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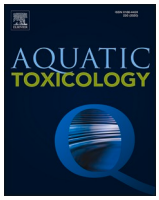

Breaking new ground: Gadolinium and Microplastics co-exposure and biochemical alterations in marine clam *Donax trunculus*

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ABSTRACT

Toxicity of single rare earth elements (REEs) or microplastics (MPs) on organisms has been reported widely, however, their combined toxicity on bivalves has limited investigation. In this study, the effects of gadolinium (Gd, 500 μg/L), microplastics (MPs, Ø ≤ 150 μm, with 0.1 μg/L and 100 μg/L) and their mixture (0.1 MPs μg/L + 500 Gd μg/L; 100 MPs μg/L + 500 Gd μg/L) were evaluated in the clam *Donax trunculus* under laboratory conditions. The impacts were assessed using a suite of biomarkers related to metabolism and energy reserves content, antioxidant and biotransformation enzymes, cellular damage and neurotoxicity. In the medium, at 500 μg/L Gd, the concentration measured after exposure was similar to the nominal concentration, whereas in the mix treatments it decreased, suggesting the adsorption of Gd onto the MPs. Regarding oxidative stress responses, MPs and Gd alone elicited distinct responses, with MPs showing mostly non-significant or inhibitory effects, while Gd increased oxidative stress. When both contaminants were combined, MPs acted as carriers for Gd, demonstrating their "Trojan horse" capability. As a consequence, the mix treatments induced the most severe oxidative stress. The toxicity order in clams was: 0.1 MPs μg/L ≈ 100 MPs μg/L < 500 Gd μg/L < 0.1 MPs μg/L + 500 Gd μg/L < 100 MPs μg/L + 500 Gd μg/L. This study advances understanding of the combined toxicity of MPs and Gd on marine bivalves, highlighting the need for further research on their interactions and broader toxic effects.

1. Introduction

Every year, approximately 300 million tons of single-use plastic products are produced worldwide for various applications across sectors like packaging, construction, electronics, and automotive (Lebreton et al., 2017). Although plastics offer benefits like flexibility, lightness, moisture resistance, and affordability, low recycling rates and improper disposal result in long-lasting marine pollution (Casabianca et al., 2021; Cau et al., 2022). Nowadays, plastic material is recognised as the most prevalent contaminant in aquatic environments (Yu and Singh, 2023), and it is estimated that 15–51 trillion plastic particles, weighing as much as 236,000 tons, are present in global marine systems (Van Sebille et al., 2015). In the literature, various designations have been created to describe the extensive magnitude of plastic pollution, such as the term "Plasticene Age" at a global scale (Rangel-Buitrago et al., 2021), "Mediterranean plastic soup" at a regional scale (Suaria et al., 2016), and "Plasticenta" at the human scale to describe the presence of plastics even in the placenta (Ragusa et al., 2021). Among the various polymers, polyethylene (PE) is the most prevalent in the environment, with average concentrations of 389 ± 377 μg m^{-3} floating in the Atlantic Ocean, followed by polypropylene (PP) at 262 ± 568 μg m^{-3} and polystyrene (PS) at 58 ± 241 μg m^{-3}) (Secco et al., 2025; Vianello, 2013; Pabortsava and Lampitt, 2020). In terms of size classification, microplastics (MPs, 1 μm–5 mm in size) (Frias and Nash, 2019) are the focus of considerable attention worldwide, given that this size range is deemed the most hazardous, with a wide range of organisms able to ingest

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particles of this dimension (Derraik, 2002). Regarding human health, MPs in the environment can enter the human body through the food chain in the form of seafood, livestock products and agricultural products (Hu et al., 2022). Due to their small size, the ingestion of MPs can lead to various physiological harms in organisms, resulting in death from false satiation (Welden and Cowie, 2016). Indoor experiments demonstrated alterations in the condition index and feeding rate of the bivalves *Mytilus edulis* and *M. galloprovincialis* in a 28-day exposure to PP and Polyethylene terephthalate (PET) polymers (Daniel et al., 2024). Biochemical parameters were analyzed by Tlili et al. (2020) in an experiment where the clam *Donax trunculus* was exposed to a PP/PE mixture (100–400 μm, 0.06 g/kg of sand) over 3 h, 1, 2, 3, 4, 7, 10, and 15 days. The results from this study indicated that the gills, rather than the digestive gland or flesh, were the primary site of MPs accumulation. MPs also caused alterations at neurotoxic levels, with significant inhibition of acetylcholinesterase (AChE) activity in both the gills and digestive gland, a typical feature of neurotoxic agents. In fact, the ingestion of MPs by filter-feeding bivalves may result in physical effects that can contribute to the disruption of neural function (Tlili et al., 2020). The relevance of MPs' dimension sizes on the adverse biological effects observed was demonstrated by Nardi et al. (2024). These authors showed that among five size MPs classes (20–1000 μm), particles in the 250–500 μm range had a greater impact on immune function, redox balance, and lipid metabolism in *M. galloprovincialis* species. This study underscored the importance of considering particle size in MPs risk assessment and monitoring beyond concentration levels. In addition to the threat posed by their size classification, another characteristic that makes MPs extremely hazardous is their ability to adsorb other environmental contaminants present in the ocean, particularly emphasized when they have rough, fragmented, and three-dimensional surfaces, leading to interactions that can result in complex toxic effects on marine organisms (Bhagat et al., 2021).

Besides MPs, another emerging environmental pollutant that is receiving growing attention is gadolinium (Gd). It belongs to the group of rare earth elements (REEs) and finds wide applications in next-generation technological and medical fields (Rogowska et al., 2018). It is the unique element of the lanthanide group for exhibiting ferromagnetic behaviour at room temperature. Above 293 K (the Curie point), it transitions to a paramagnetic state (Dan'kov et al., 1998). In the medical field, Gd chelates are essential contrast agents in magnetic resonance imaging (MRI). Traditionally considered safe when used at recommended doses (Niendorf et al., 1991), Gd has recently been found to accumulate in various organs of MRI patients, such as the brain, bones, kidneys and skin (Murata et al., 2016; Parillo et al., 2023; Ramalho et al., 2017). Its use has been linked to nephrogenic systemic fibrosis (NSF) in individuals with renal impairment (Ramalho et al., 2017), and disturbances in Ca^{2+} homeostasis (Mendichovszky et al., 2008). The widespread use and improper disposal of Gd have resulted in its detection in aquatic environments (Rogowska et al., 2018; Trapasso et al., 2021). Studies estimate that total annual Gd releases are approximately 600–918 kg in South Korea (Song et al., 2018) and around 132 kg in Germany (Kümmerer and Helmers, 2000), highlighting the significant pollution caused by this emerging contaminant in aquatic ecosystems. In general, the concentrations range from 0.35 to 80 μg/L in freshwater systems, 0.36 to 26.9 ng/L in marine environments, and up to 409.4 ng/L in a submarine outfall (Trapasso et al., 2021). Although overall environmental concentrations of Gd are typically low, significant local increases have been observed, especially in areas near urban centers or hospital effluents where Gd-based contrast agents are frequently used, reaching levels of 7078 pmol/kg in the densely populated area of Potsdam-Berlin (Lenkinski and Rofsky, 2024), and 7839 pmol/kg in River Havel (Germany) (Bau and Dulski, 1996). Due to the similarity in ionic radii between Gd^{3+} and Ca^{2+}, environmental Gd can be taken up in place of Ca^{2+}, leading to malformations in the skeletons of aquatic species (Sherry et al., 2009), as was demonstrated by Martino et al. (2017) after the exposure of Gd in the sea urchin *Paracentrotus lividus* and the gastropode *Heliocidaris tuberculata*, resulting in a range of abnormalities with a severe inhibition of skeleton growth and patterning in treated embryos. Recent indoor experiments revealed the induction of oxidative stress in early warning systems species provoked by different concentrations of Gd. Alterations in metabolic capacity and neurotoxicity were observed by Henriques et al. (2019) in *M. galloprovincialis* after exposure to 0–120 μg/L of Gd. In this case, the alteration in AChE activity may be associated with the capacity of Gd^{3+} to block K-type voltage-gated Ca^{2+} channels, also observed for other REEs. Gd can interfere with Ca^{2+} homeostasis by inhibiting Ca^{2+} binding sites, resulting in the down-regulation of Ca^{2+}-ATPase, Mg^{2+}-ATPase activity and suppression of cholinesterase function (Henriques et al., 2019). Hanana et al. (2017) reported more pronounced biochemical alterations following exposure to 0–1250 μg/L of $GdCl_3$ compared to Omniscan in *Dreissena polymorpha*, highlighting the greater hazard posed by Gd in its ionic form. Andrade et al. (2022) further observed that the biochemical response of *M. galloprovincialis* exposed to 10 μg/L of Gd changed with different salinities (20, 30, 40).

