



Effects of Microplastics and Metal Pollution on Bivalves from the Bulgarian Black Sea Sublittoral, with Comments on their Adaptive Capacity*

Georgi I. Pramatarov^{1,**}, Elina R. Tsvetanova², Vladimir M. Ilinkin³, Madlena N. Andreeva², Albena V. Alexandrova² & Nesho H. Chipev²

¹ Department of Biotechnology, Faculty of Biology, Sofia University “St. Kliment Ohridski”, 1164 Sofia, Bulgaria

² Laboratory of Free Radical Processes, Institute of Neurobiology, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria

³ National Society “Ecological Engineering and Environmental Protection”, Sofia, Bulgaria

Abstract: The present study aimed to assess the effects of microplastics (MPs) and metal bioaccumulation on the bivalve species *Donax trunculus* Linnaeus, 1758, *Cerastoderma edule* (Linnaeus, 1758), *Mya arenaria* Linnaeus, 1758 and *Mytilus galloprovincialis* Lamarck, 1819 from the Bulgarian Black Sea coast and their adaptive capacity to pollution. The MPs accumulation in the bivalves was observed under a stereomicroscope after 10% KOH tissue digestion. Metal elements (Cd, Cu, Cr, Ni, Pb, Zn and Fe) in the bivalve soft tissues were measured by EPA-METHOD 3052. The effects of pollutants on the bivalve species were assessed by oxidative stress (OS) biomarkers, which were measured spectrophotometrically. MPs were observed in all studied species but at a different ratio, pellets being the most numerous (94.7%). The accumulated MPs and metal elements induced OS but significantly correlated with different OS indices in the individual bivalve species. The principal component analysis suggested that the MPs accumulation probably leads to changes in bivalve cells similar to those caused by Pb and associated with protein oxidation and glutathione levels. In conclusion, the accumulated MPs and metal elements caused OS in all studied bivalves, which, in turn, activated their antioxidant system. This suggested the presence of adaptive potential of the bivalve species to the current ecological state of the marine environment in their habitats of the Bulgarian Black Sea sublittoral.

Key words: adaptive potential, bivalves, Bulgarian Black Sea, metal elements, microplastics, oxidative stress

Introduction

Pollution of the marine environment with microplastics (MPs) is widely recognised as a global problem. Vast amounts of plastic waste are increasingly accumulating in the environment, especially in seas and oceans. Recent data confirmed that the

Black Sea is also significantly polluted with MPs, including the Bulgarian coastline (Moncheva et al. 2016, Simeonova & Chuturkova 2019, Pojar & Stock 2019, Berov & Klayn 2020, Bobchev et al. 2024). On the other hand, trace metal elements are persistently present in the marine environment and the metal pollution is huge. Thus, metal elements to-

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**Corresponding author: georgipr@abv.bg

gether with MPs seem to be the most prominent pollutants threatening the marine environment worldwide at present (Liu et al. 2021).

Metal pollution in the Black Sea has long been considered a significant environmental problem. Various sources, including natural geological processes, river inflows, industrial wastewater discharge rich in metals, etc., contribute to the presence of metals in the marine environment (Mitryasova et al. 2020). Review and research articles have shown the metal accumulation in marine sediments, water bodies, algae, benthic organisms and fish from Turkish, Romanian, Ukrainian and Bulgarian parts of the Black Sea (Manev et al. 2020, 2021, Oros et al. 2024, Damir et al. 2024, Bat et al. 2024a, 2024b). The toxic effects of metal elements and their combinations on marine organisms have well-studied impacts on biomolecules, cellular structures and their functions leading to consequences such as oxidative stress (OS), disruption of metabolism, impaired growth, development, behaviour and reproduction (Jamil Emon et al. 2023, Santhosh et al. 2024, Kumar et al. 2024).

Pollution of the ocean and seas with MPs at present poses significant risk to the marine organisms' and human health. Organisms can accumulate MPs in their tissues, which causes negative effects on their health, with a subsequent influence at the higher systemic levels, also at the ecosystem level. MPs have been found in the tissues of several species along the Bulgarian Black Sea coast, specifically white mussels *Donax trunculus* Linnaeus, 1758, black mussels *Mytilus galloprovincialis* Lamarck, 1819, cockles *Cerastoderma glaucum* (Bruguère, 1789) and key fish species (Alexandrova et al. 2022, 2024, Ibryamova et al. 2023, Mihova et al. 2024). A comprehensive review summarized the effects of MPs on aquatic invertebrates and vertebrates, such as changes in feeding behaviour, decline in fertility, retardation in larval growth and development, increased oxygen consumption, excessive generation of reactive oxygen species (ROS) (Zolotova et al. 2022).

