic compounds, and pesticides [102,105–107]. All of these pollutants can lead to ROS formation through various chemical reactions. For instance, it was found that when polycyclic aromatic hydrocarbons (PAHs) adsorb onto MPs surfaces, ROS can be produced via photochemical reactions under UV radiation [106,108]. Another mechanism could be the Fenton reaction, which involves a transition metal ion, such as iron or copper, to produce hydroxyl radicals [102,106].

In this study, quite different ratios of OS parameters (Figures 3 and 4), together with different MPs accumulation in the studied species, were present. In _P. adspersus_ high MPs accumulation was found together with high OS stress level. In _B. reticulatum_ low MPs accumulation was present and OS was low compared to the other studied invertebrates. Interestingly, the recently settled in the Bulgarian Black Sea foreign species _M. gigas_ had the highest number of accumulated MPs, but low OS due to the activity of the anti-stress cell system. In the studied fish species, the ratios of OS parameters (means in liver and gills) and MPs accumulation showed differences, but the highest OS in _S. sprattus_ was accompanied by the highest MPs accumulation. Here, the GST activity was also high indicating an increased level of the 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 [109].

The cluster analyses we performed (Figures 5 and 6) confirmed the presence of relations between MPs' content and the measured OS indicators. In most of the studied species, a closer relation between MPs' 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 [110]. Catalase is an essential enzyme that decomposes specifically 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 of showed a time-dependent decrease in the activities of SOD, GPx, and CAT and an increase in LPO levels [111]. Evidence also suggests that MPs may directly interact with CAT [110]. Therefore, MPs themselves or through toxic substances or pathogens carried on their surface are really a significant pro-oxidant and, upon prolonged exposure, can lead to depletion or inhibition of antioxidants in organisms [110,111].

The OS and response processes are also specific for different organs and have different effects. The gills and liver are most susceptible to OS. For example, the liver is a major metabolic and detoxifying organ of the body, and during these processes, ROS can be generated as byproducts [112]. The liver also plays a crucial role in immune responses by activating immune cells upon encountering pathogens or toxins [113]. This activation can trigger inflammation and the subsequent excess production of ROS. Disruption of the redox balance causes OS stress and contributes to cellular damage, which affects liver function modulation. The results obtained by us proved that in the gills of the studied fish species, grouping of MPs with closely associated OS variables was present. The gills are in direct contact with the aquatic environment, being the primary target of pollutants, are

richly blood-supplied, and have a high surface area, where gas exchange and filtration occur [114]. Research on contamination in marine fishes found that MPs were present in the gills, dorsal muscle, and gastrointestinal tract. MPs contaminated fish had increased LPO levels in the brain, dorsal muscle, and gills, and higher AChE activity in the brain [115].

Recently, efforts to determine the ecological impacts of MPs pollutants have increased because of the increasing plastic contamination of the marine environment. However, recent review papers and reports have identified current research gaps [116–118]. The ecological risks of plastic contamination can be defined as the likelihood of adverse health effects on biota and their ecological consequences as a result of the organisms' exposure to MPs. The changes caused by OS at the cellular level can subsequently affect higher hierarchical levels, which is defined as "stress ecology" [64,65].

In this study, we used a previously introduced SOS index as a general composite risk indicator to measure the complex effects of multiple environmental stressors, including plastics, on the studied species (Table 5). Two of the species (*B. reticulatum* and *M. barbatus*) showed low levels of OS. Most of the species (*R. venosa, M. galloprovincialis, M. gigas, P. adspersus, M. batrachocephalus*) were in stress balance, as the OS was actively compensated by the anti-stress cell system. A really high OS was present only in *S. sprattus* which the antistress system seems to not effectively compensate. This is also the species with the highest MPs accumulation. The SOS index proved that it can serve as an integral measure of the OS level in the organisms. However, the SOS index cannot differentiate the specific factor/s causing OS changes, which also applies to MPs accumulation.

5. Conclusions

Much remains unknown regarding the environmental consequences of MPs pollution, and more detailed studies are required to assess the ecological and human health risks. The specific OS (SOS) index showed that OS levels were linked to both habitat conditions and species-specific antioxidant defense. These findings confirmed the priority role of environmental conditions (including MPs pollution) in OS, and the evolutionarily developed adaptation capacity and resilience to induce stress in marine organisms. This study emphasizes the increasing need for specific monitoring of MPs and OS of marine organisms to better assess ecological risks. Further research is strongly recommended in order to determine ecosystem-level effects of MPs.

Abbreviations

The following abbreviations are used in this manuscript:

AChE   Acetylcholinesterase
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
ROS   Reactive Oxygen Species
SOD   Superoxide Dismutase
SOS   Specific Oxidative Stress Index
ST   Soft tissues
W   Total weight

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