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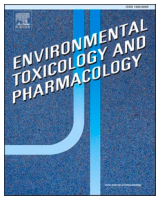

Evaluating the impact of gadolinium contamination on the marine bivalve *Donax trunculus*: Implications for environmental health

Silvia Secco [a], Marta Cunha [b], Giovanni Libralato [c], Marco Trifuoggi [d], Antonella Giarra [d], Amadeu M.V.M. Soares [b], Rosa Freitas [b,*], Massimiliano Scalici [a]

[a] Department of Sciences, University of Roma Tre, Viale Guglielmo Marconi, 446, Rome 00146, Italy
[b] Department of Biology & CESAM, University of Aveiro, Aveiro 3810-193, Portugal
[c] Department of Biology, University of Naples Federico II, Complesso Universitario of Monte Sant'Angelo, Via Vicinale Cupa Cintia 26, Napoli 80126, Italy
[d] Department of Chemical Sciences, University of Naples Federico II, Complesso Universitario di Monte Sant'Angelo, Via Vicinale Cupa Cintia 26, Napoli 80126, Italy

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ABSTRACT

Gadolinium (Gd), commonly used in contrast agents for medical imaging, has been detected in hospital wastewater and aquatic environments, raising environmental concerns. This study examined the accumulation and cellular impacts of Gd in the clam species *Donax trunculus*, commonly used as bioindicator of contamination. Gadolinium accumulation in clams increased with exposure and over time. Biological responses varied with Gd levels: low concentrations (10 and 50 μg/L) led to low metabolic activity and glycogen content, but high antioxidant activities and lipid peroxidation levels (LPO); high concentrations (250 and 500 μg/L) resulted in increased metabolic activity, while antioxidant enzyme activity was inhibited and LPO levels were the lowest. Metabolic activity decreased after two weeks, suggesting limited long-term metabolic resilience. The study underscores *D. trunculus* as an effective early warning species for Gd pollution and highlights the ecological risks of rising Gd levels, emphasizing the need for environmental monitoring and regulation.

1. Introduction

Gadolinium (Gd) is one of the 17 rare earth elements (REEs), increasingly employed since the 1980s in daily life objects. This is due to its unique properties, such as ferromagnetic behavior near room temperature and paramagnetic characteristics above 293 K (Curie point – Tc). Magnetic garnets, compact disks, computer memories, recording heads for video recorders, phosphorous for color TV tubes, garnets for microwave, and control rods for nuclear reactors are some of the applications of this element in the technological field (Rogowska et al., 2018). In the medical field, Gd chelates assume a pivotal role as contrasting agent in magnetic resonance imaging (MRI) medical exams, since this compound has historically been considered to be safe and well tolerated by humans when used at the recommended dose levels (Niendorf et al., 1991). Nevertheless, studies reported that the use of Gd in patients was associated with nephrogenic systemic fibrosis (NSF) disease, with a higher incidence in those patients with renal failure or insufficiency (Ramalho et al., 2017) and disturbance of calcium homeostasis (Mendichovszky et al., 2008). Additionally Kümmerer and Helmers (2000) reported that after the administration of 1.1 g of Gd for MRI, it remains in the patients' bodies for a long time, recording levels in the urines around 7 μg/L after 39 days, while other studies reported that the accumulation and deposition of this contaminant was also observed in the brain, bones (Murata et al., 2016), kidneys (Ramalho et al., 2017), and skin (Parillo et al., 2023) of the patients submitted to MRI.

Gadolinium can be identified in different environmental compartments, with the highest concentrations in hospital effluents. The total release of this metal by German hospitals is approximately 132 kg per year (Kümmerer and Helmers, 2000), while in the Han River (South Korea) Gd fluxes are estimated at 600–918 kg per year (Song et al., 2017). Furthermore, several studies have demonstrated that Gd is not efficiently removed by any of the wastewater treatment plant (WWTP) processes used, illustrating the potential persistence of this contaminant in the aquatic environment (Barber et al., 2015). In this context, Parant et al. (2018) reported anomalies of Gd concentrations with peaks of 80 μg/L of Gd in contaminated areas of the WWTPS of the Lorrain region River and other WWTP effluents in France, where Gd concentrations have been estimated to be as high as 100 μg/L (Dulio et al., 2009).

* Correspondence to: Departamento de Biologia & CESAM, Universidade de Aveiro, Campus Universitário de Santiago, Aveiro 3810-193, Portugal.
E-mail address: rosafreitas@ua.pt (R. Freitas).

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Anthropogenic Gd complexes are expected to have a longer environmental half-life due to their high stability. Indeed the presence of this contaminant has also been found in marine and coastal environments reaching levels of 112 pmol/kg in San Francisco Bay (USA) (Hatje et al., 2016), and 409.6 ng/L at a marine outfall in Northeast Brazil (Pedreira et al., 2018). The presence of Gd has also been detected in some wells providing drinking water in Southern France, with concentrations up to 2.4 ng/L (Rabiet et al., 2009).

The rising concern about this element's toxicity has led to the development of ecotoxicological studies using freshwater and marine species, including commonly used bioindicator bivalves and their biological responses were evaluated using biochemical markers. Henriques et al. (2019) investigated the toxic effects of Gd on the bivalve *Mytilus galloprovincialis* at increasing concentrations, namely 15, 30, 60, and 120 μg/L, affirming the capacity of this contaminant to induce oxidative stress and neurotoxicity as well as to reduce the metabolic capacity of the mussels. Hannana et al. (2017) investigated two forms of Gd ($GdCl_3$ and Omniscan) at 10, 50, 250 and 1250 μg/L on the freshwater bivalve *Dreissena polymorpha*, and the results showed alterations in the mRNA level of metallothionein, on the antioxidant enzymes, and in cyclooxygenase activity in both of the studied Gd compounds. Another experiment conducted by Andrade et al. (2022a) studied the oxidative stress effects under 10 μg/L of Gd at different levels of salinity (20, 30 and 40) on *M. galloprovincialis*, observing that this metal caused cellular damage at all salinities, although mussels revealed different biochemical responses according to the salinity considered. Trapasso et al. (2021b) exposed *M. galloprovincialis* to 50 μg/L of Gd in the presence and absence / of the algae *Ulva lactuca* to assess the role of the macroalgae in preventing oxidative impacts. The results showed the accumulation and toxic effects of Gd in *M. galloprovincialis*, which were reduced in the presence of *U. lactuca*