Given the property of MPs to act as "Trojan horses" carrying environmental contaminants in the environment (Hu et al., 2022), co-exposure experiments with bivalves have been widely conducted using MPs combined with pollutants, such as metals, POPs, PAHs, pharmaceuticals, pesticides, and additives (Bhagat et al., 2021). The toxicological outcomes of co-exposure to toxic pollutants and MPs can be divided into additive, synergistic, potentiating, or even antagonistic effects, which depend on the composition of the mixture and the mechanism of toxicity of the individual contaminant (Bhagat et al., 2021). The potential for interaction between these two contaminants is high in the environment, as both can occur at varying concentrations. The metallic nature of Gd favors its adhesion to MPs, while the ubiquity of MPs (Trujillo et al., 2024), along with their hydrophobic character, rough fragmented surfaces, and high surface-to-volume ratio, further promotes this interaction (Hu et al., 2022). Regarding Gd, Trujillo et al. (2024) demonstrated the ability of this contaminant to be adsorbed by plastic particles. The adsorption capacity depends on the chemical structure of the Gd compound (favouring Gd^{3+} ions over chelated complexes), as well as abiotic factors such as pH, contact time (adsorption begins after only 30 s of exposure to the contaminants), ionic strength, and the characteristics of the plastic particles, with smaller, three-dimensional particles featuring rough surfaces and COOH- functional groups being more conducive to adsorption. The rising levels of Gd in aquatic environments may indicate its potential interactions and adsorption by MPs within the ecosystem, creating a significant and stringent risk for biota. Considering that MPs can act as "Trojan horses" for metals, including Gd, the present study aims to investigate the type of interaction that occurs from the mix of MPs + Gd, and particularly the biochemical alterations induced by selecting two concentrations of MPs (0.1 and 100 μg/L), one of Gd (500 μg/L) and the mixture resulting from the combinations of them (0.1 MPs + 500 Gd μg/L, 100 MPs + 500 Gd μg/L) in the clam *D. trunculus* metabolic, oxidative and neurotoxic status after a 14-days exposure of these contaminants.

2. Material and methods

2.1. Experimental conditions

In this study, the Mediterranean wedge clam, *Donax trunculus*, was selected as the biological model. Adult specimens (length: 1.85 ± 0.5 cm; width: 1.07 ± 0.07 cm) were collected from Passoscuro beach in Lazio (Italy) and immediately transported to the laboratory. Upon arrival, the clams were placed in aerated tanks for one week of depuration and acclimation. During this period, the water in the tanks was renewed daily with distilled water mixed with "Royal Nature Premium Tropical Sea Salt". The temperature and salinity of the tanks were carefully controlled to replicate the natural conditions of the collection

site, maintained at 18 ± 1.0 °C and 35 ± 1, respectively, to reduce stress on the organisms in the laboratory setting. The clams were fed on a diet of *Spirulina sp.* and *Artemia sp.* 3 h before each daily water change, with a feeding rate of 5 mg per tank.

After acclimation, 12 *D. trunculus* individuals were placed in 6 L tanks containing artificial seawater, with each clam allocated 0.5 L of water, and three replicate tanks were prepared for each experimental treatment. The following clam exposure treatments were included in the experiment: 1) control (without contaminants), 2) MPs (0.1 or 100 μg/L), 3) Gd (500 μg/L), 4) mixture of contaminants (0.1 MPs + 500 Gd μg/L; 100 MPs + 500 Gd μg/L). Gadolinium standard for ICP (TraceCERT®, 1 g/L Gd in nitric acid (HNO_3), SigmaAldrich) was used in the present study. As regards the choice of MPs, PE fragments of 150–250 μm were selected as model particles in this study, as they are among the most commonly detected types of MPs, both in terms of shape and polymer composition (Nardi et al., 2024). PE-MPs were obtained from a commercial powder of PE fragments supplied by an Italian Institute of Research (ENEA-National Agency for New Technologies, Energy and Sustainable Economic Development). The selected polymer (ultra-high molecular weight polyethylene, UHMW-PE) consisted of irregular fragments in powder form, as this shape is more commonly found in the environment. The polymer used in this study is non-functionalized and composed exclusively of $–CH_2–$ repeating units, with no reactive functional groups present. Before use, the powder was sieved using two certified stainless steel sieves of 250 μm and 150 μm. Additionally, the MPs were washed thoroughly with ultrapure water before use to remove any possible surface contaminants or residues and avoid exogenous MPs contamination. The PE-MPs were analyzed by Fourier Transform Infra-Red Spectroscopy (FT-IR) analysis (analysis was done by an external independent certified laboratory) to confirm polymer composition before the exposure test.

The exposure period lasted for 14 days, with weekly water renewal to restore the physicochemical parameters and Gd concentrations. As for the acclimation period, the wedge clams were fed 3 h before each water renewal. After each week of exposure, six clams per replicate/tank were collected, promptly frozen and stored at −80 °C for subsequent analyses. For both biochemical analysis and Gd quantification, the six *D. trunculus* individuals collected from each tank were grouped into two pools of three individuals each.

Water samples from each tank were collected weekly, immediately after spiking for Gd quantification, aiming to compare real with nominal concentrations. The stability of Gd over one week of exposure was already demonstrated by Andrade et al. (2022).

2.2. Microplastics quantifications in water and clam tissues

For each treatment, three clams per week (one per tank) were processed according to the validated protocol (Tlili et al., 2020). Briefly, samples were digested at room temperature using a 30 % hydrogen peroxide (H_2O_2) solution prepared in ultrapure water and filtered before use. The digestates were then vacuum filtered using 0.7 μm mesh glass microfiber filters (Whatman GF/F 47 mm) and subsequently observed under a stereomicroscope (Stemi 305, Zeiss) to isolate extracted fragments.

2.3. Gadolinium quantification in water and clams' tissues

The water samples were acidified with HNO_3 2 % until pH < 2. The concentration of Gd in the water samples was quantified using inductively coupled plasma mass spectrometry (ICP-MS) with an Aurora M90 Brucker instrument. The same ICP-MS system was also employed to determine the total Gd concentration in the clams' tissues. Microwave-assisted acid digestion was performed in a Teflon container, where 0.1–1 g of freeze-dried samples were weighed, and then 1 mL of 67–69 % (v/v) HNO_3 was added. The samples were transferred to a microwave with a temperature of 90 °C for 3 h. After cooling to room temperature, the samples were placed in PE vials, and ultrapure water was added to a final volume of 10 mL. Blanks (vessels without samples) and duplicates were included for quality control. The limit of detection (LOD) for Gd is 0.003 and the limit for quantification (LOQ) is 0.01 mg/kg. The blanks were always below the LOQ.

2.4. Biomarker analysis

To assess cellular alterations in *D. trunculus* induced by MPs, Gd, and the combination of both, a set of biochemical parameters was measured. Metabolic capacity and energy reserves were evaluated by quantifying the electron transport system (ETS) activity, glycogen (GLY) and protein (PROT). The clams' antioxidant capacity was assessed by measuring superoxide dismutase (SOD) enzyme activity and total antioxidant capacity (TAC) content. The detoxification mechanisms were quantified by examining the activities of the enzymes carboxylesterases (CbEs) and glutathione S-transferases (GSTs), representing phase I and phase II enzymes, respectively. Cellular damage was assessed by measuring lipid peroxidation (LPO) levels, and neurotransmission was evaluated through the activity of the enzyme acetylcholinesterase (AChE). After the experimental period and before biochemical analyses, clams frozen tissues were homogenized using an Elvehjem potter. Tissue pools were prepared from three individual wedge clams per pool (two pools per tank, six per treatment). For the extraction, 0.5 g (fresh weight, FW) of homogenized soft tissue per pool was mixed with specific buffers in a 1:2 (w/v) ratio: magnesium sulfate buffer (0.1 M Tris–HCl, 15 % (w/v) PVP, 153 μM $MgSO_4$, 0.2 % (v/v) Triton X-100, pH 8.5) for ETS, and potassium phosphate buffer (50 mM potassium phosphate, 1 mM EDTA, 1 mM DTT, 1 % (v/v) Triton X-100, pH 7) for GLY, PROT, SOD, TAC, CbEs, GSTs, LPO and AChE. The extraction was carried out using a TissueLyser II (Qiagen) for 90 s, followed by centrifugation for 20 min at 10,000 *g* (or 3000 *g* for ETS) at 4 °C. The supernatants were collected, and the samples were analyzed in duplicate using a microplate reader (BioTek) (Cunha et al., 2022).

2.4.1. Metabolic capacity and energy reserves

The ETS activity was assessed following the methods of King and Packard (1975) and the modifications introduced by De Coen and Janssen (1997). Additionally, NAD(P)H (comprising NADH and NADPH) and p-iodonitrotetrazolium were added. Absorbance was measured over a 10-min period at 490 nm, with readings taken every 25 s. The extinction coefficient (ε) of 15.9 mM^{-1} cm^{-1} was used to calculate the amount of formazan produced. Results were expressed in nmol/min/g of FW.

The PROT content was measured using the biuret spectrophotometric method as described by Robinson and Hogden (1940). Bovine serum albumin (40 mg/mL) was used to prepare standards ranging from 0 to 40 mg/mL, creating a calibration curve. The colorimetric reaction was allowed to develop for 10 min at 30 °C, and absorbance was recorded at 540 nm. Results were expressed in mg/g of FW.

The sulfuric acid method outlined by DuBois et al. (1956) was used for quantifying GLY content. This method relies on the ability of phenol and sulfuric acid to facilitate quantitative colourimetric micro-analysis of sugars, where the colour intensity, maintained at a constant phenol concentration, directly correlates with sugar content. Glucose standards ranging from 0 to 5 mg/mL were employed to create a calibration curve. After a 30-min incubation at room temperature, absorbance was measured at 492 nm. The results were reported in mg/g of FW.