Recent data confirmed that the Bulgarian sector of the Black Sea is significantly polluted with plastics, including MPs and nanoplastics. The first studies on MPs in the Bulgarian Black Sea waters reported high concentrations of MPs ranging from 1.14×10^4 to 1.91×10^5 items/km² (0.33–490.52 g/km²). These amounts place the degree of pollution in the same range as that of the Baltic Sea and the Mediterranean Sea, which are considered some of the most polluted in the world (Berov & Klayn 2020). A recent survey of coastal Bulgarian waters from Durankulak to Burgas Bay showed MPs pres-

ence in 22 from a total of 23 samples with mean concentrations of MPs particles ranging from 1.3 items/L (Cape Kaliakra) to 16.3 items/L (Pasha Dere near Varna) (Georgieva et al. 2023).

Due to their large specific surface area because of the very small size and hydrophobic nature, MPs can adsorb and accumulate toxic metals from the marine environment on their surface and can also form microbial biofilms (Liu et al. 2021, Xiang et al. 2022, Adeleye et al. 2024). Recent studies have shown the adsorption capacity of both new and aged MPs for metals such as lead (Pb), chromium (Cr), iron (Fe), zinc (Zn), tin (Sn), titanium (Ti), manganese (Mn), aluminum (Al), copper (Cu), cadmium (Cd) and nickel (Ni) in aqueous environments (Khalid et al. 2021, Vedolin et al. 2018, Zou et al. 2020). Previous studies found that the interactions of MPs with other environmental pollutants could significantly alter their environmental behaviour, toxicity and bioavailability (Alimi et al. 2018, Wang et al. 2020, Zhang et al. 2020b). The adsorption of metals onto the surface of MPs in aquatic environments provides a convenient pathway for metals to migrate more easily from the environment into organisms. The coexistence of MPs and metals is believed to increase the overall toxicity of these pollutants to marine organisms; some data have already indicated that the combination of MPs and metals can enhance toxicity in fish, affecting their survival rate (Miranda et al. 2019, Zhang et al. 2020).

Thus, a general question arises, are there joint effects of MPs and metals accumulated in a marine organism and are these effects additive, synergistic or antagonistic? Currently, there are no data from field or laboratory studies on the combined effects of accumulated MPs and metals on marine organisms. To the best of our knowledge, there is only one study (Oliveira et al. 2013) providing some evidence of synergistic toxicity of microplastics and pyrene, highlighting changes in behaviour and biochemical responses in marine fish. Therefore, it is crucial to perform comprehensive studies on the possible joint effects of MPs with other bioaccumulated pollutants on the health of marine organisms.

This study aimed to assess the presence of joint effects of bioaccumulated MPs and metals, as the main pollutants in the Black Sea, on the stress and health status of key food web component species of bivalves. These are *Donax trunculus* Linnaeus, 1758, *Cerastoderma edule* (Linnaeus, 1758), *Mya arenaria* Linnaeus, 1758 and *Mytilus galloprovincialis* Lamarck, 1819 from various localities along the Bulgarian Black Sea coast and their adaptive capacity to the MPs and metal pollution.



Fig. 1. Localities and sampling sites of bivalve species along the Bulgarian Black Sea coast

Materials and Methods

Bivalves and sampling area

The studied bivalve species were collected from various localities of the Bulgarian Black Sea coast (Fig. 1). Specimens of *D. trunculus* (23–35 mm) were obtained from commercial providers or collected manually from their natural shallow sublittoral sandy habitats from the Bulgarian Black Sea coast from a depth of 0.5–3.0 m. The samples of *M. arenaria*, *M. galloprovincialis* and *C. edule* were collected from Varna Lake from a depth of 0.15–0.20 m, 0.50–1.00 m and 1.00–1.20 m, respectively (Fig. 1). All bivalves analysed in this study were collected in early autumn (September). The bivalves from each sampling site were transported in dry ice to the laboratory where they were stored at -80°C until biochemical analyses.

Microplastics detection

The MPs accumulation in the bivalves was observed under a stereomicroscope after 10% potassium hydroxide (KOH) tissue digestion (Lusher et al. 2018). In brief, the soft tissue of each specimen was removed

from the shell, cut into small pieces and 10% KOH was added. The samples were covered with aluminium foil to prevent contamination and evaporation and incubated at 60°C for 24 h with continuous agitation at 125 rpm. After cooling, the solutions were filtered through nitrocellulose filters (\varnothing 47 mm, pore size 8 μm , Sartorius Stedim Biotech, Gottingen, Germany), which were then dried for 24 h at 37°C .

The MPs identification was performed by visual observation of filters under a stereo microscope coupled with a digital camera (Zeiss Stemi 508, Carl Zeiss Microscopy GmbH, Jena, Germany). The digital images were examined using the image analysis program Image J and the MPs were quantified by size ($\leq 25 \mu\text{m}$, 25–100 μm and 100–200 μm), based on their largest cross section.

To prevent MPs contamination, work was undertaken with metal tools in glassware, rinsed with bi-distilled filtered water. The reagents used in the analysis were tested for the MPs presence.

