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Multi-Biomarker Responses of Asian Clam *Corbicula fluminea* (Bivalvia, Corbiculidea) to Cadmium and Microplastics Pollutants

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Abstract: One of the most widespread aquatic organisms in the rivers and estuarine ecosystems, in the world, is Asian clam *Corbicula fluminea*. This clam, that can adapt to environmental changes, is an invasive species in several areas and it was adopted as a model for toxicity tests. This study evaluated the effects of the exposure to cadmium (Cd), to microplastics (MPs) and their mixtures on *C. fluminea*. The oxidative stress responses, lipid peroxidation (LPO), changes in the activity of energy-related enzymes and neurotoxicity were assessed on the gill, digestive gland and gonad. The results show that Cd, MPs and their mixtures cause oxidative stress, damage and neurotoxicity. The enzymes superoxide dismutase (SOD), glutathione S-transferase (GST), acetylcholinesterase (AChE) and the LPO levels could be chosen as biomarkers of Cd pollution. Exposure to MPs induced an increase in reduced/oxidized glutathione (GSH/GSSG) ratio and increased AChE activity. The combined exposure to Cd and MPs caused a synergetic effect in gill and gonad, while an antagonism response was recorded in the digestive gland. The results provide new insights for unveiling the biologic effects of heavy metal, microplastics and their mixtures on *C. fluminea*. Besides, we demonstrated that the Asian clam is a good bioindicator of microplastic pollution that can occur in aquatic environments.

Keywords: bioindicator; stress responses; freshwater bivalves; gill; digestive gland; gonad

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1. Introduction

Bioindicators have a key role in understanding pollution levels, bioavailability and the ecological risks of contaminants, fulfilling their goal of monitoring environmental status [1]. Bivalves, among invertebrates, acts as environmental logbooks (valuable sentinel organisms) for indicating levels of different pollutants in the environment [2,3]. These organisms can concentrate and accumulate pollutants substantially above background environmental levels. Since they are filtering organisms, with low excretion rates, they act as traps, storing pollutants [4]. These characteristics make bivalves a good tool for biomonitoring organic contaminants, metals [5] and microplastics (MPs) [6]. According to Rochman et al. [6], bivalves are good bioindicators not only because of their ability to ingest MPs but also for their importance considering food security issues, in what concerns a seafood diet. However, studies of microplastics in freshwater ecosystems are taking their first steps compared to those that have been carried out in the marine environment, which are much more consolidated. Human consumption of these organisms makes them vulnerable to damage that can potentially result from exposure to MPs through this route. However, and as Seltenrich [7] points out, although there are currently no figures on the

precise dose of MPs that cause direct damage to human health, concerns about the risk associated with MPs for humans have been increasing.

The freshwater bivalve Asian clams (*C. fluminea*) have successfully been used to monitor pollution levels in aquatic ecosystems, with in situ toxicity tests (e.g., nanoparticles, toxic trace elements) [8,9], and to study the toxicological effects of MPs in the laboratory [10]. This benthic mollusc has also been regularly adopted as a model due to its strong ability to bioaccumulate metals [8,11,12], and among the tested metals, cadmium (Cd) is the most noxious for freshwater organisms, including *C. fluminea* [13].

The environmental contamination by Cd can be a consequence of anthropogenic activities or natural changes, such as the volcanic eruption, that releases this heavy metal to the atmosphere, surface water and surface soil [14]. Among the anthropogenic sources, the industrial applications (batteries, pigments, chemical stabilizers, coatings and alloys) contribute about 13,000 tons, yearly, worldwide [15]. Also, the phosphorus fertilizers, used in agricultural activities, contain this contaminant, contributing to increase its availability in the environment [16]. Even at very low concentrations, Cd has been regarded as one of the most toxic metals to aquatic organisms, bioaccumulating and leading to increased mortality [17]. A study with Asian clam exposed to Cd revealed the induction of morphological damages and significant increases in catalase (CAT) activity in the digestive gland [18].

In addition to heavy metals, other contaminants, such as MPs, have attracted the attention and concern of researchers and policymakers, due to its ubiquity, potential for bioaccumulation and toxicity [19]. Although constituting an emerging area of research, there is still limited knowledge about their toxic effects on biological organisms.

Microplastics are an anthropogenic pollutant derived from different sources: primary MPs, directly manufactured with this specific size (such as cleansers and cosmetics), mainly released from wastewater treatment plants, and secondary MPs, that result from the fragmentation of macroplastics into smaller items, by several processes (like erosion, abrasion, photodegradation, thermo-oxidative degradation, hydrolysis and microorganisms' actions [20]). Due to their accumulation, and persistence, MPs have become a major environmental problem in today's world, being available even to lower trophic organisms [21]. Indeed, Rochman et al. [10] observed different types of MPs across several levels of biological organization. In the same study, *C. fluminea*, as a prey, showed MPs-pieces in the gastrointestinal tract. Other studies [22,23] demonstrated the presence of MPs in the gut, lumen of the digestive gland, connective tissue, hemolymphatic sinuses and gills' surface. Exposure to these small particles may induce toxic effects to organisms, such as inhibition of the acetylcholinesterase activity, increased lipid peroxidation (LPO) levels and degeneration of the digestive gland [10,22,23].

Moreover, previous studies have addressed the ability of MPs surface to sorb trace metals from aquatic and sedimentary environments [24,25]. Ashton et al. [26] noted that pellets from four beaches, in the southwest England, were enriched with Cd and lead (Pb), with concentrations approaching those of sediment and algal fragments. As a consequence, MPs can increase metal bioavailability to organisms, with unpredictable ecological consequences. The focus of the cited work has been on the accumulation levels, with little attention being paid on characterizing the biological toxicity of the combined action of metals and MPs. Accordingly, the present study aims to carry out a multi-biomarker approach to assess the effects of exposure to Cd, MPs and their mixture, using the macroinvertebrate *C. fluminea* as a biological model.