Considering the toxic effects already described for Gd and the need to understand potential environmental threats towards marine wildlife resulting from the increasing concentrations of this contaminant in aquatic environments worldwide (Cunha et al., 2022; Rogowska et al., 2018), this study aimed to investigate the effects of a range of Gd concentrations, resembling low to highly contaminated areas, in the bivalve species *Donax trunculus*, focusing on its accumulation and clams' biochemical performance. This study also investigated if biochemical responses were time-dependent to understand possible clams' status after Gd discharges into the aquatic environment. This species is typical from sandy bottoms of the Mediterranean and Atlantic Sea, also presenting commercial significance. While *D. trunculus* has previously served as an early warning sentinel for investigating the toxic effects of metals (Lamine et al., 2023), toxins (Botelho et al., 2018) and microplastics (Ben-Haddad et al., 2022), this study represents the first investigation considering the effects of Gd using this species.

2. Material and methods

2.1. Experimental conditions

The Mediterranean wedge clam *Donax trunculus* was selected as a biological model for the present study. Adult individuals of *D. trunculus* were collected along the beach of Passoscuro site (Lazio, Italy) and transferred immediately to the laboratory. Wedge clams were placed in tanks under constant aeration for a period of depuration and acclimation of one week. During this period, water was renewed every day with distilled water and marine salt "Royal Nature Premium Tropical Sea Salt". The temperature and the salinity were adjusted to replicate field conditions, and set respectively at 18±1.0 °C and 35±1, minimizing stress on the organisms under the new laboratory setting. Wedge clams were fed 3 hours before the renewal of water with 5 mg of a mixture of *Spirulina sp.* and *Artemia sp* .

After the acclimation period, twelve individuals of *D. trunculus* were placed in tanks of 6 L of artificial seawater (0.5 L of water per individual), with three replicate aquaria per treatment and five treatments, corresponding to five Gd exposure concentrations: CTL-0, 10, 50, 250, and 500 μg/L. Gadolinium Standard for ICP (*Trace***CERT**®, 1 g/L Gd in nitric acid (HNO_3), SigmaAldrich) was used in the present study. The selected range of concentrations was based on environmental concentrations reported previously in contaminated areas in France where Gd concentrations have been estimated as high as 100 μg/L (Dulio et al., 2009) and concentrations tested under laboratory conditions (Hanana et al., 2017; Henriques et al., 2019).

The exposure period lasted for 14 days, and during this period water was renewed every week, with the reestablishment of the physicochemical parameters and Gd concentrations. Similarly to the acclimation period, wedge clams were fed three hours before the water renewal. Per replicate/tank six clams were collected after each exposure week and immediately frozen and stored at −80 °C for further analyses. Both for the biochemical analysis and Gd quantification, the six individuals of *D. trunculus* collected per tank were divided into pools of three.

Water samples from each tank were also collected 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. (2022a).

2.2. Gadolinium quantification in water and in clams' tissues

The water samples were acidified with HNO_3 2 % until pH <2. The quantification of Gd in the water samples was performed by inductively coupled plasma mass spectrometry (ICP-MS) on a Aurora M90 Brucker apparatus. The ICP-MS equipment was also used to measure the total concentration of Gd in the tissues of the clams. 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 temperature raised up to 90 °C for 3 h. After achieving room temperature, the samples were placed in polyethylene vials and ultrapure water was added to a final volume of 10 mL. Blanks (vessels without sample) and duplicates were included for quality control. The blanks were always below the limit of quantification.

2.3. Biomarkers analyses

To assess the impacts of Gd on clams, different biochemical parameters related to metabolic and oxidative status were measured. Alterations in organisms' metabolic capacity and energy reserves content can be associated with their capacity to respond to stress conditions (Sokolova, 2013). In the present study, such alterations were assessed by measuring the Electron Transport System (ETS) activity, the Glycogen (GLY) and Protein (PROT) contents. Beyond metabolic adjustments, organisms can activate their antioxidant defence mechanisms to fight against the excess of reactive oxygen species (ROS) normally associated with stress conditions (Regoli and Giuliani, 2014). Here, clams' antioxidant capacity was evaluated by quantifying the activity of the enzymes Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx) as well as clams' Total Antioxidant Capacity (TAC) after each exposure week. Besides a general oxidative stress generated when in the presence of contaminants, organisms might fight against xenobiotics through their mechanisms of detoxification, including phase I and phase II enzymes, such as Carboxylesterases (CbEs) and Glutathione S-transferases (GSTs), respectively. In this study, these enzymes were measured in clams after both experimental periods. Cellular damage, here represented by Lipid Peroxidation (LPO), was measured and associated with clams' defence efficiency, namely in terms of ROS elimination by antioxidant mechanisms.

After the experimental period, and prior to the biochemical analyses, clams' frozen tissues were subjected to homogenization using an Elvehjem potter. Pools of 3 individuals of wedge clams (2 pools per tank,

6 per treatment) were used. For the extraction procedure, 0.5 g (fresh weight, FW) of homogenized soft tissue per pool were prepared with specific buffers in a ratio 1:2 Magnesium Sulfate Buffer (0.1 mM Tris-HCl, with 15 % (w v^{-1}) PVP, 153 mM $MgSO_4$, 0.2 % (v v-1) Triton X-100, pH 8.5) for ETS and Potassium Phosphate Buffer (50 mM potassium phosphate, 1 mM EDTA, 1 mM DTT, Triton X-100 1 %(v v^{-1}), pH 7) for GLY, PROT, SOD, GPx, TAC, CbEs, GSTs, and LPO. The extraction procedure was done using a TissueLyser II (Qiagen) for 90 s and centrifuged for 20 min at 10,000 *g* (or 3,000 *g* for ETS) at 4 °C. Supernatants were collected and the samples were analyzed in duplicate using a microplate reader (BioTek) (Cunha et al., 2022).

2.3.1. Metabolic capacity and energy reserves

The ETS activity was measured based on King and Packard (1975) and the modifications performed by De Coen and Janssen (1997). A buffered substrate (Tris-HCl buffer with TritonX-100, pH 8.5) was added to each specimen. In addition, NAD(P)H (consisting of NADH and NADPH) and p-iodonitrotetrazolium were added. Absorbance was monitored over a 10 min period at 490 nm with intervals of 25 s, and the extinction coefficient (ε) of 15.9 mM^{-1} cm^{-1} was applied to determine the formazan formed. The results were reported in nmol/min/g of FW.