Metal elements analysis

Metal elements (Cd, Cu, Cr, Ni, Pb, As, Hg, Zn, and Fe) in the bivalves' soft tissues were measured by

EPA-METHOD 3052. A representative sample of up to 0.5 g was digested in 9 mL of concentrated nitric acid and 3 mL of hydrochloric acid for 15 min using microwave heating. The temperature profile was specified to permit specific reactions and incorporates reaching $180\pm5^{\circ}\text{C}$ in approximately less than 5.5 min and remaining at the same temperature for 9.5 min for the completion of specific reactions (<https://www.epa.gov/sites/default/files/2015-12/documents/3052.pdf>). After cooling, the vessel contents were allowed to settle and then decanted, diluted to volume and analysed by inductively coupled plasma mass spectrometry (ICP-MS). For generation of the calibration curves standard solutions was used. For ensuring the quality, measurements were performed in triplicate.

Tissue preparation for biochemical analyses

On the day of the analyses, the bivalves were thawed, their soft tissues extracted and homogenized with cold 100 mM K-PO_4 buffer, pH 7.4. The resulting homogenates were centrifuged at 3000 rpm for 10 min at 4°C to obtain a post-nuclear fraction in which lipid peroxidation (LPO), protein oxidation (PO) 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 activities of the antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase (GST) were assayed.

Biochemical analyses

The protein concentrations of the tissue post-nuclear and post-mitochondrial fractions were measured according to Lowry et al. (1951) using bovine serum albumin for generating the standard curve. The oxidative stress parameters were measured using kits purchased from Sigma-Aldrich Co. LLC (USA): Lipid Peroxidation (MDA) Assay Kit MAK085, Protein Carbonyl Content Assay Kit MAK094, Glutathione Assay Kit CS0260, Catalase Assay Kit CAT100, Glutathione Reductase Assay Kit GRSA, Glutathione Peroxidase Cellular Activity Assay CGP1 and Glutathione-S-Transferase Assay Kit CS0410.

The acetylcholinesterase (AChE) activity was measured using the method described by Ellman et al. (1961), which is based on the production of thiocholine. The reaction mixture consisted of 100 mM potassium phosphate (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 the

bivalves. 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).

Statistical analysis

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 range coefficient. To identify meaningful predictors of dependent variables multiple analysis of relationships was carried out using Principle Component Analysis (PCA). The calculations were carried out using the STATISTICA 10 package (Stat Soft Inc., USA).

Results

The measured values of the bioaccumulation of MPs, trace metal elements and the OS bioindicators in the studied bivalves from the different regions of the Bulgarian Black Sea coast are presented in Table 1. The results obtained showed the presence of significant differences in the accumulation of metal elements, MPs and the OS markers among the bivalve species from the different sampling localities. In all studied bivalve individuals, MPs with the smallest sizes ($\leq 25\text{ }\mu\text{m}$) predominated. A high number of MPs particles was found in clams sampled from the regions of Varna, Shkorpilovtsi, St. Konstantin & Elena, Irakli, St. Vlas, Pomorie and Ahtopol (Table 1).

Different concentrations of metal elements were found in the bivalve species across the studied localities. The highest concentrations of accumulated metal elements were observed in the bivalves from Varna Lake. Only for Cu, higher levels were detected in *D. trunculus* from Varna, St. Vlas and Slunchev Bryag. Elevated Cd levels were measured in *D. trunculus* sampled from Slunchev Bryag, St. Vlas and Obzor. The highest Pb concentration was present in clams from Duni and Fe in the clams from Slunchev Bryag. Of the three bivalve species studied from Varna Lake, *M. galloprovincialis* showed the highest accumulation of Cd, while in *C. glaucum* the greatest accumulation of Cr, Ni, Pb, Hg and Fe was found (Table 1).

Concerning the OS indicators in the studied bivalves, high LPO levels were found in *D. trunculus* from St. Konstantin & Elena and in *M. galloprovincialis* from Lake Varna. In a large part of the studied bivalves (i.e., *D. trunculus* samples from St. Konstantin & Elena, Varna, St. Vlas, Slunchev

Table 1. The accumulation of microplastics and trace metal elements, and the oxidative stress markers in bivalve species from representative locations of the Bulgarian Black Sea coastal zone (** – significance $p < 0.05$; * – significance $p < 0.1$)