2. Materials and Methods

2.1. Chemicals

For Cd exposure, cadmium chloride ($CdCl_2$) at analytical grade (99.5% pure), purchased from Sigma-Aldrich (Steinheim, Germany), was dissolved to a concentration of 10 μg Cd L^{-1}. The Cd concentration was chosen according to the levels observed in the environment (0.5–10 μg Cd^{2+} L^{-1}) and shown to exert toxic effects in marine organisms [27].

The exposure solutions were prepared with milli-Q water. All the other chemicals used were of the highest analytical grade available and purchased from Sigma-Aldrich (Germany), Merck (Germany) and Bio-Rad Laboratories (Germany). MPs were obtained by polystyrene (PS) trituration, sieved and particles in a range of sizes up to 200 µm were chosen, to reflect the characteristics of food particles ingested in the natural environment.

2.2. Experimental Design

Healthy clam *C. fluminea* were obtained from the Fluvial Beach of Miradeses in Tua River, a clean site of the northwest (NW) of the Iberian Peninsula (41°33′57.3″ N 7°15′29.4″ W). The bivalves were transported to the University of Trás-os-Montes and Alto Douro (Vila Real, Portugal), in aerated river water, and allowed to acclimatize during a month in a 100 L static tank with dechlorinated tap water (pH 7.3–7.5) and constant aeration, at room temperature (20 °C) and a 14 h light:10 h dark cycle. The water in the aquarium was renewed with a rate of 2/3 every 48 h, to ensure the constancy of water parameters.

During the acclimation period, bivalves were fed every day with a mixture of cultivated *Chlorella* sp. and *Chlamydomonas* sp. microalgae, as described by Oliveira et al. [23], and were monitored daily to check mortality. Only healthy clams with uniform size were used for the assay. Following the criteria for harvesting wild bivalves for human consumption, only clams with intact well-closed shells, evidencing no fissures, were chosen. The selected clams were placed on trays for a few minutes to ensure that they put their feet out and that, after stirring the water, firmly closed the shells.

After the acclimation period, 5 specimens per liter (n = 300) of *C. fluminea* (length: 1.5 ± 0.2 cm) were randomly selected and moved to 5 L glass tanks. Three glass tanks were used per experimental group. The bioassay was carried out over 7 days and included the following four treatments, in triplicate: control, cadmium (10 µg Cd L^{-1}), MPs (2 mg MPs L^{-1}) and the mixture of Cd (10 µg Cd L^{-1}) and MPs (2 mg MPs L^{-1}). The specimens were not fed during the experiment to avoid the interactions of MPs and food. After 7 days of exposure, water samples and mollusks (n = 3) were collected to determine the concentrations of cadmium. All samples for Cd determinations were immediately collected to analyze, as indicated below. Gill, digestive gland and gonad were excised from two clams per replica (3 replicas per treatment; n = 6). Gill is the first organ exposed to environmental contaminants, while the digestive gland is the major organ for the accumulation of metals and other contaminants [28]. The gonad was selected to improve the knowledge about the reproductive toxicity in bivalves, which remains scarce. All excised organs were homogenized in cold buffer (0.32 mM of sucrose, 20 mM of 4-(-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer), 1 mM of $MgCl_2$ and 0.5 mM of phenylmethyl sulfonylfluoride (PMSF), pH 7.4), centrifuged at 15,000× g for 20 min at 4 °C (Sigma model 3K30, Osterode, Germany), and the post-mitochondrial supernatant (PMS) was collected and stored at −80 °C until further analysis.

2.3. Cd Quantification in Water and Bivalve Organs

At the end of the experiment, water samples of each treatment were collected from the tanks and sent for metal analysis. The samples were acidified (65% HNO_3, Merck, Darmstadt, Germany) and stored at 4 °C until analysis. The whole bodies of Asian clam were collected from each treatment and stored at −20 °C until analysis. For the analysis of Cd concentrations, each sample was first digested with 2 mL of 65% p.a. (pro analysis) of nitric acid (Merck, Darmstadt, Germany) and 1 mL of 30% of hydrogen peroxide (Merck, Darmstadt, Germany), incubated at room temperature during 24 h and then heated until the solution was clear. The samples were then dried at 155 °C and cooled to room temperature. In the end, 5 mL of HNO_3 matrix solution was added to the digested samples and stirred. The Cd content in the water and the digested samples was quantified using electrothermal atomic absorption spectrometry (Unicam 939 Spectrometer, GF90 furnace). All samples were analyzed in duplicate, ultrapure water was used to prepare the standard solution and

other reagents and the freshly prepared working solutions were diluted with 0.5% (*v*/*v*) HNO_3 solution to obtain a series of working solutions for plotting the calibration curves, safeguarding quality assurance/quality control (QA/QC). The Cd concentrations in water and organisms are shown as the mean ± standard deviation (SD).

2.4. MPs Scanning Electron Microscopy plus Energy-Dispersive X-Ray Spectroscopy (SEM/EDS) Analysis

Polystyrene (PS) microparticles were characterized using scanning electron microscopy plus energy-dispersive X-ray spectroscopy (SEM/EDS). SEM generates high-resolution imaging of particles while EDS allows the identification of chemical elements in MPs samples with the distinction of non-plastic interferences [28]. This technique has been used to perceive MPs particles in the sample [29–31]. SEM/EDS was conducted using a SEM in environmental mode (SEM/E-SEM FEI Quanta-400) equipped with an EDS System. PS samples were mounted on double-sided adhesive carbon tabs on an aluminum SEM stub. Samples were imaged at 50×–400× using the microscope's backscattered electron (BSE) detector, with a resolution of approximately 0.1 µm. After imaging, the samples underwent electron dispersive X-ray spectroscopy (EDS) analysis for the determination of elemental composition. The polymers were identified based on the matching with the spectral database and the presence of featured adsorption bands [32].