For GLY quantification the sulfuric acid method described by DuBois et al. (1956) was employed. This method is based on the fact that phenol with sulfuric acid enables quantitative colourimetric micro-analysis of sugars; so the colour intensity, under constant phenol concentration, directly correlates with the sugar content. Glucose standards ranging from 0 to 10 mg/mL were utilized to establish a calibration curve. Absorbance was measured at 492 nm after incubation for 30 min at room temperature. The outcomes were expressed in mg/g FW.

The protein (PROT) content was measured following the Biuret spectrophotometric method, as outlined by Robinson and Hogden (1940). A standard curve was generated using bovine serum albumin (BSA) at concentrations ranging from 0 to 40 mg/L. This technique relies on complex formation in an alkaline solution, where copper (II) ions react with peptide bonds in proteins. Before measuring absorbance, the solution was incubated for 10 min at 30 °C in darkness, then read at 540 nm. Following Beer's Law, the absorbance is directly proportional to protein concentration in the sample, with results expressed in mg/ g FW.

2.3.2. Antioxidant capacity

The superoxide dismutase (SOD) activity was quantified using the method described by Magnani et al. (2000). The activity of SOD (Cu-Zn) was determined by a simple and rapid method. It is based on the ability of the enzyme to inhibit the autoxidation of pyrogallol. The autoxidation of pyrogallol in the presence of EDTA at pH 8.2 is 50 %. The principle of the method is based on the competition between the autoxidation of pyrogallol by O_2^- and the dismutation of this radical by SOD. The mixture's absorbance was read at 420 nm. The reaction was initiated by adding pyrogallol solution (in 0.01 mmol/L HCl). Following a one-min waiting period, the absorbance was read at 420 nm. The results were expressed as nmol/min/g FW.

The activity of GPx was quantified according to the method of Paglia and Valentine (1967). The reaction was monitored at 340 nm at 15 s intervals for 5 min, and enzymatic activity was determined using the extinction coefficient (ε) of 6.22 mM^{-1} cm^{-1}. The outcomes were expressed as U/g FW, where one unit (U) represents the amount of enzyme causing the formation of 1.0 mmol NADPH oxidized per min.

The TAC content was assessed using the method described by Benzie and Strain (1996) with adaptations performed by Wootton et al. (2021). A standard curve was generated using TAC standards ranging from 0 mM to 1000 mM. The change in absorbance between 0 and 10 min was used as an indicator of the amount of antioxidant activity. Results are reported in μmol/g FW.

2.3.3. Detoxification mechanism

The activity of GSTs was assessed following the protocol outlined by Habig et al. (1976) with certain adaptations as detailed by Carregosa et al. (2014). The sample was mixed with the reaction solution, consisting of potassium phosphate buffer at pH 6.5, containing 1-chloro-2, 4-dinitrobenzene and GSH. The absorbance was recorded at 340 nm, and the GSTs activity was determined using the extinction coefficient (ε) of 9.6 mM^{-1} cm^{-1}. The enzymatic activity was reported in U/g FW, where one unit (U) is defined as the amount of enzyme catalyzing the formation of 1 mmol of dinitrophenyl thioether per min.

The activity of CbEs was assessed using the method described by Hosokawa and Satoh (2001) with alterations made by Solé et al. (2018). The pace of pNPB hydrolysis was measured by monitoring absorbance at 405 nm for 5 min at 15 s intervals, employing an extinction coefficient (ε) of 18 $mM^{-1}cm^{-1}$. The findings were expressed as nmol/min/g FW.

2.3.4. Lipid peroxidation levels

The levels of lipid peroxidation (LPO) were evaluated by measuring the malondialdehyde (MDA) content, following the method described by Ohkawa et al. (1979). The estimation was carried out by quantifying thiobarbituric acid reactive substances (TBARS), which are formed in the reaction between LPO by-products such as MDA and 2-thiobarbituric acid (TBA). Absorbance was recorded at 532 nm, and the LPO levels were determined using the extinction coefficient (ε) of 156 mM^{-1} cm^{-1}. The results were reported in nmol MDA/ g FW.

2.4. Data analysis

For each specific treatment, the bioaccumulation factor (BCF) was computed, following the calculation of Arnot and Gobas (2006), by dividing the mean concentration of Gd detected in the tissues of *D. trunculus* at the end of the experimental period by the mean Gd value observed in seawater measured immediately after spiking, representing the authentic exposure concentration.

Statistical hypothesis testing + add-on in PRIMER v6 (Anderson, 2008) was performed on all biochemical results (ETS, PROT, GLY, SOD, GPx, TAC, CbEs, GSTs, LPO) and Gd concentrations obtained from each exposure condition. The pseudo-F *p*-values in the main PERMANOVA tests were evaluated in terms of significance. Pairwise comparisons were performed when significant differences were observed in the main test. Values with $p < 0.05$ were considered significantly different. The null hypothesis tested was: for each biomarker and exposure period (weeks 1 and 2), no significant differences existed among exposure concentrations (0, 10, 50, 250, 500 μg/L). For each biomarker significant differences among treatments in week 1 were represented in figures with lowercase letters, while in week 2 were represented with uppercase letters. Significant differences between week 1 and week 2 were represented with asterisks.

The matrix containing biochemical descriptors, along with Gd concentrations in wedge clam's tissues per treatment, was employed to compute the Euclidean distance similarity matrix. This matrix was then simplified by calculating the distance between centroids based on the condition, which was subsequently subjected to ordination analysis using Principal Coordinates (PCO). Pearson correlation vectors of biochemical descriptors (with correlation coefficients >0.75) were overlaid onto the PCO graph.