Microplastics			Oxidative Stress Markers										Trace Elements								
	≤25 µm	25- 100 µm	101- 200 µm	LPO	PO	GSH	SOD	CAT	GPx	GR	GST	AChE	As	Cd	Cu	Cr	Ni	Pb	Hg	Zn	Fe
Donax trunculus																					
St. Konstantin & Elena																					
Mean	10.89**	0.11	0	2.00*	9.90**	391.0*	17.89**	2.56**	1.40	21.69**	114.16	83.93	NA	0.054	19.23*	0.378	0.104	0.240	NA	8.47	137.3
SD	1.25	0	0	0.34	1.95	50.48	4.51	0.69	0.38	6.66	29.80	26.89		0.006	2.71	0.11	0.025	0.090		0.074	44.12
Varna																					
Mean	14.67**	8.0**	5**	0.75	10.01**	450.5**	4.800	0.99	4.187	4.170	156.593	11.429	NA	0.055	27.09**	0.145	0.071	0.275	NA	14.92	53.00
SD	5.19	0.8	0.81	0.10	1.13	42.73	0.46	0.15	0.642	0.49	17.58	0.74		0.004	0.300	0.004	0.001	0.019		0.001	0.73
Shkorpilovtsi																					
Mean	10.79**	0.78	1.0**	0.36	7.43	392.3**	0.59	1.17	3.45	7.23*	114.98	37.88	NA	0.077	18.14*	0.259	0.055	0.350	NA	11.04	114.65
SD	3.36	0.16	0.71	0.02	0.35	70.41	0.04	0.14	0.71	0.48	11.34	1.05		0.00	0.95	0.02	0.02	0.03		0.28	12.14
Byala																					
Mean	4.25	0.09	0	0.65	3.91	120.8	15.69**	1.86*	12.68**	5.05	106.03	134.36**	NA	0.073	6.79	0.113	0.000	0.039	NA	8.74	95.79
SD	1.08	0.02	0	0.08	0.43	9.96	1.07	0.30	2.53	1.10	21.44	11.88		0.001	0.013	0.001	0.000	0.002		0.044	0.57
Obzor																					
Mean	3.74	0.37	0	0.55	3.32	129.3	18.70**	1.62*	11.76**	7.24*	121.52	116.11**	NA	0.097*	7.79	0.025	0	0.0326	NA	10.22	27.86
SD	1.25	0	0	0.03	0.96	12.69	2.28	0.11	0.57	2.38	26.65	9.78		0.001	0.046	0.002	0	0.002		0.023	0.10
Irakli																					
Mean	9.0**	3.0*	1.0**	0.80	4.17	456.5**	2.13	2.55**	0.75	5.90	141.89	73.45	NA	0.06	11.13	0.34	0.00	0.01	NA	18.04	163.49
SD	0.9	0.33	0.1	0.09	2.16	20.38	1.51	0.52	0.71	1.97	14.31	8.21		0.00	0.09	0.00	0.00	0.00		0.06	1.35
St. Vlas																					
Mean	10.0**	2.85*	0.5	0.99	9.06**	370.8**	5.18	2.59**	1.343	3.07	153.32	11.677	NA	0.119*	29.71**	0.298	0.121	0.025	NA	14.01	166.94
SD	2.36	1.52	0.35	0.07	0.51	39.00	0.59	0.17	0.24	0.76	7.84	1.29		0.001	0.255	0.001	0.002	0.002		0.027	0.193
Slunchev Bryag																					
Mean	4.0	1.0	0	0.89	11.17**	330.7*	2.755	2.83**	0.703	3.36	197.32**	14.793	NA	0.127*	21.20**	0.579	0.164	0.163	NA	14.10	275.89*
SD	1.41	0	0	0.05	1.12	28.00	0.24	0.25	0.23	0.53	14.88	0.99		0.012	2.590	0.062	0.009	0.006		1.430	34.446

Table 1. Continuation.