2.5. Biochemical Analyses

The evaluated parameters reflect the effects of the pollutants on the individual's health, with consequences on the development, longevity and reproduction, encompassing: (i) production of damaging reactive oxygen species (ROS), (ii) repair mechanisms for oxidized components, including superoxide dismutase (SOD), catalase (CAT) and glutathione-s-transferase (GST), (iii) indicators of redox homeostasis, such as reduced (GSH) and oxidized (GSSG) glutathione levels, (iv) indicators of cell damage, such as lipid peroxidation (LPO), and metabolism parameters, like lactate dehydrogenase (LDH) activity and (v) neurotoxicity, evaluated through acetylcholinesterase (AChE) activity. The activity of AChE has been widely studied in the brain and muscle, but little studied in other organs, and bivalves have high levels of AChE activity in the gills and digestive gland [33,34]. The ROS was measured fluorometrically by comparing with the dichlorofluorescein (DCF) standard curve at 485 nm excitation/530 nm emission, according to Deng et al. [35]. SOD activity was determined at 560 nm, according to Durak et al. [36], by measuring the superoxide anion generated by the xanthine oxidase/hypoxanthine through the inhibition of the nitroblue tetrazolium (NBT) reduction. SOD from bovine erythrocytes was used to construct a standard curve (0–3.75 U mL^{-1}). Catalase activity was measured at 240 nm, following the method of Claiborne [37], based on the decomposition of hydrogen peroxide. Bovine CAT was used as a standard (0–5 UmL^{-1}). GST was determined at a wavelength of 340 nm, using the method described by Habig and Jakoby [38], following the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH. The glutathione levels were determined by measuring both GSH and GSSG [39], using the fluorochrome ortho-phthalaldehyde (OPA), at 320 nm and 420 nm, for excitation and emission wavelengths. The GSH and GSS concentration (in nmol/mg protein) was determined from a standard curve of pure GSH and GSSG respectively, which was linear in the range of 0–10 mM. The oxidative stress index (OSI) was determined by the ratio between GSH and GSSG. LPO levels were assessed through the quantification of thiobarbituric acid reactive substances (TBARS) at 535 nm, based on Gartaganis et al. [39]. AChE activity was determined at 405 nm, according to the method of Rodriguez-Fuentes [40], described for microplates, using the 5-thio-2-nitrobenzoic acid (TNB) extinction coefficient of 13.6 $mM^{-1}cm^{-1}$, and expressed as µmol TNB/min.mg protein. For LDH activity, the determination was performed according to Domingues et al. [41] at 340 nm using the extinction coefficient of 6.22 $mM^{-1}cm^{-1}$. The total protein content of homogenates was determined according to Bradford [42] at 595 nm using bovine serum albumin as the standard (0–1.4 mg/mL^{-1}). The assays were carried in duplicate as described by Felix et al. [43], at 30 °C using a Power Wave XS2

microplate scanning spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) or a Varian Cary Eclipse (Varian, Palo Alto, CA, USA) spectrofluorometer, equipped with a microplate reader.

2.6. Statistical Analyses

Assumptions of normality and homogeneity of variances were assessed using Shapiro–Wilk's and Levene's test, respectively. Data was evaluated through analysis of variance (one way-ANOVA) followed by Tukey's multiple comparison test, to detect differences in biological parameters among fish from four experimental groups. Data are expressed as mean ± standard deviation (SD). If ANOVA assumptions failed, groups were compared by a non-parametric independent samples test, Kruskal–Wallis, followed by Dunn's pairwise comparison tests. $p \leq 0.05$ values were considered significant and all statistical analyses were performed using Sigma-Plot 12.0.

To test the influence of environmental variables (Cd and MPs concentrations) in the different studied biomarkers, in exposed *C. fluminea*, we performed a multivariate analysis. Since the response of data have a gradient of 1.4 SD units long and contain 6.9% of zero values, a linear method is recommended. The principal component analysis (PCA) of the studied variables was applied to reduce the dimensionality and extract the dominant factors. Correlation coefficients between each variable and principal component are known as loadings, and these explain the correlativity of the original variables and the principal components. Variables were standardized before all analyses to preserve the original scale. The PCA was carried out using CANOCO 5 software (version 5.12) [44].

3. Results

3.1. Cadmium Accumulation

Cd concentrations of water samples from the tanks and accumulation in bivalve tissues after single and combined exposure (Cd and MPs) are shown in Table 1. Considering water samples, Cd concentrations increased in the order of control < MPs < mixture < Cd. Likewise, in body tissues, compared to the control and MPs groups, Cd bioaccumulation significantly increased in animals exposed to Cd, single and in mixture with MPs. However, contrary to what was observed in the water samples, in bivalve tissues, the highest Cd levels (1.86 ± 0.37 $mg{\cdot}g^{-1}$ live weight—LW) were found in the groups exposed to the mixture of Cd and MPs.

Table 1. Cadmium (Cd) concentrations determined in water and clams from control, Cd, microplastics (MPs) and Mix groups, after 7-days of exposure.

Cd Levels	Control	Cd	MPs	Cd + MPs
Water ($\mu g\ L^{-1}$)	0.35 ± 0.01	6.73 ± 0.10	0.29 ± 0.10	4.94 ± 0.24
Clam (μg Cd g^{-1} LW)	0.51 ± 0.20	1.35 ± 0.12	0.70 ± 0.05	1.86 ± 0.37

3.2. Characterization of MPs Particles

The fragmented MPs samples had an irregular shape and a cracked surface, with many small fragments, resultant from the mechanical action (Figure 1A–C). This plastic debris showed a wavy appearance, exposing a smooth surface. EDS spectra of particles reveled a significant carbon peak (Figure 1D), which was consistent with the infrared identification of polystyrene (PS). Also, the particles exhibited small peaks of titanium (Ti), suggesting the presence of pigments made of titanium oxides. The titanium oxides in these particles are consistent with their white color.