2.4.2. Antioxidant capacity

The activity of SOD was quantified using the method outlined by Magnani et al. (2000). This is a simple and rapid approach based on the enzyme's ability to inhibit the autoxidation of pyrogallol. In the presence of EDTA at pH 8.2, the autoxidation of pyrogallol is 50 %. The method's principle relies on the competition between pyrogallol autoxidation by O_2^- and the dismutation of this radical by SOD. The

absorbance of the mixture was first measured at 420 nm before the addition of the pyrogallol solution. Then, 1 min after the pyrogallol solution (prepared in 0.01 M HCl) was added, the absorbance was measured again. Results were expressed as U/g of FW, where one unit (U) of enzyme activity represents the inhibition of 50 % of pyrogallol autoxidation.

Total antioxidant capacity (TAC) content was evaluated using the method described by Benzie and Strain (1996), with adaptations by Wootton et al. (2021). A standard curve was created using iron sulphate heptahydrate ($FeSO_4$) standards ranging from 0 to 1000 mM. After 10 min of incubation, the absorbance was read at 593 nm. The results were reported in μmol/g of FW.

2.4.3. Detoxification mechanism

The activity of GSTs was evaluated using the protocol described by Habig et al. (1976), with modifications detailed by Carregosa et al. (2014). The sample was combined with a reaction solution containing potassium phosphate buffer at pH 6.5, 1-chloro-2,4-dinitrobenzene, and reduced glutathione. Absorbance was measured at 340 nm for 5 min at 15 s intervals, and GSTs activity was calculated using the extinction coefficient (ε) of 9.6 mM^{-1} cm^{-1}. Enzymatic activity was reported in U/g of FW, where one unit (U) is defined as the amount of enzyme that catalyzes the formation of 1 nmol of dinitrophenyl thioether per min.

The activity of CbEs was measured using p-nitrophenyl butyrate (*p*NPB) substrate, following the method described by Hosokawa and Satoh (2001), with modifications by Solé et al. (2018). The rate of *p*NPB hydrolysis was monitored by measuring absorbance at 405 nm over a 5 min period at 15 s intervals, employing an extinction coefficient (ε) of 18 mM^{-1} cm^{-1}. Results were expressed as nmol/min/g of FW.

2.4.4. Cellular damage

The levels of LPO were assessed by measuring malondialdehyde (MDA) content, following the method outlined by Ohkawa et al. (1979). This estimation involved quantifying thiobarbituric acid reactive substances (TBARS), which are produced from the reaction between LPO by-products, including MDA, and 2-thiobarbituric acid (TBA). Absorbance was measured at 532 nm, and LPO levels were calculated using the extinction coefficient (ε) of 156 mM^{-1} cm^{-1}. The results were expressed in nmol MDA/g of FW.

2.4.5. Neurotransmission

The activity of AChE was evaluated using acetylthiocholine iodide as the substrate, following the modified method of Ellman et al. (1961) as described by Mennillo et al. (2017). The reaction was monitored spectrophotometrically at 412 nm over a 5 min period, with readings taken at 15 s intervals. Results were expressed as nmol/min/g of FW.

2.5. Data analysis

The bioconcentration factor (BCF) for each treatment was calculated according to the method of Arnot and Gobas (2006). This was done by dividing the average concentration of Gd found in the tissues of *D. trunculus* after the experiment by the mean of Gd concentration in seawater immediately after spiking, which represented the actual exposure level.

Statistical hypothesis testing, along with the add-on in PRIMER v6 (Anderson, 2008), was applied to all biochemical results (ETS, PROT, GLY, SOD, TAC, CbEs, GSTs, LPO, AChE) and the concentrations MPs (0.1 and 100 μg/L), Gd (500 μg/L), mix MPs + Gd (0.1 MPs + 500 Gd μg/L; 100 MPs + 500 Gd μg/L) across each exposure treatment in water and tissue. The significance of the pseudo-F *p*-values in the primary PERMANOVA tests was assessed. When significant differences were detected in the main test, pairwise comparisons were conducted. A *p*-value of lower then 0.05 was considered indicative of statistical significance. The null hypothesis tested was: for each biomarker and exposure period (weeks 1 and 2), there were no significant differences among exposure treatments (0-CTL, 0.1 MPs, 100 MPs, 500 Gd, 0.1 MPs + 500 Gd, 100 MPs + 500 Gd μg/L). In the figures, for each biomarker, significant differences among treatments in week 1 were shown in lowercase letters and week 2 in uppercase letters. Asterisks indicate significant differences between week 1 and week 2 for a given treatment.

The matrix containing the biochemical descriptors, together with the Gd concentrations in the tissues of the wedge clams per treatment, was used to calculate the Euclidean distance similarity matrix. This matrix was then simplified by calculating the distance between centroids based on the treatments, which was then subjected to ordination analysis using Principal Coordinates Ordination (PCO). Pearson correlation vectors of biochemical descriptors (with correlation coefficients > 0.75) were superimposed on the PCO graph.

2.6. Integrated Biomarker Response (IBR) Index

The biochemical parameter-derived data were consolidated through the application of the Integrated Biomarker Response index version 2 (IBRvs2), as originally proposed by Beliaeff and Burgeot (2002) and later refined by Sanchez et al. (2013). This method was employed to integrate and visualize the biochemical responses of clams exposed to different treatments involving Gd, MPs, and their combinations over two time points (week 1 and week 2). The analysis was based on the deviation of each treatment group from the control condition (CTL - week 1), with separate calculations performed for week 1 and week 2. To minimize data variance prior to IBR computation, a log transformation was applied using the formula $Yi = \log(Xi / X0)$, where Xi denotes the individual biomarker value under treatment and X0 represents the mean value of the corresponding control group. The overall mean (μ) and standard deviation (σ) of the Yi values were calculated, followed by standardization using $Zi = (Yi - \mu) / \sigma$. A biomarker deviation index ($A = Zi - Z0$) was then derived to express individual biomarker shifts relative to the reference. The IBRvs2 value was obtained as $IBRvs2 = |A|$, considering all evaluated biochemical parameters. Higher IBRvs2 values corresponded to increased disturbance.

3. Results

3.1. MPs and Gd concentrations in seawater and wedge clams' tissues

Regarding Gd concentrations in seawater from the experimental treatments, values obtained from water samples collected immediately after spiking both in week 1 and week 2 showed that measured and nominal concentrations were similar to the Gd treatment (500 μg/L). In the mixture treatment of 0.1 MPs + 500 Gd μg/L, Gd concentrations decreased significantly to 185.2 μg/L and 182.3 μg/L in the 1st and 2nd weeks, respectively, compared to the Gd treatment. In the treatment with 100 MPs + 500 Gd μg/L, Gd concentrations fell to 50.7 μg/L and 37.2 μg/L in the 1st and 2nd weeks, respectively. Regarding the Gd concentrations in clams, the concentration of this element in the tissues decreased significantly following the order of 500 Gd >mix (0.1 MPs + 500 Gd) μg/L > mix (100 MPs + 500 Gd) μg/L (Table 1).

As regards MPs in the CTL, no MPs were found in the tissues of *D. trunculus* as expected both in weeks 1 and 2, while the treatments with MPs and mix confirmed the ingestion of MPs (Table 2) with several items varying from 1 to 2 in the MPs treatment at 0.1 μg/L and 0.1 MPs + 500 Gd μg/L, and from 3 to 8 in the treatment of 100 μg/L and 100 MPs + 500 Gd μg/L after the 2nd week (Table 2).

3.2. Biological responses: biochemical parameters

Regarding metabolic activity, ETS values increased significantly in all treatments after the 1st week of exposure compared to the control treatment. In week 2, the activity of ETS decreased significantly at 100 μg/L MPs, while increased significantly in 500 Gd μg/L and both mix treatments. Comparing week 1 and week 2, significantly higher ETS

Table 1
Concentration of gadolinium (Gd) in water samples (μg L^{-1}) immediately after spiking and *Donax trunculus* tissues (μg g^{-1}, dry weight) collected after week 1 (7 days) and week 2 (14 days) and the respective bioconcentration factor (BCF) for clams (L kg^{-1}). Values are the mean (3 replicates per treatment) ± standard deviation.

	Treatment Gd	Water (μg l^{-1})	*Donax trunculus*	
			μg g^{-1}	BCF (L kg^{-1})
1 Week	CTL	$< 0.01^{a}$	$< 0.01^{a}$	–
	Gd 500 μg/L	505.0^{b}	44.48 ± 24.84^{b}	88.08
	Mix (0.1 MPs + 500 Gd) μg/L	185.2^{b}	23.5^{b}	127.03
	Mix (100 MPs + 500 Gd) μg/L	50.7^{a}	9.12 ± 6.26^{a}	179.88
2 Week	CTL	$< 0.01^{a}$	$< 0.01^{a}$	–
	Gd 500 μg/L	488.10^{b}	66.52 ± 11.96^{b}	136.28
	Mix (0.1 MPs + 500 Gd) μg/L	182.3^{b}	30.4^{b}	166.76
	Mix (100 MPs + 500 Gd) μg/L	37.2^{a}	22.0^{a}	591.40

Table 2
Number of MPs (mean number of MPs/clams) in *Donax trunculus* collected after week 1 (7 days) and week 2 (14 days) of exposure. Values are the mean (3 replicates per treatment) ± standard deviation.