Microplastics				Oxidative Stress Markers								Trace Elements									
	≤25 µm	25- 100 µm	101- 200 µm	LPO	PO	GSH	SOD	CAT	GPx	GR	GST	AChE	As	Cd	Cu	Cr	Ni	Pb	Hg	Zn	Fe
Pomorie																					
Mean	8.62**	0.55	0	0.54	4.166	200.69	15.17**	2.70**	10.03**	6.52	98.033	73.580	NA	0.047	7.68	0.065	0.186	0.172	NA	13.41	46.613
SD	4.35	0	0	0.03	0.35	16.15	0.15	0.09	1.53	0.44	10.10	6.93		0.003	0.017	0.001	0.008	0.109		0.036	0.260
Sozopol																					
Mean	6.0*	2.0	0	0.66	5.223	177.48	19.40**	3.33**	14.20**	8.55*	111.387	85.728	NA	0.214*	5.77	0.066	0.015	0.0685	NA	12.61	57.073
SD	2.25	1	0	0.05	0.58	12.00	1.72	0.19	0.96	0.98	11.93	6.75		0.129	0.032	0.000	0.002	0.001		0.028	0.574
Duni																					
Mean	0.84	0.11	0	0.71	4.922	179.81	21.84**	2.71**	11.81**	4.91	113.379	86.414	NA	0.049	6.39	0.029	2.765	1.866*	NA	12.93	24.145
SD	0.15	0	0	0.09	0.68	28.00	1.91	0.19	1.21	0.72	13.49	11.13		0.002	0.054	0.002	0.057	0.008		0.051	0.037
Arkutino																					
Mean	2.39	0.11	0	0.60	4.179	423.89**	2.853	2.21**	1.480	9.46*	93.921	11.108	NA	0.054	5.98	0.053	0.016	0.068	NA	10.22	46.241
SD	0.2	0	0	0.05	0.81	22.00	0.44	0.13	0.37	1.80	18.17	1.67		0.002	0.015	0.002	0.003	0.004		0.042	0.197
Ahtopol																					
Mean	8.42**	1.08	0.72**	0.69	9.882**	370.33*	2.224	2.72**	2.264	4.09	116.581	19.207	NA	0.230	18.71*	0.101	0.143	0.306	NA	10.07	41.553
SD	3.67	0.37	0.52	0.02	0.75	18.00	0.28	0.13	0.38	0.58	10.08	1.67		0.10438	0.43641	0.00265	0.01428	0.00786		0.04	0.14739
Cerastoderma glaucum – Varna Lake																					
Mean	1.40	0.49	0.40	0.66	8.08**	217.68	4.820	2.22*	3.755	7.74*	167.06	27.496	4.32	0.590*	13.89*	4.80**	24.52**	4.45**	1.66**	66.08*	957.57**
SD	0.22	0.22	0.18	0.63	1.81	12.57	2.36	1.06	1.26	1.78	9.18	6.19	0.37	0.02	0.18	0.05	0.55	0.28	0.10	0.71	37.95
Mya arenaria – Varna Lake																					
Mean	2.47	0.53	0.07	0.46	6.34	270.67	2.975	1.973	1.552	34.76	203.83	18.53	3.33	0.570	14.83	3.27	3.68	3.85	0.23	184.11	322.02
SD	0.48	0.16	0.007	0.07	0.65	27.67	0.328	0.287	0.157	2.112	10.18	1.08	0.59	0.07	2.45	0.38	0.66	0.53	0.05	32.41	17.88
Mytilus galloprovincialis – Varna Lake																					
Mean	1.44	1.11	0.33	3.93	5.71	209.22	1.09	0.380	4.10	5.09	193.7	11.83	2.44	6.825	8.03	2.18	2.76	3.05	0.26	150.61	683.44
SD	0.67	0.29	0.16	0.37	0.38	52.25	0.09	0.06	0.23	0.4	0.88	1.17	0.37	5.925	2.61	0.07	0.92	0.26	0.01	49.43	451.84

*Units: LPO [nmoles MDA/mg protein]; PO [nmoles PC/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] and metal concentration [ppm].

Table 2. Estimated values of the correlation coefficient (Spearman) of correlation between trace metal elements and indicators of oxidative stress in mussels (* - significant at $p < 0.05$)

	Cd	Cu	Cr	Ni	Pb	Zn	Fe
LPO	-0.10	0.43*	-0.31	0.33	0.64*	-0.41	-0.07
PO	-0.29	-0.07	0.02	-0.23	0.67*	-0.27	-0.52*
GSH	-0.22	-0.41	-0.39	-0.65*	0.48	-0.35	-0.17
SOD	-0.11	0.64*	-0.31	0.33	0.29	-0.21	-0.06
CAT	-0.23	0.21	-0.28	0.44	-0.20	0.10	0.19
GPx	0.59*	0.32	0.33	-0.16	-0.39	0.19	0.04
GR	-0.42	0.01	-0.29	0.05	0.21	-0.25	-0.28
GST	0.22	0.11	0.24	-0.01	0.02	-0.15	0.45
AChE	-0.36	0.26	-0.08	0.51*	-0.18	0.25	0.31

Table 3. Spearman Correlation Coefficients of the interdependence between total microplastic particles in the studied bivalve species and stress indicators (* - significant at $p < 0.05$)

	<i>M. arenaria</i>	<i>M. galloprovincialis</i>	<i>C. glaucum</i>	<i>D. trunculus</i>
	MPs/ind	MPs/ind	MPs/ind	MPs/ind
LPO	-0.123	-0.273	0.447	0.070
PO	-0.493	0.698	0.670	0.890*
GSH	-0.925*	-0.212	-0.223	0.710
SOD	0.185	0.333	-0.223	-0.760
CAT	0.586	-0.576	-0.892*	0.340
GPx	0.308	-0.212	0.894*	-0.730
GR	0.360	0.880*	-0.223	-0.290
GST	-0.370	-0.151	0.223	0.110
AChE	0.655	0.162	-0.223	-0.770

Bryag, Ahtopol and *C. glaucum* from Varna Lake), relatively high levels of PO were measured (Table 1).

High GSH concentrations were found in *D. trunculus* from St. Konstantin & Elena, Varna, Shkorpilovtsi and Arkutino, as well as from St. Vlas and Slunchev Bryag. The lowest GSH levels were observed in clams from Obzor and Byala (Table 1).