The integrated biomarker response index version 2 (IBRvs2), developed by Beliaeff and Burgeot (2002) and revised by Sanchez et al. (2013), was used to synthesize data from various biochemical markers, illustrating the overall biochemical effects of different treatments on *D. trunculus*. Responses to each treatment (10, 50, 250, 500 μg/L of Gd) were compared with the control (CTL-0 μg/L). A logarithmic transformation was applied to reduce variance, and the data were normalized and used to calculate the biomarker deviation index (A), with a central reference of 0. The IBRvs2 was then determined as the sum of the absolute deviations (Σ |A|). Results were visualized in a star plot, where areas beyond the reference line indicated biomarker induction, and within it indicated inhibition, with higher IBRvs2 values showing

greater biochemical responses in treated mussels.

3. Results

3.1. Gadolinium concentrations in seawater and wedge clams' tissues

Regarding Gd concentrations in seawater from the experimental conditions, values obtained from water samples collected immediately after spiking both in week 1 and week 2 showed that measured and nominal concentrations were similar for all conditions and weeks, confirming the Gd spiking process (Table 1). Regardless of the exposure week, Gd concentrations in clams increased along the exposure gradient, with significant differences among treatments (Table 1).

3.2. Biological responses: biochemical parameters

3.2.1. Metabolic capacity and energy reserves

Regarding metabolic activity, after the first week of exposure, the levels of ETS increased significantly in all Gd concentrations compared to CTL values. In week 2 the same increasing trend was observed, with significantly high levels in clams exposed to 50 and 250 μg/L of Gd in comparison to CTL, 10 and 500 μg/L of Gd. Comparing week 1 and week 2, significantly higher ETS activity was observed after the first week in clams exposed to 10 and 50 μg/L of Gd (Fig. 1A).

In week 1 the PROT levels decreased significantly at the highest concentration compared to the two lowest concentrations. In contrast, in week 2 the PROT content decreased in all the analysed concentrations compared to CTL clams, with significant differences at 10, 50 and 500 μg/L of Gd. No significant differences were observed between exposure weeks (Fig. 1B).

Regarding the GLY content, after the first week of exposure, clams showed higher values along the increasing concentration range, with significant differences between clams exposed to 10, 250 and 500 μg/L of Gd and control ones. A similar pattern was observed after the second week of exposure, with significant differences between clams exposed to the highest concentrations (250 and 500 μg/L) of Gd and the remaining treatments. Differences between weeks were observed at the lowest and the highest concentrations with higher GLY values after week 1 (Fig. 1C).

3.2.2. Antioxidant enzymes

Regarding antioxidant enzymes, in week 1 SOD activity increased significantly at the lowest Gd concentration (10 μg/L) compared to the CTL and 500 μg/L exposed clams. No differences were observed among the remaining treatments. In week 2 a similar trend was observed, and the levels of SOD increased at the lowest concentrations of 10 μg/L and 50 μg/L and decreased at the highest concentrations of 250 μg/L and 500 μg/L. Significant differences between weeks were only observed at the lowest exposure concentration, with higher SOD activity after week 1 (Fig. 2A).

Regarding GPx, after both exposure weeks, the activity decreased significantly along the increasing gradient of Gd concentrations, with significantly lower values in all exposure concentrations compared to the CTL. No significant differences were observed between weeks (Fig. 2B).

In week 1, TAC content was the lowest at the highest exposure concentration, with significant differences to CTL and 10 μg/L exposed clams. Also, in week 2 the levels of TAC decreased along the increasing concentrations with significant differences between CTL and 10 μg/L, 250 μg/L, and 500 μg/L exposed clams. No significant differences were observed between exposure weeks (Fig. 2C).

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

Exposure periods	Treatments	Water	*Donax trunculus*	BCF
Week 1	CTL	< 0.01	$< 0.01^{a}$	-
	Gd 10 μg/L	10.18	0.41 ± 0.12^{a}	40.28
	Gd 50 μg/L	51.50	3.66 ± 0.81^{b}	71.07
	Gd 250 μg/L	248.90	9.12 ± 6.26^{c}	76.82
	Gd 500 μg/L	505.0	44.48 ± 24.84^{d}	88.08
Week 2	CTL	< 0.01	$< 0.01^{a}$	-
	Gd 10 μμg/L	9.50	0.72 ± 0.80^{a}	75.79
	Gd 50 μg/L	56.50	4.61 ± 1.26^{b}	81.59
	Gd 250 μg/L	249.90	34.03 ± 7.74^{c}	136.17
	Gd 500 μg/L	488.10	66.52 ± 11.96^{d}	136.28