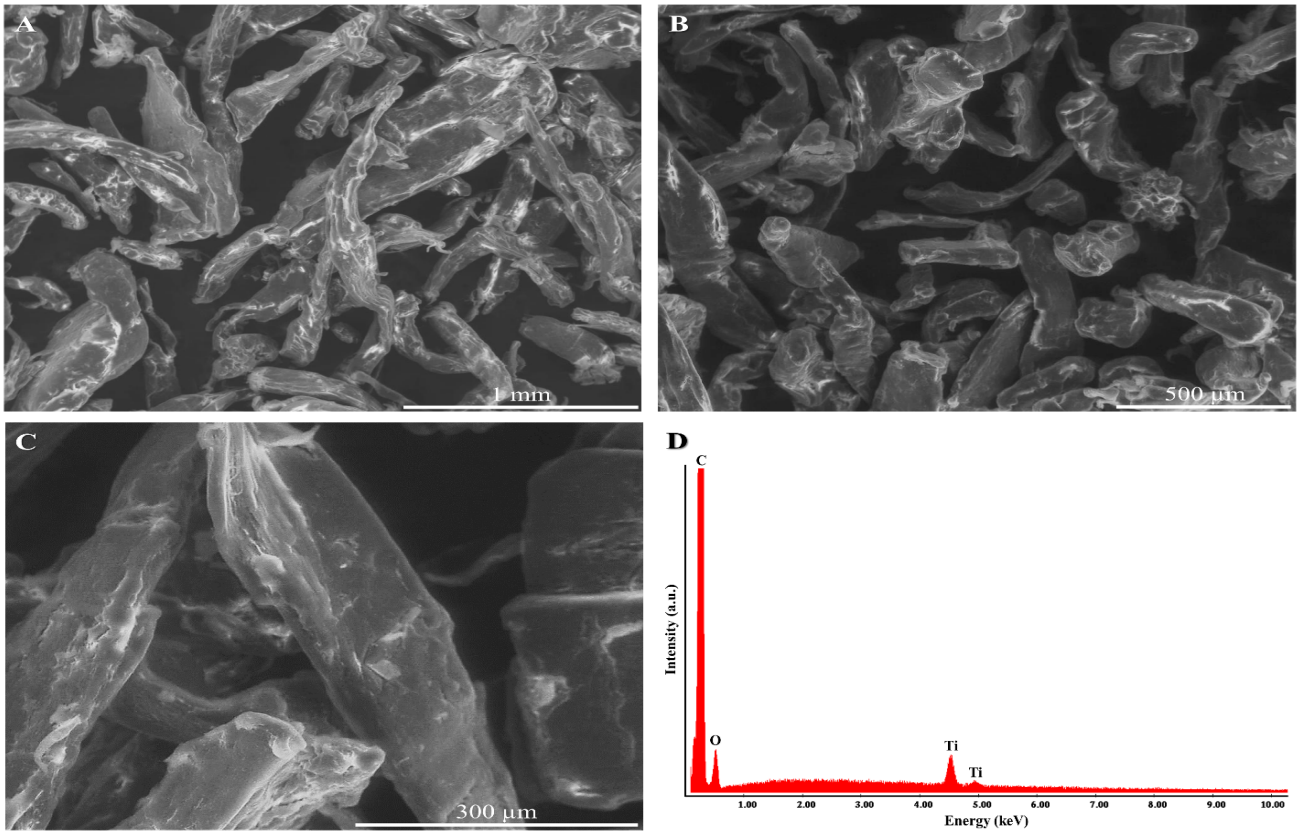

Figure 1. Scanning electron microscopy (SEM) images of microplastic particles showing the triturated surface cracks from low to high magnification (from (**A**–**C**)). Electron dispersive X-ray spectroscopy (EDS) spectrum (**C**) showing C and O peaks as expected from the types of polystyrene and showing titanium (Ti) indicating the presence of white pigments (**D**).

3.3. Oxidative Stress

The biochemical responses to the exposure to Cd and MPs, alone or in the mixture, are presented in Table 2.

In gill, exposure to Cd, alone or in the mixture with MPs, induced a significant increase ($p < 0.01$) in reactive oxygene species (ROS)production. However, exposure to MPs alone did not affect ($p > 0.05$) ROS levels, when comparing to the ones observed in animals from the control group. Considering SOD activity, exposure to both Cd and MPs caused a significant ($p < 0.01$) inhibition in the enzyme activity, that was much more noticeable in animals exposed to Cd, alone and in mixture with MPs. On the other hand, the CAT activity slightly increased in the gill of clams submitted to waterborne Cd. This increase was statistically different ($p < 0.01$) from the control values in animals exposed to MPs and the mixture of Cd with MPs. The ratio GSH/GSSG, indicative of the cells' oxidative stress level, significantly decreased ($p < 0.01$) in gill, after 7 days of exposure to Cd, alone or in mixture with MPs, while exposure to MPs alone significantly increased ($p < 0.01$) this ratio. In line with these results, the LPO levels significantly increased ($p < 0.01$) in gills of animals exposed to Cd, alone and in mixture with MPs. MPs also significantly increased the LPO levels, although not as pronounced as that observed in clams exposed to Cd.

In digestive gland, exposure to Cd, alone or in mixture with MPs, induced a significant increase ($p < 0.05$) in ROS production. However, exposure to MPs alone did not affect ($p > 0.05$) ROS levels, when comparing to the ones observed in animals from the control group. Considering SOD activity, exposure to Cd, alone or in mixture to MPs, caused a significant inhibition ($p < 0.01$) in the enzyme activity, while for MPs treatment, no significant changes ($p > 0.05$) occurred. On the other hand, the CAT activity increased significantly ($p < 0.05$) in the digestive gland of clams exposed to Cd, MPs and the mixture of

Cd with MPs, but the increase was more pronounced in the Cd group. The ratio GSH/GSSG significantly decreased ($p < 0.01$) in the digestive gland, after 7 days of exposure to Cd, alone or in mixture with MPs, while exposure to MPs alone did not induce significant changes ($p > 0.05$) in this ratio. In line with these results, the LPO levels significantly increased ($p < 0.01$) in the digestive gland of animals exposed to Cd, alone and in mixture with MPs, while exposure to MPs alone did not induce significant changes ($p > 0.05$) in LPO levels.

Table 2. Biochemical biomarkers activities in gill, digestive gland and gonad of *Corbicula fluminea* after exposure to Cd and/or MPs for 7 days.