In a large part of the studied samples, high activities of SOD, CAT and GPx were present, for example, in *D. trunculus* from St. Konstantin & Elena, Byala, Obzor, Pomorie, Sozopol and Duni. High GST activities were found in all studied bivalves from Varna Lake (Table 1).

Higher AChE values were measured in clams from Obzor and Byala compared to those from the other studied locations, suggesting a relatively clean marine environment, since AChE inhibition is a classical biomarker for monitoring contamination and intoxication of a diverse spectrum of organic environmental pollutants.

The relationship between the metal elements and different OS biomarkers was studied by cor-

relation analysis. The estimated correlations in all studied bivalves are presented in Table 2. The data showed that the accumulated metal elements in all bivalves showed a significant correlation with different OS indicators.

Lead content was significantly correlated with the prooxidative stress markers, namely LPO and PO. Copper showed significant correlation with SOD, Ni had significant correlation with GSH and AChE and Fe was correlated with PO. Two of the studied metal elements, i.e. Cr and Zn, did not show a significant relation with the OS markers.

The data from the measured correlations between accumulated MPs and OS markers in bivalves are presented in Table 3. The results showed that MPs particles in the individual species of bivalves had significant correlations with various indicators of the prooxidant and the antioxidant biomarkers of the bivalves.

In *M. arenaria*, the number of MPs was highly and significantly correlated with GSH and a relatively high correlation was present (although not

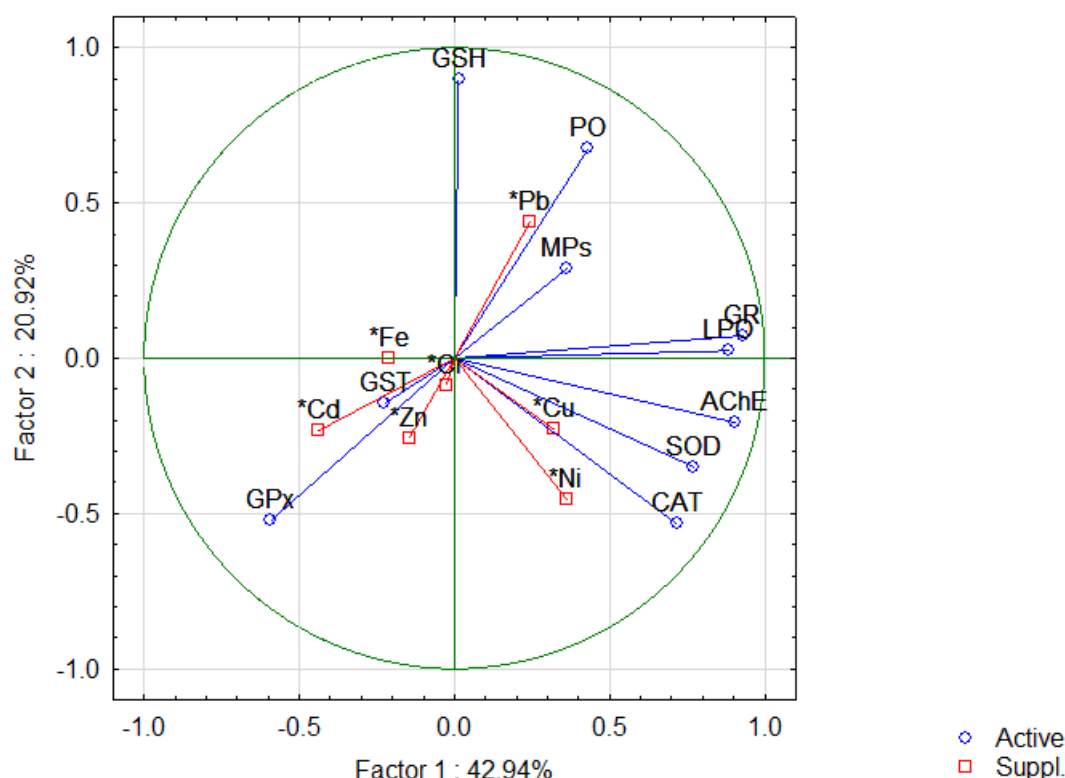


Fig. 2. Factor plot of Principle Component Analysis of the correlations of microplastics, metal elements and oxidative stress indicators in the studied bivalve species

statistically significant) with AChE. In *M. galloprovincialis*, there was a significant correlation with GR and relatively high one (not statistically significant) with PO. In *C. glaucum*, significant correlations were observed with CAT and GPx, as well as a relatively high correlation also with PO. In *D. trunculus*, the amount of microplastic particles was significant and positively correlated with PO, in addition, a high positive correlation (although not statistically significant) with GSH and negative correlations with SOD and AChE activity were measured.