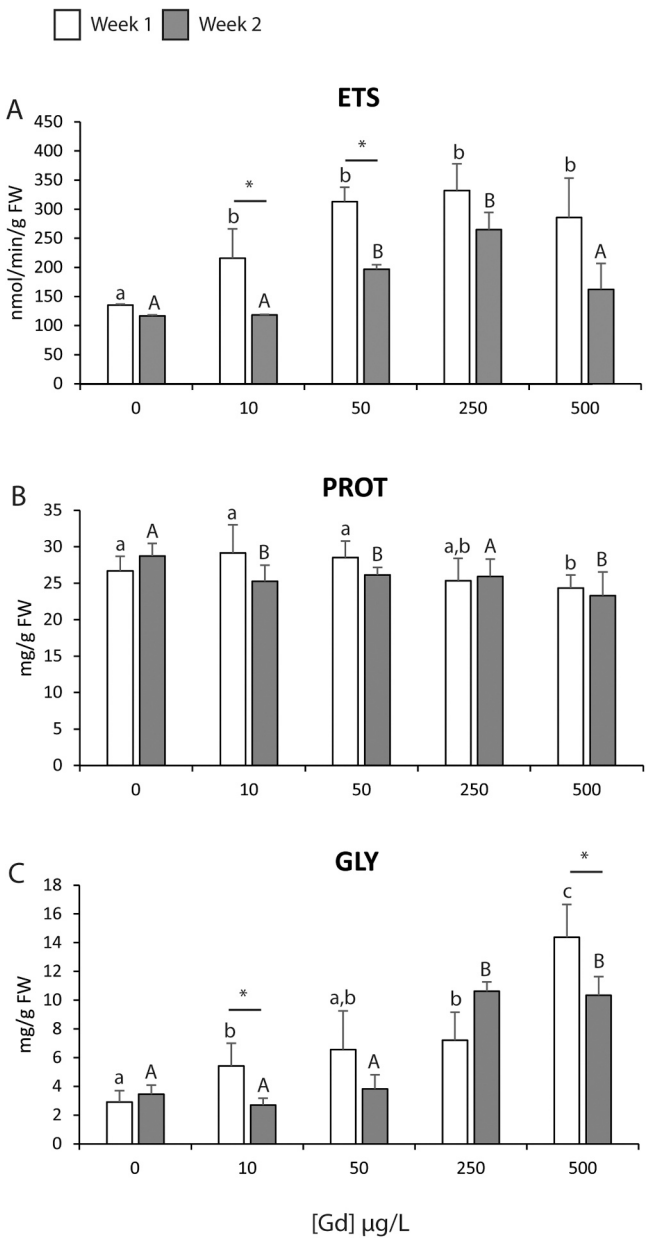

Fig. 1. A: Electron transport system (ETS) activity; **B:** Glycogen (GLY) content; **C:** Protein (PROT) content, in *Donax trunculus* exposed to gadolinium (CTL-0, 10, 50, 250 and 500 μg/L) for 7 days (week 1) and 14 days (week 2). For week 1, significant differences among treatments are identified with lowercase letters; for week 2 significant differences among treatments are identified with uppercase letters. Differences between weeks are identified with an asterisk.

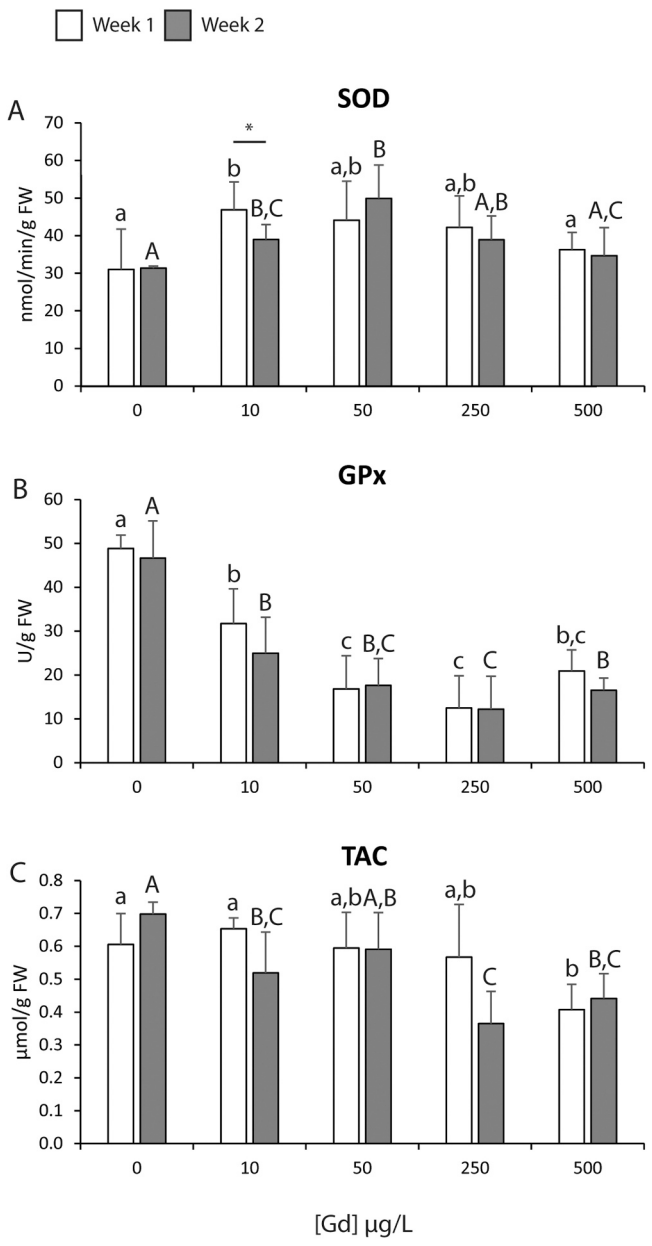

Fig. 2. A: Superoxide dismutase (SOD) activity; **B:** Glutathaione peroxidase (GPx) activity; **C:** Total Antioxidant Capacity (TAC) content, in *Donax trunculus* exposed to gadolinium (CTL-0, 10, 50, 250 and 500 µg/L) for 7 days (week 1) and 14 days (week 2). For week 1, significant differences among treatments are identified with lowercase letters; for week 2 significant differences among treatments are identified with uppercase letters. Differences between weeks are identified with an asterisk.

3.2.3. *Biotransformation enzymes*

Concerning biotransformation enzymes, in week 1 CbEs activity showed a significant difference between 50 µg/L and 500 µg/L with higher values at 50 µg/L. In week 2 no significant differences were observed among the treatments. Between exposure weeks, significantly lower values were observed in week 2 compared to week 1 for 10 µg/L and 50 µg/L treatments (Fig. 3A).

Regardless of the exposure week, no significant differences were observed among treatments in GSTs activity. No significant differences were observed between weeks, regardless of the exposure treatment (Fig. 3B).

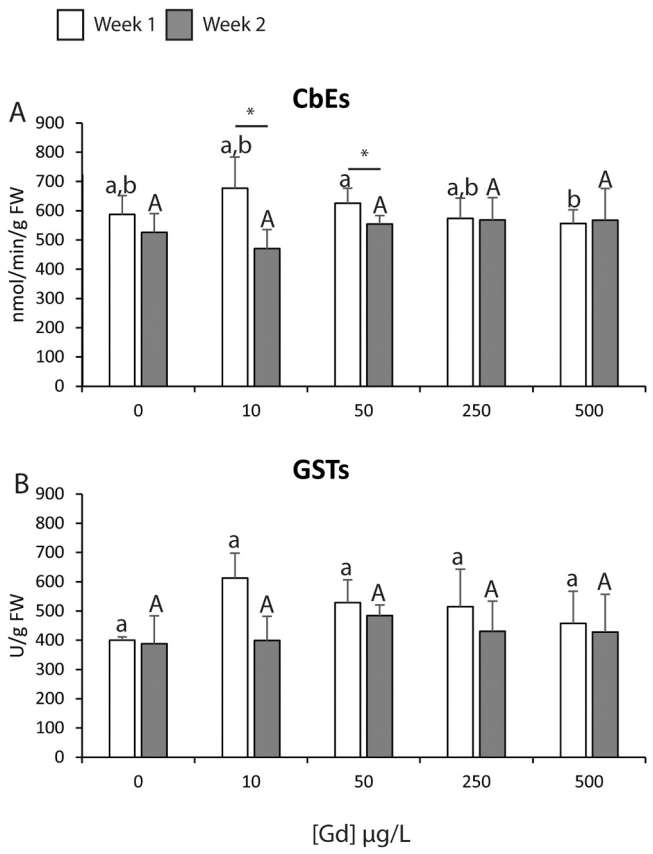

Fig. 3. A: Carboxylesterases (CbEs) activity; **B:** Glutathione S-tranferases (GSTs) activity, in *Donax trunculus* exposed to gadolinium (CTL-0, 10, 50, 250 and 500 µg/L) for 7 days (week 1) and 14 days (week 2). For week 1, significant differences among treatments are identified with lowercase letters; for week 2 significant differences among treatments are identified with uppercase letters. Differences between weeks are identified with an asterisk.