	Treatment MPs	*Donax trunculus* Mean number of MPs/clams ± SD
1 Week	CTL	0^{a}
	MPs 0.1 μg/L	1 ± 1^{a}
	MPs 100 μg/L	3 ± 1^{b}
	Mix (0.1 MPs + 500 Gd) μg/L	1.3 ± 1.2^{a}
	Mix (100 MPs + 500 Gd) μg/L	5.0 ± 2^{b}
2 Week	CTL	0^{a}
	MPs 0.1 μg/L	2 ± 1^{a}
	MPs 100 μg/L	6.7 ± 1.5^{b}
	Mix (0.1 MPs + 500 Gd) μg/L	1.3 ± 0.6^{a}
	Mix (100 MPs + 500 Gd) μg/L	8 ± 2^{b}

activity was observed after the 1st week in clams exposed to 0.1 and 100 μg/L MPs, 500 μg/L Gd and 0.1 MPs + 500 Gd μg/L treatments (Fig. 1A).

Regarding PROT, after both exposure weeks, the content increased in both mix treatments, with significant differences observed in week 1. Furthermore, in week 2 the content of PROT decreased significantly in 0.1 and 100 MPs μg/L while increased in both mix treatments. Significant differences between weeks were recorded at 0.1 MPs μg/L, 100 MPs μg/L and 0.1 MPs + 500 Gd μg/L treatments, with higher values after week 1 when clams were exposed to MPs alone and significantly lower values in week 1 at 0.1 MPs + 500 Gd μg/L treatment (Fig. 1B).

Regarding the GLY content, after the 1st week of exposure, clams showed a significant increase in 100 MPs μg/L and 500 Gd μg/L. In the 2nd week, a decreasing trend was observed in 0.1 MPs μg/L and 100 MPs μg/L, while an increase was observed at 500 Gd μg/L and 100 MPs + 500 μg/L. Significant differences between weeks were recorded in 0.1 MPs μg/L and 100 MPs μg/L, with lower levels in the 2nd week compared to the 1st week (Fig. 1C).

Regarding antioxidant enzyme SOD, in both week 1 and week 2 the activity decreased significantly in both MPs treatments, while increasing significantly in 500 Gd μg/L, and both the mix treatments. Between the weeks, significant differences were observed in the treatment of 0.1 MPs μg/L + 500 Gd μg/L with higher SOD activity after week 2 compared to week 1 (Fig. 2A).

In week 1, TAC content decreased significantly in the two MPs treatments. After the 2nd week a significant decrease in both MPs concentrations was observed, while clams showed significantly higher TAC levels in both mix treatments. Significant differences between weeks were recorded in the treatment 100 MPs + 500 Gd μg/L, with higher values after week 2 (Fig. 2B).

Concerning biotransformation enzymes, after both exposure weeks, the activity of CbEs increased significantly in both MPs and 500 Gd μg/L treatments. No significant differences were observed between weeks, except for 0.1 μg/L of MPs (Fig. 3A).

The activity of GSTs showed a significant decreasing trend in 100 MPs μg/L in week 1, while a significant increasing trend was observed in 500 Gd μg/L, and both the mix treatments. The same pattern was observed after the 2nd week of exposure. Between exposure weeks, significantly lower values were observed in week 2 compared to week 1 in 100 MPs μg/L, 500 Gd μg/L, and 0.1 MPs + 500 Gd μg/L treatments (Fig. 3B).

In terms of cellular damage, in week 1, the levels of LPO decreased significantly in clams exposed to 0.1 MPs μg/L and 500 Gd μg/L treatments. The same pattern was observed after the 2nd week of exposure, with decreased levels in 0.1 MPs μg/L and 500 Gd μg/L treatments, with a significant increase in the treatment 0.1 MPs + 500 Gd μg/L. No significant differences were observed between weeks (Fig. 4A).

Regarding neurotransmission, a decreasing activity of AChE was observed in Gd treatment, and both mix treatments after the 1st week of exposure, and in the week 2 in 0.1 MPs + 500 Gd. Between weeks, significant differences were observed in 0.1 MPs μg/L with a higher activity after the 2nd week compared to the 1st week (Fig. 5A).

3.3. Multivariate analysis

The Principal Coordinates Ordination (PCO) plot illustrates the multivariate biochemical response patterns in clams exposed to different treatments across two time points (week 1 and week 2), accounting for 79.4% of the total variation (Fig. 6A). PCO1 explained 49.9 % of the total variation separating all the treatments of MPs and control treatments of both weeks, on the positive side of PCO1, from both the mix and Gd treatments of week 1 and week 2 on the negative side of PCO1 (Fig. 6). The variables that better explained the variation of PCO1 were PROT ($r = -0.87$), SOD ($r = -0.91$), TAC ($r = -0.91$), GSTs ($r = -0.81$), all located on the negative side of the PCO1. The PCO vertical dimension (PCO2) explained 29.5 % of the total variation separating organisms exposed to Gd of both weeks and MPs treatments of week 1 on the negative side, from mix, CTL, and MPs treatments of week 2 in the positive side. The vector that best explained this distinction was LPO ($r = 0.90$) on the positive side of PCO2.

3.4. Integrated Biomarker Response (IBR) index

The IBR values reveal clear differences in organismal stress responses across single and combined exposures of Gd and MPs (Fig. 6B). In week 1, Gd alone (500 Gd) induced the highest IBR value (11.02), indicating a strongest biological response. In contrast, exposures to MPs alone (0.1 and 100 MPs) resulted in lower IBR values (8.07 and 7.04, respectively), suggesting a comparatively moderate effect. The combined treatments (Gd + MPs) did not exceed the response to Gd alone; instead, the IBR values for 500 Gd + 0.1 MPs (8.46) and 500 Gd + 100 MPs (6.87) were lower. In week 2, in general higher IBR values were observed ranging from 6.32 to 10.12. The mixture treatments (9.24 for 500 Gd + 0.1 MPs and 9.77 for 500 Gd + 100 MPs) reached values comparable to Gd alone (9.96) and 100 MPs alone (10.12).

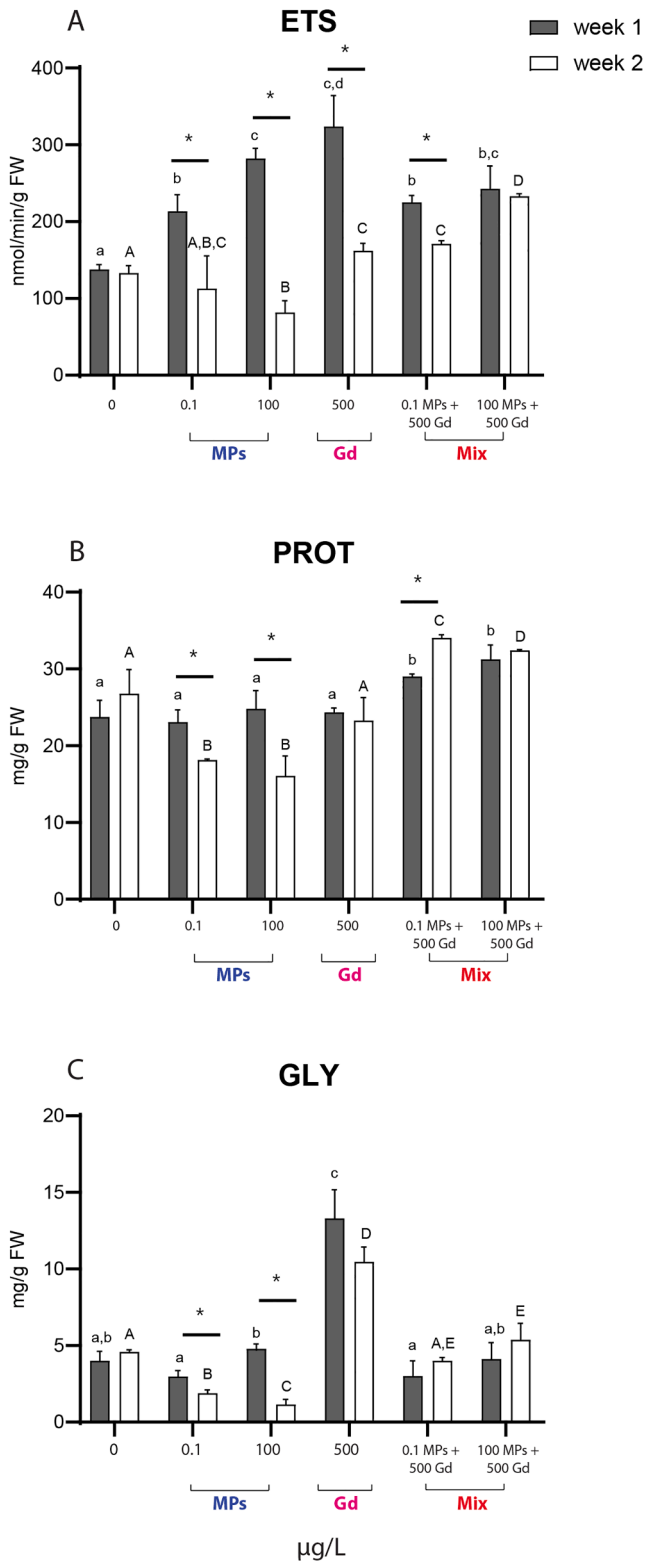

Fig. 1. A: Electron transport system (ETS) activity; **B:** Protein (PROT) content; **C:** Glycogen (GLY) content in *Donax trunculus* exposed to CTL-0, MPs (0.1, 100) µg/L, Gd (500 µg/L), and mix (0.1 MPs+500 Gd; 100 MPs+500 Gd) µg/L for 7 days (week 1) and 14 days (week 2). After 7 days, differences among the treatments are identified with lowercase letters. After 14 days, differences among the treatments are identified with uppercase letters. Differences between weeks are identified with an asterisk (*).