To evaluate the multiple relations and the potential significant joint effects of the accumulated MPs and metal elements on redox status in the studied bivalves, a principal component analysis (PCA) was performed using all the measured variables (Fig. 2).

Although the total variation explained was 63.86%, the results of the analysis clearly indicated the presence of three main groups of inter-correlated variables. Along the main horizontal axis (factor 1), explaining 42.94% of the correlation data, two well-defined groups of related variables were identified. The first group (left part of the plot) consisted of Cd, Zn, Fe and Cr correlated with the OS components GPx and GST. The second group (the right part of the plot) included the remaining components and the

number of microplastic particles was present here. Along the second principal component axis (factor 2), explaining 20.92% of the data (vertical axis), two groups of correlated variables could be identified. The first group (upper part of the ordinate) includes Pb, MPs, PO, LPO, GR and GSH. The second group consists of correlated OS markers CAT, SOD and AChE together with two of the metal elements, Cu and Ni, also correlated with them.

Discussion

The results from this study showed the presence of differences in the accumulation of both metal elements and microplastics, as well as differences in the OS reactions in the studied bivalves depending on the biotic and abiotic characteristics of the marine environment of the locations from which they were collected. The different habitats and locations where the studied bivalve species live are characterised by the presence of multiple environmental stressors, incl. degree of anthropogenic pollution, natural factors, food resources, etc.

The highest accumulation of metal elements in the tissues of bivalves was found in Varna Lake which is characterized by a high degree of industrial and shipping pollution and, respectively, high con-

centrations of metals (especially As, Fe, Cu and Pb) and petroleum products in the sediments and water (Ganchev et al. 2023). The bivalves from Varna Lake showed also interspecies differences in the accumulated metal elements. Specifically, in the soft tissues of *C. glaucum*, the accumulation of Ni and Hg was significantly higher than in the soft tissues of *M. arenaria* and *M. galloprovincialis*. This may be due, at least in part, to the feeding of *C. glaucum* on detritus, whereas *M. galloprovincialis* and *M. arenaria* feed on plankton. There is some evidence that detritus has the highest overall Hg content compared to sediments and organs of aquatic plants (Brahmstedt et al. 2021) and the accumulation of metal elements in feeding on detritus organisms is significantly higher (Windom et al. 1982). In *M. galloprovincialis* significantly higher Cd accumulation was present compared to *C. glaucum* and *M. arenaria* from the same habitats. Cadmium is among the metal elements that can accumulate in high amounts in *M. galloprovincialis* (Oros et al. 2024). Relatively high concentrations of some metal elements (especially Cu) were also established in *D. trunculus* from the water areas of the seaside resorts (i.e. St. Vlas, Slunchev Bryag, Varna, Ahtopol, Shkorpilovtsi, St. Konstantin & Elena). The high accumulation of Cu found in bivalves from sites near large resorts could be primarily caused by high contamination levels due to the combination of runoff from landscape areas and urban runoff, sewage discharge, swimming pool maintenance and boat-related activities (anti-fouling paints).

Concerning MPs, we established high concentrations in all bivalves studied and especially in the clams from the coastal areas of the seaside resorts (Alexandrova et al. 2022). Several studies have also shown a high degree of MPs pollution of recreational beaches (Jaubet et al. 2021, Wu et al. 2021, Hines et al. 2023, Franco et al. 2023). During the active tourist season, the population in the Bulgarian resort complexes more than doubles, which can lead to a significant increase in the flow from the wastewater treatment plants (WWTP). Research has strongly indicated that the increased human population because of summer tourism is associated with increases in MPs in wastewater from WWTP, thus becoming a significant source of MPs in marine ecosystems (Franco et al. 2023).

Environmental factors (including anthropogenic pressures) are known to cause and shape the OS responses of marine organisms (Lushchak 2011, Costantini 2014). Several studies have shown that both metals and MPs, as main pollutants, can induce the generation of reactive oxygen species (ROS)

(Mejdoub et al. 2017, Kournouto et al. 2020, Moraes et al. 2023, Ferreira et al. 2023, Kadac-Czapska et al. 2023, Das 2023). High levels of ROS lead to oxidative changes in biomolecules and disruption of the structure and functions of the cellular structures they build, resulting in damage of cells and organisms in general, including also death. Moderate levels of ROS production activate signalling pathways that lead organisms to compensate OS and adapt to changing environmental conditions and anchor themselves in different ecological niches. Specifically, ROS act as a direct signal that leads to the release of nuclear factor erythroid 2-related factor 2 (Nrf2) from Keap1, allowing Nrf2 to initiate the transcription of antioxidant and protective genes which regulates the expression of more than 200 genes that encode proteins involved in antioxidant defence (Forman et al. 2021).