Biochemical Parameters	Analyzed Organs	Control	Cd	MPs	Cd + MPs
ROS µmol DCF/mg of protein	Gill	20.06 ± 3.7 a	31.25 ± 4.5 b	18.65 ± 1.7 a	29.76 ± 3.8 b
	Digestive gland	49.2 ± 5.2 a	103.0 ± 9.8 b	51.8 ± 4.8 a	62.8 ± 9.6 c
	Gonad	72.1 ± 4.7 a	54.0 ± 4.0 b	41.5 ± 6.5 c	53.3 ± 6.2 b
SOD U/mg of protein	Gill	661.3 ± 75.5 a	3.8 ± 0.8 b	351.6 ± 12.5 c	5.7 ± 1.3 b
	Digestive gland	483.0 ± 19.7 a	6.9 ± 1.0 b	465.7 ± 28.6 a	13.1 ± 2.3 b
	Gonad	2040.4 ± 194.8 a	5.4 ± 1.3 b	406.7 ± 25.8 c	3.4 ± 0.4 b
CAT U/mg of protein	Gill	65.5 ± 9.3 a	72.8 ± 7.4 a	91.6 ± 10.4 b	84.0 ± 6.6 b
	Digestive gland	71.2 ± 9.1 a	237.2 ± 21.3 b	91.0 ± 6.5 c	111.3 ± 3.8 d
	Gonad	315.9 ± 31.6 a	309.9 ± 35.0 a	91.4 ± 8.4 b	544.4 ± 67.3 c
GST nmol CDNB/min.mg of protein	Gill	10.6 ± 1.6 a	22.7 ± 2.0 b	11.0 ± 4.6 a	39.7 ± 3.5 c
	Digestive gland	25.5 ± 3.1 a	173.0 ± 14.7 b	33.5 ± 1.5 a	220.6 ± 10.9 c
	Gonad	44.0 ± 10.2 a	159.3 ± 19.8 b	29.0 ± 2.8 a	135.9 ± 11.2 c
Reduced/Oxidized Glutathione Ratio	Gill	0.8 ± 0.03 a	0.1 ± 0.01 b	1.2 ± 0.1 c	0.1 ± 0.02 b
	Digestive gland	0.6 ± 0.02 a	0.1 ± 0.01 b	0.6 ± 0.08 a	0.1 ± 0.01 b
	Gonad	1.1 ± 0.1 a	0.1 ± 0.02 b	1.6 ± 0.2 c	0.08 ± 0.03 b
LPO nmol MDA/mg protein	Gill	0.2 ± 0.03 a	0.7 ± 0.08 b	0.4 ± 0.05 c	1.0 ± 0.1 d
	Digestive gland	0.3 ± 0.03 a	1.2 ± 0.1 b	0.4 ± 0.1 a	0.8 ± 0.03 c
	Gonad	0.5 ± 0.05 a	0.7 ± 0.04 b	0.3 ± 0.03 c	0.3 ± 0.01 c

DCF—dichlorofluorescein, U—Units, MDA—malonaldehyde, CDNB—1-chloro-2,4-dinitrobenzene. Data are expressed as mean ± standard deviation (SD) from four experimental groups. Different lowercase letters indicate significant differences between groups ($p < 0.05$).

In gonad, exposure to Cd, alone or in a mixture with MPs and MPs alone, induced a significant decrease in ROS production ($p < 0.01$). Regarding SOD activity, the exposure of both Cd and MPs caused a significant inhibition ($p < 0.01$) in the enzyme activity. A significant increase ($p < 0.01$) in the CAT activity was observed in the gonad of clams exposed to the mixture of Cd and MPs, while a significant decrease ($p < 0.01$) was observed in the gonad of organisms exposed to MPs alone. The ratio GSH/GSSG exhibited a significant decrease ($p < 0.01$) in the gonad, after 7 days of exposure to Cd, alone or in mixture with MPs, and the exposure to MPs alone induced a significant increase ($p < 0.01$) in this ratio. The results showed a significant increase ($p < 0.01$) of LPO levels in the gonad of animals exposed to Cd alone, however MPs, alone and in mixture, induced a significant decrease ($p < 0.01$) in LPO levels.

3.4. Metabolic Changes

All LDH results are accessible in Figure 2. In gill, digestive gland and gonad, a significant reduction ($p < 0.05$) of LDH activity was observed in animals exposed to Cd, alone or in the mixture to MPs, and MPs alone.