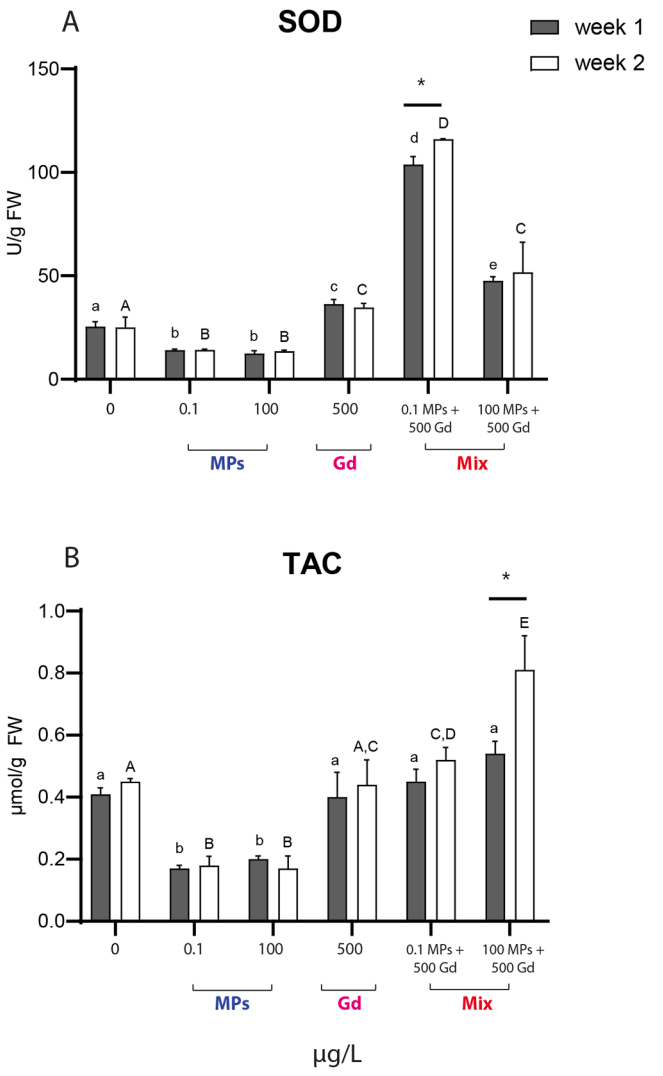

Fig. 2. A: Superoxide dismutase (SOD) activity; **B:** Total antioxidant capacity (TAC) in *Donax trunculus* exposed to CTL-0, MPs (0.1, 100) µg/L, Gd (500 µg/L), and mix (0.1 MPs+500 Gd; 100 MPs+500 Gd) µg/L for 7 days (week 1) and 14 days (week 2). After 7 days, differences among the treatments are identified with lowercase letters. After 14 days, differences among the treatments are identified with uppercase letters. Differences between weeks are identified with an asterisk (*).

4. Discussion

4.1. MPs and Gd concentration in water and clams' tissues

Donax trunculus was previously used as an early warning species of different hazardous contaminants such as plastics (Ben-Haddad et al., 2022; Secco et al., 2025), metals (Belabed and Soltani, 2018; Merad et al., 2018) and toxins (Botelho et al., 2018). The pioneering study presented here investigated the accumulation capacity in *D. trunculus* after the exposure of two contaminants of emerging concern, selecting two concentrations of MPs (0.1 and 100 µg/L), one of Gd (500 µg/L) and the mixture resulting from the combinations of them (0.1 MPs + 500 Gd µg/L, 100 MPs + 500 Gd µg/L). The concentration of Gd in the water matrix exhibited significant variation across the treatment of Gd alone (500 µg/L) and the two mix treatments, with the presence of MPs in the aquaria identified as a critical influencing factor. Notably, in the treatments involving Gd alone, the concentration within the aquarium corresponded closely to the administered concentration of 500 µg/L. In contrast, Gd concentrations in the mixed treatments (0.1 MPs + 500

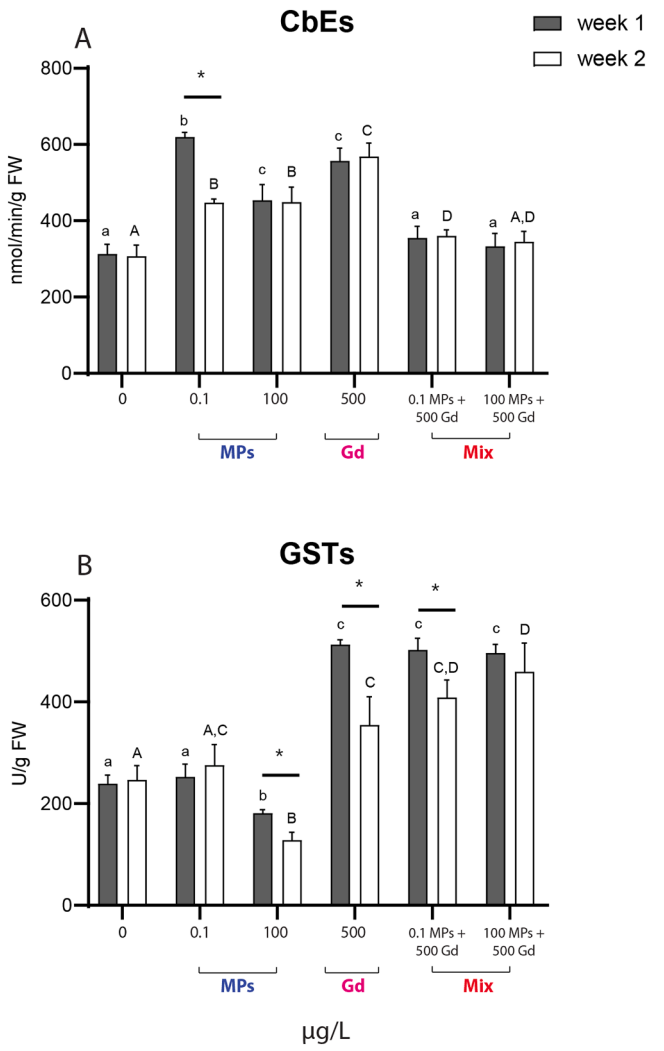

Fig. 3. A: Carboxylesterases (CbEs) activity; **B:** Glutathione S-tranferases (GSTs) activity in *Donax trunculus* exposed to CTL-0, MPs (0.1, 100) µg/L, Gd (500 µg/L), and mix (0.1 MPs+500 Gd; 100 MPs+500 Gd) µg/L for 7 days (week 1) and 14 days (week 2). After 7 days, differences among the treatments are identified with lowercase letters. After 14 days, differences among the treatments are identified with uppercase letters. Differences between weeks are identified with an asterisk (*).

µg/L Gd and 100 MPs + 500 µg/L Gd) showed a marked decrease, particularly at the highest administered concentration of MPs (100 µg/L). It is well known that plastics have the property of adsorbing contaminants from the surrounding environment, such as metals, persistent organic pollutants (POPs), pharmaceuticals, pesticides, and additives (Bhagat et al., 2021; Brennecke et al., 2016; Rochman et al., 2013), and recently the adsorption of Gd by nanoplastics (NPs) has been demonstrated (Trujillo et al., 2024). Additionally, the plastics particles used in this experiment have two properties that facilitate the adsorption of contaminants. Firstly, they are MPs, which have a significant surface-to-volume ratio compared to larger dimensional ranges (Alimi et al., 2018). As a result, this translates into a greater surface availability for adsorbing pollutants (Bhagat et al., 2021), in this case, Gd. Secondly, they are fragmented with rough surfaces, which promotes the tridimensionality of the plastics and the available spaces for adsorbing pollutants (Casabianca et al., 2021). Other studies have highlighted an "antagonistic" effect of MPs on adsorbed contaminants in the aquaria, reducing the concentration of pollutants in the exposure medium (Bhagat et al., 2021). Dibutyl phthalate (DBP), a plasticizer used in the production of plastics, has shown antagonistic interaction with MPs at a

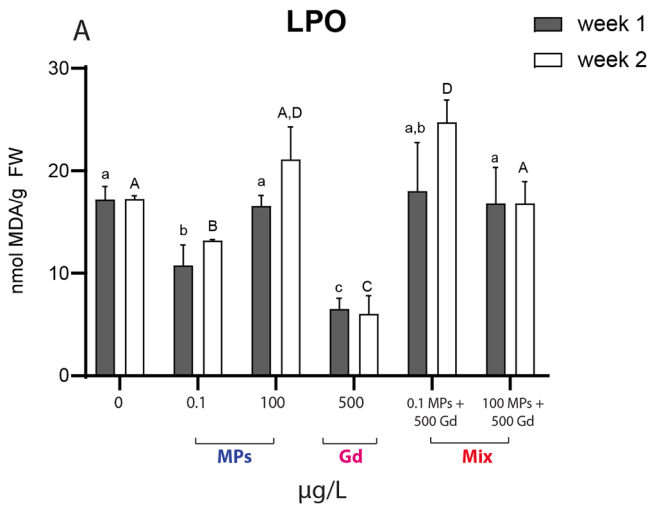

Fig. 4. A: Lipid peroxidation (LPO) levels in *Donax trunculus* exposed to CTL-0, MPs (0.1, 100) µg/L, Gd (500 µg/L), and mix (0.1 MPs+500 Gd; 100 MPs+500 Gd) µg/L for 7 days (week 1) and 14 days (week 2). After 7 days, differences among the treatments are identified with lowercase letters. After 14 days, differences among the treatments are identified with uppercase letters. Differences between weeks are identified with an asterisk (*).