3.2.4. *Oxidative damage*

In week 1, levels of LPO decreased significantly in clams exposed to the highest Gd concentration (500 µg/L). A similar trend was observed after the 2nd week, but in this case, clams showed significantly higher LPO levels at 10 µg/L and significantly lower values at 250 and 500 µg/L compared to the remaining treatments. Significant differences between weeks were recorded at the lowest exposure concentration, with higher values after week 2 (Fig. 4).

3.3. *Multivariate analysis*

As regards PCO analysis, PCO1 explained 50.2 % of the total variation separating the lowest concentrations of Gd exposure (0-CTL and 10 µg/L for weeks 1 and 2 and 50 µg/L for week 2) in the positive side of PCO1, from the highest concentrations of Gd treatments in the negative side of PCO1 (Fig. 5). The variables that better explained the variation of PCO1 were GPx ($p = 0.85$), TAC ($p = 0.89$) and GLY ($p = -0.92$), with the antioxidant enzymes on the positive side (control and lower exposure concentrations) and the GLY content associated with the negative side (higher exposure concentrations). The PCO vertical dimension (PCO2) explained 27.9 % of the total variation separating organisms exposed to the lowest and highest concentrations (positive side) from the organisms under intermediate concentrations (negative side). The vector that best explain this distinction was GSTs ($p = -0.84$).

3.4. *Integrated biomarker response*

In IBRvs2, organisms exposed to Gd for one week revealed higher

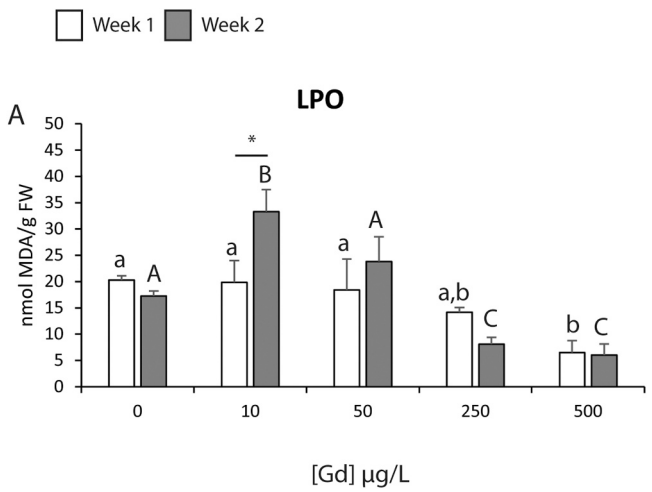

Fig. 4. Lipid Peroxidation (LPO) levels, in *Donax trunculus* exposed to gadolinium (CTL-0, 10, 50, 250 and 500 µg/L) for 7 days (week 1) and 14 days (week 2). For week 1, significant differences among treatments are identified with lowercase letters; for week 2 significant differences among treatments are identified with uppercase letters. Differences between weeks are identified with an asterisk.

scores (15.5, 12.8, 9.4 and 8.3 for 10, 50, 250, 500 µg/L respectively) than organisms subjected to Gd for two weeks (8.3, 7.9, 10.1, 7.6 and 5.6 for CTL,10, 50, 250, 500 µg/L, respectively). Higher scores obtained after week 1 are associated with greater alterations observed in clams' metabolic capacity, while lower IBR scores after week 2 were related mainly to reduced antioxidant capacity.

4. Discussion

4.1. Gadolinium concentration

Bivalves are commonly used as sentinel species and recent studies demonstrated their ability also to biomonitor REEs, such as Lanthanum (Andrade et al., 2022b), Neodymium (Freitas et al., 2020b), Dysprosium (Freitas et al., 2020a), and Gd (Hanana et al., 2017a; Henriques et al., 2019). The study presented here investigated for the first time the accumulation capacity and cellular impacts in the clam species *D. trunculus* after exposure to Gd, over 1 and 2 weeks. *D. trunculus* was previously used as a bioindicator of other emerging/hazardous contaminants such as plastics (Ben-Haddad et al., 2022), metals (Lamine et al., 2023) and toxins (Botelho et al., 2018).

In terms of accumulation, *D. trunculus* demonstrated to be an excellent sentinel species as the level of Gd in their tissues increased significantly with the increasing gradient of concentrations. The present findings also suggest that wedge clams demonstrated a rising capacity for Gd accumulation over time, with higher concentrations after 2 weeks of exposure. Indeed also the BCF factor revealed the capacity of Gd accumulation in the wedge clams, which increased along the rising tested concentrations and over exposure time. Several authors confirm the high capacity of bivalves to accumulate Gd, such as Henriques et al. (2019) which exposed *M. galloprovincialis* to Gd (15–120 µg/L) for 28 days. Their results demonstrated the capacity of mussels to accumulate Gd, with a direct relationship between accumulated and exposure concentration. Indeed mussels at the lowest tested concentration accumulated a lower Gd level than the LOQ which was 0.38 µg/g; while at the highest treatment (120 µg/L), a concentration of 2.5±0.50 µg/g was observed. Trapasso et al. (2021) demonstrated that after 21 days at 50 µg/L Gd *M. galloprovincialis* accumulated 2.2±0.81 µg/g of this