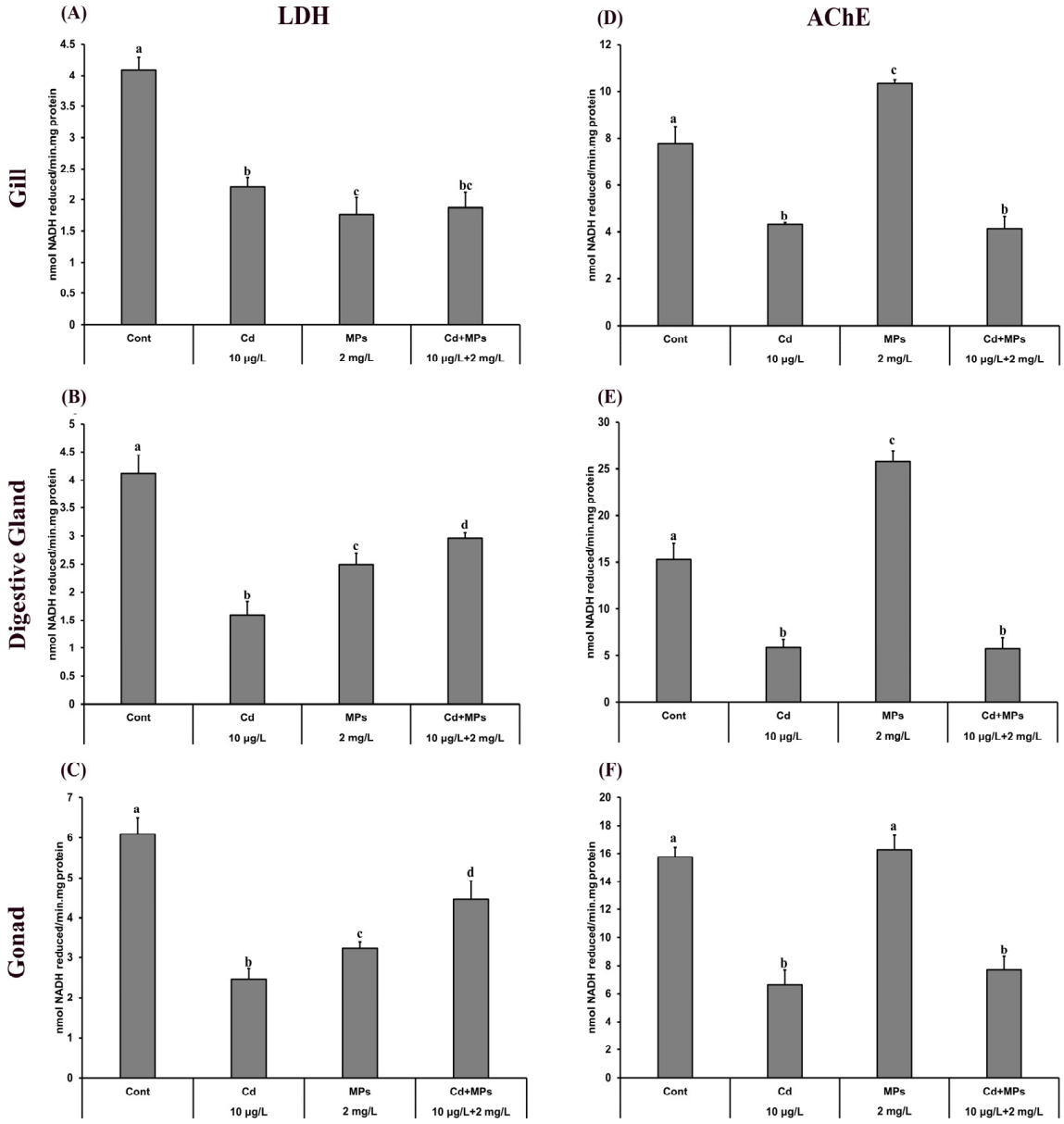

Figure 2. Lactate dehydrogenase (LDH) levels and acetylcholinesterase (AChE) activity determined in the gill (**A**,**D**), digestive gland (**B**,**E**)or gonad (**C**,**F**) of *Corbicula fluminea* after exposure to cadmium (Cd) and/or microplastics (MPs) for 7 days. Data are expressed as mean ± SD from four experimental groups. Different lowercase letters indicate significant differences between groups ($p < 0.05$).

3.5. Neurotoxicity

The neurotoxicity evaluation in clam tissues is shown in Figure 2. Relative to the control group, animals exposed to Cd, alone and in mixture with MPs, showed a significant reduction ($p < 0.01$) of AChE activity in gill, digestive gland and gonad. In gill and digestive gland, bivalves exposed to MPs alone showed a significant increase of AChE activity ($p < 0.01$), but in gonad, no significant changes ($p > 0.05$) occurred in AChE activity.

3.6. Principal Component Analysis (PCA)

The PCA results show that the studied variables related to biochemistry, namely the enzymatic activity on different *C. fluminea* organs, were more sensitive to the concentration of Cd than to the MPs concentration (Figure 3A,B). The first principal component

explained 59.90% of the overall variance, and the first two principal components explained 80.41%. For all the variables, the highest contribution of the individual response to the ordinations axis was SOD (0.0962), CAT (0.8830) and GST (0.507) (Figure 3A). While the PC1 axis variation is more interrelated with the different treatments applied, the PC2 axis is more associated with organ differences. In other words, axis PC1 is negatively correlated with the gradient of Cd concentration and positively correlated with MPs concentration. The samples located in the upper left quadrant (mainly gill, Figure 3C) of the plot were associated with treatments of Cd and mixture of Cd with MPs. Also, they are associated with inhibition of SOD activity, together with higher levels of LPO. The samples located in the lower left quadrant (mainly digestive gland and gonad) of the plot were also associated with treatments of Cd and its mixture with MPs. Furthermore, in this same quadrant, samples were associated with induction of GST and increased levels of ROS and LPO, together with lower levels of GSH/GSSG ratio and AChE. The samples located in the upper right quadrant (mainly gill) of the plot were associated with treatments of control and MPs alone and also associated with increased levels of GSH/GSSG ratio and AChE, together with lower levels of ROS and LPO. The samples located in the lower right quadrant (mainly gonad) of the plot were associated with treatments of control and MPs alone and also with increased levels of GSH/GSSG ratio. The results show that levels of ROS and LPO have the opposite behavior of levels of GSH/GSSG ratio and enzymatic activity of AChE. Moreover, the enzyme CAT is highly negatively correlated with axis PC2. The activity of SOD and LPO levels are more positively and negatively associated with axis PC1, respectively.