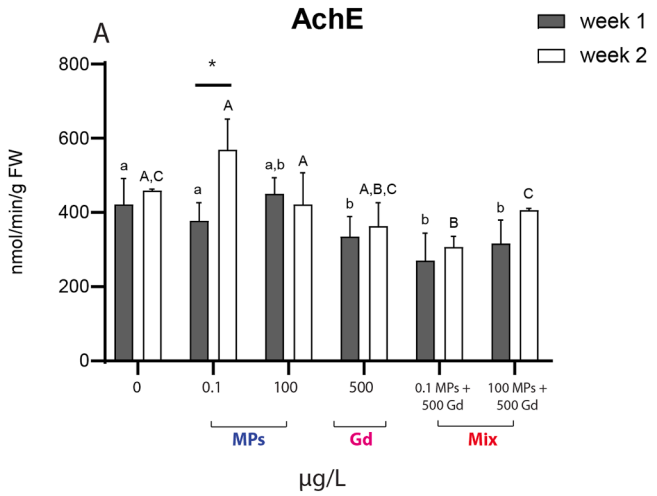

Fig. 5. Acetylcholinesterase (AChE) activity in *Donax trunculus* exposed to CTL-0, MPs (0.1, 100) µg/L, Gd (500 µg/L), and mix (0.1 MPs+500 Gd; 100 MPs+500 Gd) µg/L for 7 days (week 1) and 14 days (week 2). After 7 days, differences among the treatments are identified with lowercase letters. After 14 days, differences among the treatments are identified with uppercase letters. Differences between weeks are identified with an asterisk (*).

lower concentration of DBP, while, at relatively high concentrations of DBP, the interaction was found to be synergistic (Li et al., 2020). Similar studies were reported with gold nanoparticles and MPs, synergism was reported at higher concentrations of the mixture, whereas antagonism was observed at lower concentrations (Pacheco et al., 2018).

As regards concentrations of Gd in *D. trunculus*, in the treatment with only Gd, the clams accumulated a larger amount of this contaminant, while between the two mix treatments with MPs and Gd, the clams accumulated more Gd in the 0.1 µg/L MPs + 500 µg/L Gd treatment compared to the 100 µg/L MPs + 500 µg/L Gd treatment. As stated above, this difference may show an antagonistic effect, so in the 100 µg/L MPs + 500 µg/L Gd treatment, a higher quantity of MPs in the medium may have adsorbed Gd on their surfaces, thus making it less available to the *D. trunculus* clams. Other exposure experiments reported a reduction

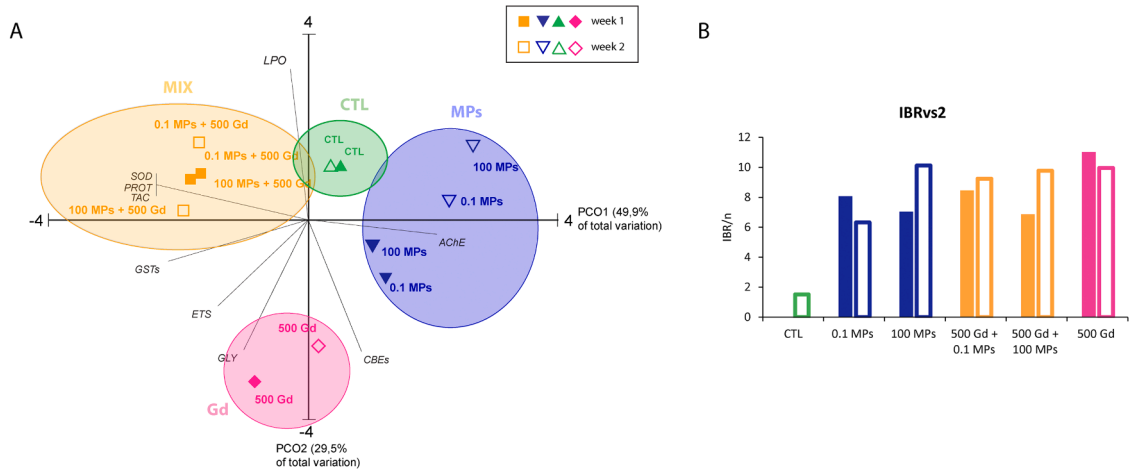

Fig. 6. A: Centroids ordination diagram (PCO) based on Gd concentration in wedge clams' tissue and biochemical parameters assessed in *Donax trunculus* exposed to exposed to CTL-0, MPs (0.1, 100) μg/L, Gd (500 μg/L), and mix (0.1 MPs+500 Gd; 100 MPs+500 Gd) μg/L. Pearson correlation vectors are superimposed as supplementary variables, namely biochemical data ($r > 0.75$): Gadolinium (Gd), Microplastics (MPs), electron transport system (ETS) activity, glycogen (GLY) content, protein (PROT) content, superoxide dismutase (SOD) activity, total antioxidant capacity (TAC), carboxylesterases (CbEs) activity, glutathione S-tranferases (GSTs) activity, lipid peroxidation (LPO) levels, acetylcholinesterase (AChE) activity. **B**: Integrated Biological Response (IBR) Index for each treatment (CTL-0, MPs (0.1, 100) μg/L, Gd (500 μg/L), and mix (0.1 MPs+500 Gd; 100 MPs+500 Gd) μg/L) and week (week 1 and week 2).

in the accumulation of other contaminants such as arsenic (Wang et al., 2020), mercury (Oliveira et al., 2017) and benzo[a]pyrene (Pittura et al., 2018) in mussels in the presence of MPs, confirming that the adsorption of contaminants by MPs in the medium can influence their bioaccumulation. Regarding the exposure duration, it can be observed that more Gd was accumulated after the 2nd week compared to the 1st, indicating that the clams did not reach a threshold beyond which they were no longer able to accumulate Gd in their tissues, but continued to accumulate it throughout the experiment.

Regarding plastic accumulation, the study confirmed that *D. trunculus* successfully ingested MPs smaller than 250 μm at both 0.1 and 100 μg/L concentrations, with a maximum of 3 MPs detected at the lowest concentration and up to 8 MPs at the highest concentration. MPs typically enter clams through the inhalant siphons during water filtration and can either be expelled through faeces or translocated to the circulatory system and other tissues, where they induce oxidative stress (Nardi et al., 2024). Tlili et al., (2020) observed that after 15 days of exposure to 0.06 g/Kg of sand, MPs accumulated in both gills and digestive gland, with a total of 28 ± 4 MPs ingested. Similarly, Nardi et al. (2024) reported an average of 21.83 ± 15.02 MPs (< 250 μm) after 14 days of exposure. A lower number of MPs detected in *D. trunculus* recorded in this study may be due to the relatively low exposure concentrations, indicating that longer exposure periods may be necessary to achieve higher ingestion levels at such concentrations. Since wedge clams did not accumulate significant amounts of MPs, it is possible that MPs did not enhance the accumulation of Gd in their tissues. Instead, the MPs with adsorbed Gd may have remained in the medium. The biochemical response perfectly reflects the accumulation of contaminants in the tissues of *D. trunculus*. Specifically, in the Gd treatment and the mix treatments, glutathione S-transferases (GSTs) levels are elevated compared to the control, indicating enhanced detoxification activity triggered by the accumulation of this contaminant. In contrast, the lowest GSTs levels observed in the MPs treatments suggest a reduced detoxification capacity, likely due to the low number of MPs within the clams.