In this study, the activation of the antioxidant defence system of bivalves from the different locations in response to environmental factors, incl. metal and MPs pollution, was assessed. The results indicated the presence of adaptive potential to the ecological state of the marine environment in the habitats of the Bulgarian Black Sea sublittoral. Superoxide dismutase, CAT, GPx, GR and GST are enzymes balancing the generation of ROS in living organisms. They interact to scavenge ROS and none of them can fulfil their function independently (Yan et al. 2008, Ighodaro & Akinloye 2018). Superoxide dismutase, CAT and GPx serve as the first line of defence against OS neutralizing the molecules with the potential of developing into a free radical or any free radical with the ability to induce the production of other radicals (Ighodaro & Akinloye 2018, Jomova et al. 2024).

Correlation analyses of metal elements and OS showed the presence of a significant relationship between Pb content in the bivalves and the pro-oxidative stress markers, namely LPO and PO (see Table 2). Lead is known to increase LPO, especially of polyunsaturated fatty acids with more than two double bonds (Yiin et al. 1995) and mussels, incl. those from the Bulgarian Black Sea coast are rich in Eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (Peycheva et al. 2021, 2022). Lead is also known to bind to sulfhydryl (-SH) groups in proteins, which can lead to inhibition of enzyme activity, incl. antioxidants such as SOD, CAT and GPx (Ito et al. 1985, Kasperczyk et al. 2004, Patil et al. 2006). In turn, the inhibitory effects of Pb on enzymes may cause cells to be more susceptible to oxidative insult (Patra et al. 2011). It is known that heavy metal ions, such as Cd²⁺, Hg²⁺ and Pb²⁺ form highly stable

complexes with sulphur, nitrogen and oxygen atoms of proteins thus disrupting their biological functions (Tamás et al. 2014). In addition, metals can interfere with protein folding in vivo and cause protein aggregation in living cells (Sharma et al. 2008, Tamás et al. 2014). Protein oxidation can arise from metal ion binding at side chains, with radical formation occurring in proximity to these residues (Stadtman 1993, Stadtman & Levine 2003). According to this mechanism, it was demonstrated that the complex formed by the binding of Fe^{2+} to the ϵ -amine group in lysine can react with H_2O_2 to form hydroxyl radicals ($\bullet\text{OH}$) via the Fenton reaction, leading to the final production of carbonyls and other products. Other amino-acid residues that can bind Fe^{2+} to generate $\bullet\text{OH}$ are proline, histidine, arginine and cysteine (Welch et al. 2002).

In the present study for the first time, the interactions between MPs and metal elements in the aquatic environment of the Bulgarian Black Sea coast were assessed. The results obtained in this study show that MPs accumulated in individual bivalve species had statistically significant correlations with metal elements and different OS indicators (see Table 3). There was a significant positive correlation between PO and MPs accumulation in *D. trunculus* and a definite (although not highly significant) positive correlation in *M. galloprovincialis* and *C. glaucum*. Our data indicated a clear relationship between metals and MPs accumulated in the studied bivalves. The PCA analysis showed significant correlations between Pb, PO and MPs, which formed a distinct group (see Fig. 2). It can be assumed that the toxic effect of MPs, like that of Pb, is mediated by PO. Bearing in mind that the obtained results do not show a highly significant correlation between LPO and MPs accumulation in the studied bivalves, hypothetically it can be assumed that MPs toxicity could be ascribed to the chemicals, pathogens and microbial biofilms adsorbed on them, which seemed to affect mainly proteins. Some studies revealed that the growth of biofilms can positively affect the adsorption of heavy metals and concentration of heavy metals on MPs will increase as the biofilm matures (Richard et al. 2019, Qi et al. 2021). It was also found that polyvinyl chloride (PVC)- and polypropylene (PP)-MPs adsorb Pb, Cu and Cd to a greater extent than other plastic types (Gao et al. 2014). Recent studies show that after MPs with adsorbed metals enter fish, the metals are released into the organs and tissues of fish, resulting in acute heavy metal poisoning (Emenike et al. 2022, Chen et al. 2023). The low pH in guts/digestive systems of organisms could enhance the desorption of toxic metals and

leading to increased accumulation in their bodies since desorption greatly depends upon pH (Khalid et al. 2021). In fish, the toxic effects of heavy metal adsorption by MPs may affect their molecular (gene expression), cellular (cytotoxicity, oxidative damage, inflammatory response, neurotoxicity and metabolism) and individual (survival, feeding activity and swimming, energy reserves and respiration, intestinal microorganisms, development and growth and reproduction) levels (Chen et al. 2023).

Conclusion

In the present study, for the first time, significant correlations between the effects of MPs and metals accumulation on the OS reaction of bivalves from the Bulgarian Black Sea coast were demonstrated. Environmental factors such as pollution levels, temperature, pH, and microbial communities can affect the accumulation of MPs and metals in bivalves as well as the interactions between them. The obtained results suggest a possible mediation of the OS reaction of bivalves to MPs through metals absorbed by them. The combined effects of MPs and metal accumulation on the OS reaction of bivalves are largely unknown and require further in-depth research.

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