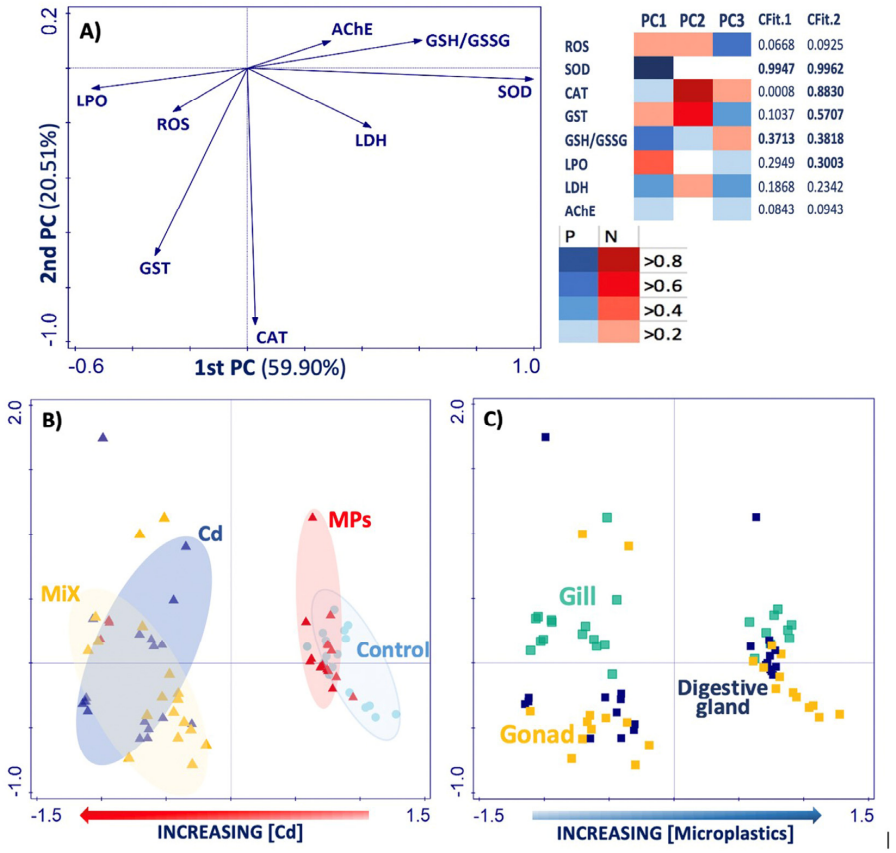

Figure 3. (**A**) Principal component analysis (PCA) based on (**B**) biochemical variables analyzed in *Corbicula fluminea* exposed to MPs and Cd, alone or combined (MIX), in different organs, (**C**) Gill, Gonad and Digestive gland. Correlation of the individual response variables to the different ordination axis, blue indicate positive, and red negative, relationship with principal components, deeper color means higher correlation. Additionally, the cumulative fraction of variation in individual response variables by the first two axes is shown.

4. Discussion

Heavy metals, such as Cd, can be accumulated by benthic marine organisms, inducing physiologically adverse effects [45]. It has been suggested that the mechanisms of acute Cd toxicity result in a stimulation of ROS generation, which can lead to enhanced oxidative stress and generate LPO [46]. Our results showed that Cd can accumulate in *C. fluminea* body tissues and generate ROS production. Bivalve molluscs produce ROS as part of their basal metabolism in response to endogenous and exogenous stimuli [47]. As noted in this research, *C. fluminea* exposed to Cd, combined with MPs and MPs alone, showed an increase in the ROS levels in gill and digestive gland. In the mixture group, the production of ROS was probably induced by Cd since the exposure to PS microparticles did not induce relevant changes in ROS production in both organs. Besides the ROS generation in *C. fluminea*, the activities of several oxidative stress-related enzymes were additionally examined, to assess the effects of combined and single Cd and MPs exposure.

SOD is one of the most active enzymes to respond to ROS [48] and a loss of SOD activity was observed in the gill of animals exposed to Cd, alone and in mixture with MPs, which reflect that the organism may have suffered some degree of damage caused by the stress induced by these pollutants. Two possible reasons may explain this response. On one hand, an exhaustion of the organism defenses due to severe stress, which reduces the scavenging capacity of SOD [49,50]. On the other hand, a direct interaction between pollutants and SOD could occur, damaging or inhibiting the enzyme. To support the last hypothesis, it is documented that the toxicity induced by Cd is involved in a reduction in SOD activity in several vertebrate organs [51,52], although these inferences should be demonstrated by further experiments. For single MPs exposure, SOD activity did not significantly differ from that of the control in digestive gland, while in gill and gonad, a significant decrease of SOD activity was observed. This suggests that MPs exposure during 7 days induced toxicity in gill and gonad, whereas the same does not occur in the digestive glands.

In gill, CAT presented a steady activity and was not affected by exposure to Cd alone. Like SOD, CAT plays an important role in protection against oxidation, which is crucial in the detoxification of oxyradicals to non-reactive molecules [48]. However, seven days of Cd exposure may not be sufficient to induce a significant response of this enzyme or there is a non-significant contribution of CAT to antioxidant defense processes in response to Cd. Also, in the gill, in response to MPs exposure (alone and in mixture), the activity of CAT increased significantly, revealing the toxicity of MPs for this organ, thus having a protective response to avoid possible oxidative damage. The same response was also noted by Ribeiro [53] in *S. plana* gills after three days of exposure to PS MPs. In the digestive gland, a significant increase in the CAT activity was observed after the exposure to Cd alone. This result is consistent with the finding of Borković-Mitić et al. [54], who observed high CAT activity in the digestive gland, compared to the gills, after exposure to metals. The authors argue that the bioavailability of pollutants entering through the digestive tract trigger a high activity of the antioxidant defense system in the digestive gland. Still, in the digestive gland, CAT activity induction was recorded after exposure to MPs, alone and in mixture with Cd. Furthermore, the negative impacts of Cd might have decreased in the presence of MPs, in mixture, due to a possible antagonistic interaction between the two stressors. Also, considering the response to MPs exposure, a higher CAT activity was observed in the gill, compared to the digestive gland. As referred by Martins et al. [55], the mechanisms of CAT response have to be more rapid and efficient in gills than in other tissues. In gonad, the exposure of Cd alone induced the same steady state of enzyme activity that was observed in the gill. In this organ, the exposure of MPs alone caused a decrease in CAT activity and while the mixture displayed a significant activity of CAT, pointing to a possible synergetic effect of Cd and MPs on the CAT activity. The divergent behavior in the CAT-oxidative stress response of both pollutants suggests different reactions among the three organs on the response to the same stress conditions.

In the gill, digestive gland and gonad, a significant increase of GST activity was found in clams exposed to Cd, single and combined, suggesting a compensatory adaptive mechanism to neutralize increased levels of ROS when SOD was depleted. The GST enzyme is involved in the detoxification mechanism of many xenobiotics, including metals, playing a critical role in the protection of tissues from oxidative stress [56]. Some studies have described the activity of GST as having a good response to environmental contamination [56,57] and it has been suggested as a useful biomarker in Cd exposure [58]. The GST activity in animals from the mixture group revealed a possible interaction between Cd and MPs, which display a synergistic toxic effect of Cd in the gill. Selvam et al. [59] results associate MPs as a major vector to transport heavy metals in the water system. However, studies on the physiologic effects of the interaction of heavy metals adsorbed by MPs are remarkably scarce. Furthermore, in the gill, digestive gland and gonad, CAT and GST function together to neutralize the generated ROS against the oxidative stress. However, the response of these enzymes, in *C. fluminea,* can be different in each organ, as observed in the present study, with gill and gonad inducing GST activity as the first response to Cd stress; while in the digestive gland, the CAT activation was the primary observed response to the same stress. The catalytic action of GST was also confirmed by the observed decrease in oxidized glutathione ratio, observed in the gill, digestive gland and gonad of clams exposed to Cd, alone and in the mixture.