4.2. Biological responses

Four distinct treatment-related clusters were evident in the multivariate analysis (Principal Coordinates Ordination, PCO). Control (CTL) samples are tightly grouped near the origin, reflecting stable baseline biomarker levels. Clams exposed to MPs (0.1 and 100 μg/L of MPs concentrations) form a well-defined cluster on the positive side of PCO1, primarily influenced by increased acetylcholinesterase (AChE) activity, suggesting a neurotoxic response. In contrast, samples treated with Gd alone are distinctly separated along PCO2, associated with higher glycogen (GLY) content and electron transport system (ETS) activity, indicating significant effects on energy metabolism. The combined exposure groups (Gd + MPs) form a separate cluster on the negative side of PCO1, driven by biomarkers such as superoxide dismutase (SOD), total protein (PROT), total antioxidant capacity (TAC), and GSTs, which reflect enhanced oxidative stress and detoxification responses. Lipid peroxidation (LPO) levels appears centrally located, contributing variably across treatments. Overall, the PCO analysis reveals treatment-specific biomarker response profiles, with each cluster exhibiting distinct biochemical signatures and time-dependent variations, particularly evident in the Gd and MP treatments when administered individually.The Integrated Biomarker Response (IBRvs2) index bar graph complements the PCO analysis by quantitatively summarizing the magnitude of the biochemical responses in clams across different treatments. The control group (CTL) displays the lowest IBR index (~2), consistent with its central and tightly clustered position in the PCO plot, indicating minimal disturbance. The MPs-only treatments (0.1 MPs and 100 MPs) present intermediate IBR responses, with values ranging from ~6 to ~10, suggesting a comparatively moderate effect. These values align with their clear separation along the positive side of PCO1, driven primarily by neurotoxicity-associated markers like AChE. In contrast, the 500 Gd treatment exhibits the highest IBR values in both weeks (~11 and ~10), aligning with its distinct separation in the PCO plot, particularly along the PCO2 axis, driven by energy-related biomarkers (GLY, ETS). Notably, while Gd alone remained among the highest in both weeks, the MPs-only and mixture treatments closed the gap in week 2, implying prolonged or delayed effects from MPs and/or enhanced interaction effects over time. The mixtures of Gd and MPs also show elevated IBR values (~8 – ~10), reflecting a high level of biochemical disturbance and corroborating their grouping on the left side of the PCO1 axis, where oxidative stress and detoxification biomarkers (SOD, PROT, TAC, GSTs) are prominent. Nevertheless, the combined treatments (Gd + MPs) did not exceed the response to Gd alone, suggesting a possible antagonistic interaction in the short term, where the presence of MPs might attenuated Gd-induced biomarker activation. Overall, the data suggest that Gd is the primary driver of biomarker response in the

early phase (week 1), while MPs contribute more significantly to stress over time. The combined exposures did not exhibit clear synergistic effects; instead, they showed time-dependent interactions. Overall, the patterns revealed by the IBR index closely mirror those identified in the PCO analysis, where treatment groups such as Gd and MPs were clearly separated, indicating distinct biomarker profiles. Similarly, the IBR values were highest in the 500 Gd and 100 MPs groups, reinforcing the separation observed in the PCO and confirming that these treatments induced the most pronounced responses. In both analyses, mixture treatments (Gd + MPs) occupied intermediate positions, suggesting partial overlap with both Gd and MPs groups, consistent with a possible antagonistic interaction or time-dependent effects.

The increase in ETS activity has been considered a key strategy used by bivalves to cope with stress, often associated with activation of defense mechanisms (Freitas et al., 2020). The results here presented demonstrated that, after the 1st week of exposure, contaminated clams significantly increase their metabolic capacity regardless of the treatment (MPs, Gd, mix), with the highest values in clams exposed to single treatments. An increase in metabolic activity has already been observed in experiments involving exposure to MPs at both low and high concentrations of 10, 10^4, and 10^6 particles /L after 14 days (Shang et al., 2021), and to Gd at a concentration of 10 μg/L (Cunha et al., 2022). Regarding both treatments, higher ETS activity was observed compared to the CTL clams, although the increase was less pronounced than in the Gd (500 μg/L) and MPs (100 μg/L) treatments. Based on this result, we can hypothesize that wedge clams were subjected to elevated stress levels, beyond their capacity to continue to raise their metabolism. Under extreme stress—whether from prolonged exposure, excessively high concentrations, or, as in this case, the simultaneous presence of two contaminants—bivalves may lose the ability to sustain their metabolic activity at optimal levels (Andrade et al., 2021; Pinto, 2019). As a result, they were unable to sustain an elevated metabolic response to the imposed stress. This finding is consistent with the results obtained by Zhang et al. (2021), who conducted an experiment exposing organisms to MPs, phenanthrene (Phe), and a combination of MPs + Phe. The authors observed an elevated ETS activity in the individual treatments of MPs and Phe alone. However, under the heightened stress caused by the contaminant mixture, metabolic enzymes were inhibited to an unnatural high degree. The metabolic capacity of contaminated clams strongly decreased after two weeks of exposure compared with values recorded after the 1st week, and only in the presence of Gd (single or combined) the ETS activity was higher than at the CTL. Such findings might indicate that under moderate stress conditions (MPs exposure) clams were able to adapt their biochemical performance after a prolonged exposure period, while at higher stressful conditions (Gd and mix treatments) clams' strategy of defense still relied on metabolic activation.

Accompanying clams' metabolic responses, the PROT content followed the same pattern, with higher values recorded in clams exposed to the mix treatments, especially after the 2nd week of exposure. This result indicates that accompanying the metabolic capacity, clams increased their PROT production more than their use, supporting the hypothesis that individuals exposed to both contaminants in the mix treatments are under significant stress, leading to increased production of proteins, namely enzymes, particularly antioxidant enzymes (Freitas et al., 2020), to combat the stress induced by the combination of the two contaminants. As shown in the PCO graph, the PROT content is closely associated with SOD and TAC biomarkers, reinforcing the idea that higher PROT content is associated with higher enzyme production and higher antioxidant defenses.

Regarding GLY content, it appears that this energy reserve is not primarily utilized by clams exposed to Gd, as levels were higher in this treatment. In the PCO graph, GLY content is closely related to Gd treatments. Nevertheless, the presence of MPs seems to trigger GLY use, with lower GLY levels observed in clams exposed to the mix treatments compared to Gd alone, and even lower levels when exposed solely to MPs. Furthermore, after the 2nd week of exposure, particularly at the highest MPs concentrations, GLY content was even lower than in week 1. This finding reinforces the idea that this stress condition leads to increased GLY expenditure. Similar results were reported by Banaei et al. (2022), who observed decreased GLY storage in hepatocytes of *Cyprinus carpio* after 30 days of exposure to PE-MPs (0–1400 μg/L). This reduction in GLY storage likely reflected its mobilization as an energy source to counteract the cytotoxic effects of MPs. In fish exposed to MPs, GLY breaks down into glucose aligned with the observed increase in blood glucose levels (Banaei et al., 2022).

When organisms are under control conditions, the harmful effects of reactive oxygen species (ROS) are mitigated by various antioxidant defense mechanisms, including SOD which is the first responsible for the removal of O^{2-} by converting it into H_2O_2. In addition to SOD, other enzymatic and non-enzymatic mechanisms contribute to ROS elimination, which can be evaluated by measuring the TAC against oxidative stress (Regoli and Giuliani, 2014), providing insights into the combined effects of organisms' antioxidants defenses and their additive, synergistic, or antagonistic interactions (Fraga et al., 2014). In this context, it is observed that the activity of SOD is inhibited and TAC content is decreased in the presence of MPs, regardless of the exposure time. This antioxidant response appears to be typical of MPs. Indeed Parra et al. (2021) demonstrated that *Corbicula fluminea* exposed to 2 mg/L MPs showed lower values for SOD both in gills and gonads in comparison to CTL clams. Other authors have reported that the presence of plastics does not significantly induce antioxidant enzyme activity, suggesting that they do not contribute to the stress response (Wang et al., 2020). Furthermore, the study by Zhang et al. (2021) suggested that the increase in SOD response was not influenced by the tested MPs concentration (1 mg/L), but rather by their size (17 and 150 μm). In the study of Zhang et al. (2021) in the size range of 100–150 μm, MPs are more toxic because they are not easily expelled by the organism, leading to an inhibitory response for SOD. This would account for the comparable level of inhibition observed in the plastics tested in the present study at both low and high (0.1 and 100 μg/L MPs) concentrations, indicating that the concentration was not fundamental in triggering a response; rather, it was the size of the administered MPs that played a crucial role.

A contrasting response, with SOD activation, was observed in the presence of Gd, reaching the highest values in the two mix treatments (0.1 MPs + 500 Gd μg/L and 100 MPs + 500 Gd μg/L), in particular when Gd was combined with the lowest MPs concentration. As demonstrated previously, in the presence of Gd, bivalves are capable of activating antioxidant defense mechanisms (Henriques et al., 2019). Published data show that bivalves can increase the activity of antioxidant enzymes also in the presence of other rare earth elements (REEs) (Pinto et al., 2019). In both mix treatments, SOD exhibited the highest values compared to the other treatments, indicating a strong stress response. However, in the 100 MPs + 500 Gd mix treatment in both weeks, there was a subsequent decrease in this parameter. As previously stated, following the results of Zhang et al. (2021) that observed lower values in antioxidant response of Phe + MPs mix treatment compared to the exposure of contaminants alone, this inhibition may reflect that organisms have undergone a certain degree of damage from the stress of pollutants exceeding a certain threshold, further supporting the 1st hypothesis. A similar trend is observed for TAC, where both treatments with MPs showed the lowest values, while the mix treatments exhibited the highest values, supporting the hypothesis that individuals exposed to both contaminants experience greater stress (Zhang et al., 2021). This suggests that while SOD activity is inhibited at the highest contaminant levels, other enzymes, including other antioxidant defenses or those involved in biotransformation, may be stimulated. In the PCO graph, SOD and TAC are strongly associated with the mix treatments, explaining the separation of these treatments from the ones only with MPs.