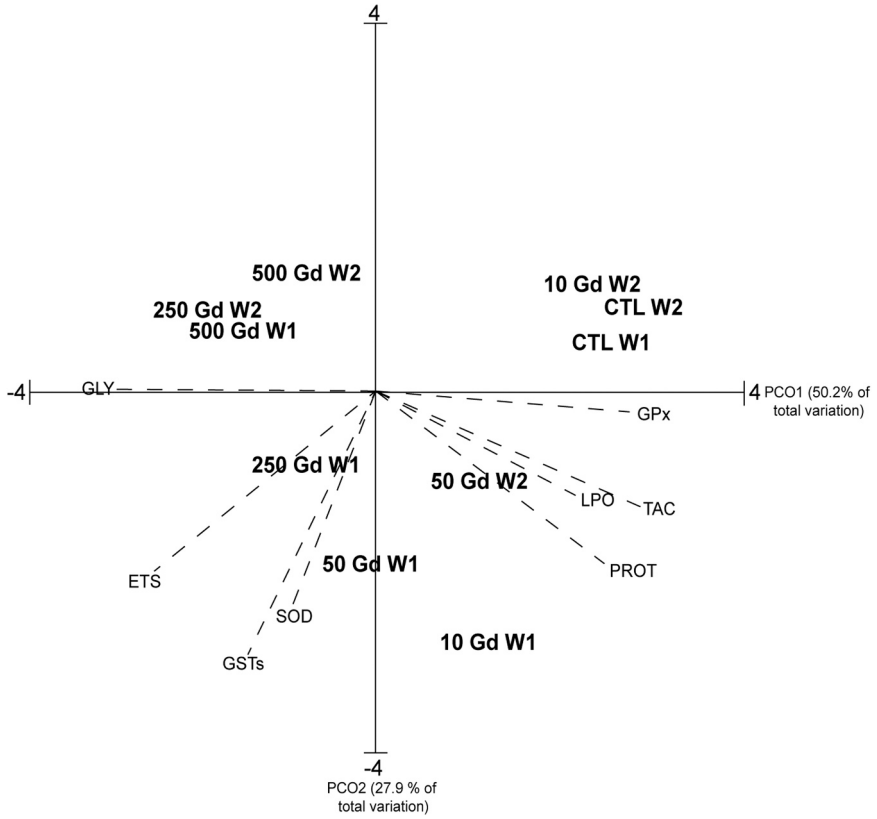

Fig. 5. Centroids ordination diagram (PCO) based on biochemical parameters assessed in *Donax trunculus* exposed to gadolinium (CTL-0, 10, 50, 250 and 500 µg/L) for 7 days (week 1) and 14 days (week 2). Pearson correlation vectors are superimposed as supplementary variables, namely biochemical data ($r > 0.75$): ETS (electron transport system activity), GLY (glycogen content), PROT (protein content), SOD (superoxide dismutase activity), GPx (glutathione peroxidase activity), TAC (total antioxidant capacity content), CbEs (carboxylesterases activity), GSTs (glutathione S-tranferases activity), LPO (lipid peroxidation levels).

element. After a shorter exposure period (14 days) Cunha et al. (2022) observed that the exposure to 10 μg/L of Gd led to an accumulation of 0.15±0.02 μg/g in *M. galloprovincialis*. These studies tested different concentrations and different exposure periods, revealing that the accumulation of Gd in *M. galloprovincialis* is proportional to the studied concentrations, even though the amounts of Gd accumulated in the tissues of *M. galloprovincialis* remain at a much lower level than the concentrations to which they were subjected. Studying the accumulation in a freshwater species, Hanana et al. (2017) showed that at the end of the experiment (28 days) *D. polymorpha* exposed to 10–1250 μg/L accumulated significantly more the ionic form of Gd in $GdCl_3$ than the chelated form of Omniscan with 0.43±0.20 μg/g and 0.03±0.03 μg/g respectively for the lowest tested concentration of 10 μg/L, 3.18 ±1.16 μg/g and 0.14±0.03 μg/g for the concentration of 250 μg/L. In the present study *D. trunculus* at the lowest concentration comparable to the previous studies of 10 μg/L accumulated 0.41 ± 0.12 μg/g after the 1st week and 0.72±0.80 μg/g after the 2nd week, while at 50 μg/L they accumulated 3.66±0.81 after 7 days and 4.61±1.26 after 14 days. Therefore, the wedge clam demonstrated exceptional bioaccumulation capabilities across a range of Gd concentrations, accumulating significantly more Gd in their tissues than the above-mentioned bivalve species when exposed for similar periods and to a similar range of concentrations.

4.2. Biological responses

In terms of biological responses, the biochemical alterations observed in *D. trunculus* organisms differed among treatments, as revealed by the PCO analysis, which reflect the Gd exposure gradient. The positive side of PCO presents the CTL and the lowest concentrations of Gd exposure (10 and 50 μg/L), associated with the highest values of LPO, GPx and TAC. On the other hand, the negative side presents the highest concentrations of Gd (250 and 500 μg/L) associated with higher values of GLY, ETS, and SOD.

An increase in ETS activity along the concentration gradient (0–500 μg/L) of Gd was observed, particularly after the 1st week of exposure, with a limited metabolic capacity noted after two weeks. This response contributed to higher IBR scores observed for the treatments after the 1st week, indicating a rapid reaction given by clams to acute Gd exposure. This response might be associated with *D. trunculus* efforts to fuel up defence mechanisms in response to short-term adverse conditions caused by increasing concentrations of the contaminant. Cunha et al. (2022) observed a similar trend in ETS activity in an experiment involving the administration of Gd (10 μg/L) in *M. galloprovincialis*. This response is considered an efficient strategy used by bivalves to fight against moderate stressful conditions, mostly accompanied by the activation of other defence mechanisms (Freitas et al., 2020b). Similarly, other studies have shown that marine bivalves (*Ruditapes philippinarum* and *M. galloprovincialis*) exhibited an increased metabolic capacity, assessed by ETS activity, after 28 days of exposure to nanoparticles (multi-walled carbon nanotubes) and drugs (salicylic acid) (De Marchi et al., 2018). However, the present results showed that after week 2 and at the highest stress levels (250 and 500 μg/L of Gd), bivalves were not able to steadily increase their metabolism against the stress imposed. Indeed although after week 2 clams were able to increase their metabolic capacity up to 250 μg/L, their ETS values were lower than the ones observed after week 1 and at the highest Gd concentration, ETS values decreased up to CTL values. Other long-term studies (28 days) have demonstrated the same tendency of bivalves to decrease their metabolic capacity when exposed to La (0–10 μg/L) with authors suggesting that after a longer exposure period and/or higher stress levels, bivalves are no longer able to keep their metabolic activity at maximum levels (Freitas et al., 2019).