The GSH/GSSG ratio is an important biomarker of oxidative stress induction [60], possibly due to GSH oxidation into the oxidized form of GSSG under oxidative stress, a strategy reported in clam *Ruditapes philippinarum* to eliminate the excess ROS [61]. Copola et al. [62] also support the use of the GSH/GSSG ratio in the assessment of impacts caused by metals, in bivalves.

LPO levels are an important indicator of oxidative damage in aquatic organisms because these animals contain high amounts of lipids with polyunsaturated fatty acid, a substrate for oxidation [63]. This parameter has been proven as a useful biomarker of oxidative damage induced by heavy metal exposure [50]. The present work was performed to compare the sensitivity of LPO as a stress biomarker of chemical stress induced by Cd and MPs. In gill, exposure to both pollutants and the mixture induce a significant increase in LPO levels in the gill of clams, as previously reported in the gill of mussels (*Mytilus galloprovincialis*) after exposure to sub-lethal concentrations of Cu, Cd, Pb and Fe [64] and in gill of *Scorbicularia plana* when exposed to virgin MPs [65]. It has been described [66] that low activity of SOD would lead to the accumulation of superoxide anion, thus producing hydroxyl radicals in the presence of environmental contaminants and causing oxidative stress. In this study, SOD activity decreases in the gill, which made clams vulnerable to oxidative damages such as LPO. In the digestive gland, the stress of Cd, single and combined, affected the levels of ROS and, probably, prompted an increase in LPO content. Similar results in *Perna viridis* digestive gland antioxidant responses show an insufficient capacity to counteract the ROS formation, leading to increased LPO levels [66]. The CAT exhibited an antagonistic response, in clams from the mixture group, which equally revealed a reduction in LPO levels, indicating the decrease of damage in the presence of MPs. In gonad, the results observed in the group of animals exposed to Cd alone are comparable to the ones observed in gill. The loss of SOD-antioxidant defense in the gonad was paralleled by higher LPO level. However, the gonad results in the treatments of MPs, alone and in the mixture, could be explained by the depletion in SOD activity, which leads to an increase of LPO content.

LDH are ubiquitous in nature, involved in balancing the equilibrium between the anabolism and catabolism of carbohydrates [67]. Induction of LDH in aquatic invertebrates and vertebrate organisms have been associated with metal and MPs contamination, which might be due to induction of an alternative anaerobic glycolytic pathway for energy production [68,69]. In gill, digestive gland and gonad, our results show a significant decrease of LDH activity caused by the administered contaminants, single and in mixture,

suggesting inhibition of anaerobic metabolism. It is reasonable to assume that Cd and MPs stress inhibits cellular oxidation, which is evident from the suppression of LDH activity.

The AChE activity suffered a significant inhibition in gill, digestive gland and gonad of animals exposed to Cd, alone and in the mixture. AChE is an essential enzyme involved in neurotransmission, at cholinergic synapses. However, exposure of organisms to neurotoxic agents inhibits its activity [70]. Accordingly, the AChE inhibition has been used as a biomarker of neurotoxicity, induced by pollutants in aquatic invertebrates [22,53,71]. Consequently, the significant reduction observed in the activity of AChE is indicative of adverse effects in cholinergic neurotransmission, and thus, potentially in nervous and neuromuscular function. Contrarily, the present study reports an induction of AChE in gill and digestive gland as a response to MPs exposure, and some authors [72,73] detected active AChE during the apoptosis process by different stimuli, but several questions still need to be addressed, and this possible process after MPs exposure needs to be studied further.

According to PCA, there is a greater response of CAT activity to stress in the digestive gland and gonad. Also, the presence of Cd mainly promotes an inhibition of the SOD and the CAT enzyme and LPO levels were induced by the presence of this contaminant. On the other hand, increasing levels of GSH/GSSG ratio and AChE are highly correlated with the control and MPs conditions. These results were generally in line with ANOVA results, indicating the serious effects of both stressors on *C. fluminea*.

5. Conclusions

The results of the study illustrate the induction of oxidative stress by inhibition of SOD activity caused by 10 μg Cd L^{-1} exposure, single and in combination with plastic microparticles. Cd exposure also generated a significant reduction in the activity of AChE, indicating adverse effects in cholinergic neurotransmission. Oxidative stress induced by cadmium exposure increased the activity of GST, indicating an attempt of the organism to detoxify the body, although the high LPO levels suggest that Cd levels led to oxidative damage. The results showed that SOD, GST and AChE enzymes and the measure of LPO levels could be chosen as biomarkers of Cd pollution. The MPs presence is related to increased levels of the ratio GSH/GSSG in the body, demonstrating oxidative stress in the system. MPs exposure also causes an induction of AChE, pointing out that the unusually high AChE activity may be an effective marker of exposure to MPs.

The combination of Cd and MPs exposure caused an antagonism response of CAT activity in the digestive gland, and on the contrary, in gonad, a synergetic effect was observed in the same treatment, revealing the divergence on biomarker response in *C. fluminea* to identical stress conditions in different organs. A synergetic effect on GST response was also observed in the gill in animals from the mixture group, pointing to MPs as a possible vector to transport Cd metal. Overall, Cd, MPs and their mixtures cause oxidative stress, damage and neurotoxicity.

These findings accentuate the concern regarding *C. fluminea*, a model of the base of the trophic network, exposed to metals and MPs in freshwater systems, and highlight the need of more research on the subject. This work also demonstrated that the Asian clam is an adequate bioindicator for MPs pollution monitoring in the aquatic environments.

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