The enzymes CbEs and GSTs are well-known for their critical roles in detoxification processes. CbEs participate in phase I biotransformation, while GSTs are involved in phase II. These enzymes help convert

harmful substances, aiding in their removal from cells (Regoli and Giuliani, 2014). Particularly in the presence of MPs and Gd single exposures, phase I of the detoxification process was stimulated, with significantly elevated CbEs levels at 0.1 and 100 μg/L MPs, and 500 μg/L Gd. In contrast, no significant variations in CbEs were observed when both contaminants were combined, across both weeks. Complementary to phase I, the enzymes involved in phase II of the detoxification process were inhibited at the highest concentration of MPs (100 μg/L). Furthermore, an increase in GSTs activity was observed in the Gd and mix treatments. Previous studies have shown similar responses. For instance, Capolupo et al. (2021) found no alterations in the gills or digestive glands of *M. galloprovincialis* exposed to PS-MPs (0–150 ng/L). Zhang et al. (2021) reported that while smaller MPs (17 μm) caused no changes, larger MPs (150 μm) inhibited GSTs activity. The observed inhibition can be attributed to the bivalves' reduced ability to expel larger MPs compared to smaller ones following ingestion. This accumulation induces physiological stress in the organisms, ultimately resulting in inhibitory effects. In contrast, GSTs levels increased in the presence of Gd, peaking in the mix treatments after one week of exposure, suggesting that the organisms were actively working to expel the accumulated contaminants. Similarly, Henriques et al. (2019) and Parant et al. (2018) reported elevated GSTs activity with increasing Gd concentrations, further supporting this enzyme's role in detoxification processes. GSTs activity reached its highest level in the mix treatments, as also demonstrated by Zhang et al. (2021) following exposure to a mix of MPs and Phe. After the 2nd week, a decrease in GSTs levels was observed in the treatments of 100 μg/L MPs, 500 μg/L Gd, and both mix treatments (0.1 MPs + 500 Gd μg/L; 100 MPs + 500 Gd μg/L), indicating that at higher MPs, Gd and combined exposure concentrations clams were no longer able to continue to increase the activity of these enzymes along with the increase of the tested conditions. Furthermore, for Gd exposure, this inhibitory response may be explained by the fact that, as shown by Kim et al. (1998) in studies conducted with rats, Gd^{3+} ions inhibit GSTs by blocking Ca^{2+} channels and alter the dose-inhibitory response curves of protein kinase C inhibitors, which are also recognized as suppressors of drug-metabolizing enzymes.

Cellular damage, such as LPO, can occur if excess ROS are not effectively neutralized by the organism's defense mechanisms (Regoli and Giuliani, 2014). MPs are known to interact with the surface receptors of cellular membranes as well as penetrate the lipid bilayer, inducing structural changes to the membrane. The MPs can then be internalized by the molluscan cells through endocytosis which can lead to high ROS production by NADPH oxidase. As for the concentration of 0.1 μg/L MPs and 500 μg/L Gd, LPO levels were lower than the ones recorded in CTL clams, while for the concentration of 100 μg/L MPs and both mix concentrations, the levels tend to be similar or higher than CTL ones. These results might indicate that: 1) LPO is not a reliable indicator of oxidative stress in the studied contaminants. This hypothesis is supported by Oliveira et al. (2017), whose study on common goby (*Pomatoschistus microps*) exposed to a mix of MPs (0–184 μg/L) and pyrene (20 and 200 μg/L) found no significant responses to either the individual contaminant concentrations or their combination; 2) the inhibition observed at the lowest MPs concentration and Gd might be associated with high ROS generation through increased ETS activity and limited defense capacity which lead to the destruction of lipid membranes. This damage occurs as ROS interact with lipids, causing oxidative stress that compromises membrane integrity, and this destructive process can result in lower levels of LPO being measured. When lipid membranes are severely compromised, the cellular components responsible for generating LPO may be diminished or destroyed, leading to reduced overall LPO levels despite the initial increase in ROS production. A similar response was observed by Pinto et al. (2019) in *M. galloprovincialis* specimens exposed to La (100–10,000 μg/L), by Henriques et al. (2019) when exposing the same mussels species to Gd (0–120 μg/L), and by Zhang et al. (2021) after exposure the clam *M. veneriformis* to polystyrene at 50 μg/L.

Neurotoxicity is typically evaluated by measuring AChE activity, with a reduction in this enzyme's activity being a recognized indicator of neurotoxic effects from specific compounds, particularly pesticides like organophosphates and carbamates (English et al., 2012; Matozzo et al., 2005). Inhibition of AChE has also been linked to exposure to a broader range of chemicals, such as pharmaceuticals, nanoparticles, metals, and REEs (Cajaraville et al., 2000; De Marchi, 2020; Matozzo et al., 2005). Thus, while MPs treatments seemed to have no negative impact on clams' neurotoxic status, Gd and mix treatments appeared to have, to a limited extent, a negative impact, highlighting the greater harmful effect of Gd and the combination of the contaminants compared to MPs alone. The PCO graph highlights the close relationship of AChE and MPs on the positive side of PCO1, with higher activity values in clams exposed to MPs alone, in contrast to mix treatments on the negative side of PCO1. AChE is essential for the release of nerve impulses, and its selective inhibition serves as the primary mode of action for many organochlorine pesticides (Capolupo et al., 2021; Colovic et al., 2013). In aquatic organisms, the downregulation of AChE is a well-known effect of toxicant exposure, which can disrupt synaptic pathways involved in muscle contraction and heart function (Valbonesi et al., 2003). Additionally, research has shown that plastic particles of different sizes impair cholinergic pathways in bivalve mollusks, with similar effects also reported in teleost fish (Avio et al., 2015). Similarly, Pinto et al. (2019) observed inhibited AChE enzyme activity in *M. galloprovincialis* after 28 days of exposure to 10 mg/L of La, indicating clear neurotoxic effects of this pollutant.

5. Conclusion

The present study investigated a suite of biomarkers in the marine bivalve *D. trunculus* after exposure to two emerging contaminants currently detected in significant quantities in aquatic environments: MPs and Gd, and their possible interaction. The presence of MPs affected the stability of Gd concentrations in the aquaria. Gadolinium remained stable in aquaria with Gd alone, but decreased under mixed exposure after the 1st and 2nd weeks, suggesting a possible interaction with MPs, which reduced Gd availability in the medium. Nonetheless, wedge clams accumulated Gd throughout the experiment, while MPs' retention remained low, likely due to the short exposure time not allowing for substantial accumulation. Regarding the biochemical response, MPs and Gd alone triggered entirely different oxidative stress responses, with MPs exhibiting non-significant or inhibitory effects for most of the biochemical parameters analysed (probably due to the relatively low number of ingested MPs and the short exposure duration). In contrast, Gd increased oxidative stress in the same studied parameters. In combined exposures, Gd mainly exhibited a stronger toxic effect on organisms, while MPs showed the carrier function of transferring pollutants, potentiating the effects of oxidative stress. Therefore, the biochemical parameters with lower levels (ETS, SOD, CbEs) indicate a high level of stress in the mix treatments, as suggested by those parameters describing an overall and complementary response to stress (PROT, TAC, GSTs), in which the levels are the highest. Regarding the time factor, all three treatments showed more severe responses in the 2nd week compared to the 1st. Treatments with MPs exhibited differences between the 1st and 2nd weeks in terms of metabolic activity and energy reserves (ETS, PROT, GLY), as well as neurotoxicity (AChE). The Gd treatment showed variations between the two weeks in biotransformation enzyme activity (GSTs), while both mix treatments displayed differences in antioxidant defenses (SOD, TAC). Accordingly, the order of single and combined toxicity in the exposure test on clams was: 0.1 MPs μg/L ≈ 100 MPs μg/L < 500 Gd μg/L < 0.1 MPs μg/L + 500 Gd μg/L < 100 MPs + 500 Gd μg/L. This study contributes to advancing knowledge on the combined toxicity of MPs and Gd in marine bivalves. Although their association has not yet been documented in natural environments, representing a limitation for the study, both contaminants are present in the marine ecosystem and possess physicochemical characteristics that make such a

link highly plausible, as already demonstrated under laboratory conditions. These findings underscore the relevance of this emerging issue and support the need for future research to assess it under real environmental scenarios. Additionally, further studies are needed to explore the interactions between MPs and other pollutants, particularly concerning their combined toxic effects as reflected in integrated biological responses.

CRediT authorship contribution statement

Silvia Secco: Writing – original draft, Methodology, Formal analysis. **Marta Cunha:** Writing – original draft, Methodology, Formal analysis. **Carla Leite:** Writing – original draft, Methodology, Formal analysis. **Giovanni Libralato:** Writing – review & editing, Resources, Methodology. **Marco Trifuoggi:** Writing – original draft, Resources, Methodology. **Antonella Giarra:** Writing – original draft, Methodology, Conceptualization. **Amadeu M.V.M. Soares:** Resources, Funding acquisition. **Rosa Freitas:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Massimiliano Scalici:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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