Following the increasing ETS activity, the results obtained showed that after the first week, the GLY content also increased along the exposure gradient, suggesting that wedge clams in the presence of Gd responded by reducing the GLY expenditure and/or increasing GLY production, which could be compensated by the use of other energy reserves sources, such as lipids (Bayne et al., 1975). A similar trend was observed after the second week of exposure but with lower GLY levels compared to the week 1, indicating that clams were no longer able to maintain GLY content at such high levels while needing to use them. Previous studies by Cunha et al. (2022) and Andrade et al. (2022a) already demonstrated that bivalves use lipids as energy resources when under stressful conditions such as the presence of Gd.

The content of PROT tended to decrease at higher Gd concentrations, especially after the 2nd week of exposure, corroborating the need for clams to use their energy reserves after a longer exposure period. The reduced PROT content after week 2 contributed to the lower IBR scores observed at this point, compared to those recorded after two weeks of exposure. Similar outcomes were observed by (Henriques et al., 2019) wherein at lower concentrations of Gd, both GLY and PROT levels increased. Conversely, at the highest Gd concentrations, both GLY and PROT decreased, suggesting that when bivalves are subjected to high stress, they start the expenditure of their energy reserves to fuel up defence mechanisms.

Regarding the antioxidant response, in general, clams exhibited a decreased capacity along the exposure gradient, especially after the second week of exposure, which resulted in lower IBR scores after week 2 in comparison to week 1. While SOD activity tended to increase up to 50 μg/L regardless of the exposure week, at higher concentrations this enzyme showed activity levels similar to control. Nevertheless, the total antioxidant capacity showed no activation (10 and 50 μg/L) or even a decrease (250 and 500 μg/L), demonstrating a lower antioxidant capacity of *D. trunculus*, as evidenced by GPx results, with a clear inhibition of this enzyme in the presence of Gd. Similarly, Henriques et al. (2019) and Hanana et al. (2017) demonstrated a comparable antioxidant response in the presence of increasing concentrations of Gd, with increasing activity of this parameter at the lowest concentration of the contaminant (15 and 10 μg/L respectively), while a decreasing level was observed at the highest concentration. Such results as well as the present findings suggest that clams can augment their antioxidant defense capacity in the presence of Gd within a certain threshold. A similar trend of the antioxidant responses has been observed with exposure to other REEs, including La (Hanana et al., 2017b) tested in the freshwater bivalve *D. polymorpha*. Freitas et al. (2020b) observed a decreasing trend in the activities of SOD and GPx, while CAT exhibited an increasing trend.

The present study revealed that neither phase I nor phase II biotransformation enzymes were significantly implicated in Gd detoxification, which may explain high Gd concentrations in clam tissues. It is known that CbEs and GSTs play a crucial role in the elimination of harmful substances, as they are involved in phase I and phase II biotransformation processes, respectively. These enzymes catalyze the conversion of contaminants, facilitating their excretion from their cells (Regoli and Giuliani, 2014). (Leite et al., 2024) conducted an experiment comparing the bioaccumulation capacity of *M. galloprovincialis* for two REEs, Praseodymium (Pr) and Europium (Eu). The study highlighted that during exposure to Pr, the mussels accumulated a higher amount of the contaminant, but biotransformation enzymes were not significantly involved in the oxidative stress response. In contrast, exposure to Eu led to an increase in detoxification enzymes, though with a lower accumulation of the contaminant. The non-activation of GSTs and CbEs can also be attributed to a relatively short exposure time (14 days). In fact, in experiments involving longer exposure periods (28 days), a higher biotransformation capacity was observed in mussels exposed to Gd (0–10 000 μg/L). Testing the impacts of similar Gd concentrations in *M. galloprovincialis,* Henriques et al. (2019) and Andrade et al. (2022a), (2023) demonstrated GSTs and CbEs enzymes were activated after 28 days of exposure. Therefore, we might hypothesise that biotransformation capacity is time-dependent, and for low exposure concentration, such as the one tested in the present study, mussels might

not be able to activate the enzymes involved in this defense capacity.

In the present study, LPO levels were higher at the lowest concentrations of Gd, which could be attributed to the limited antioxidant capacity of *D. trunculus*. However, at the highest concentration in week 1 as well as the higher ones (250 and 500 μg/L) in week 2 LPO levels were lower than the ones observed in control clams. Lower LPO levels at week 2 might be explained by a decrease in ETS activity, an attempt of clams to limit ROS production. Furthermore, when exposed to higher concentrations of Gd clams reduced their metabolic capacity, probably associated with valves' closure, to avoid Gd accumulation. This hypothesis has already been observed by Pinto et al. (2019) after exposure of *M. galloprovincialis* specimens to La (100–10000 μg/L).

5. Conclusion

This study represents the pioneering investigation utilizing the bivalve *D. trunculus* to assess the impact of Gd. This species has proven to be a good early warning sentinel species, exhibiting an increasing accumulation of administered contaminant concentrations, thereby offering a realistic depiction of environmental concentrations.

This capacity to bioaccumulate high levels of the contaminant is further supported by the minimal activity of biotransformation enzymes, which seem to play no significant role in the oxidative stress response to contaminant exposure. In this context, Gd has demonstrated a highly specific induced response, resulting in the activation of certain oxidative stress responses (ETS, GLY, SOD, LPO) while inhibiting others (GPx, TAC, GSTs, CbEs), triggering a response that also depends on the concentration of contaminant exposure. Furthermore, a time-dependent response was observed, highlighting that longer exposure periods might cause higher cellular injuries. The results obtained indicate oxidative stress effects even at lower concentrations, akin to those commonly encountered in nature. Furthermore, the increasing use of Gd in medical and technological domains may precipitate a future rise in environmental concentrations of this emerging contaminant, potentially engendering severe repercussions on the health of aquatic organisms. Human health is also imperilled, as many marine species serve as both edible commodities and subjects of commercial interest, as well as direct exposure through bodily contact with water at recreational sites.

CRediT authorship contribution statement

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

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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