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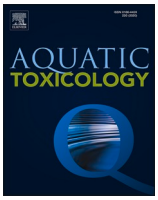

Gadolinium ecotoxicity is enhanced in a warmer and acidified changing ocean as shown by the surf clam *Spisula solida* through a multibiomarker approach

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ABSTRACT

Humans have exhaustively combusted fossil fuels, and released pollutants into the environment, at continuously faster rates resulting in global average temperature increase and seawater pH decrease. Climate change is forecasted to exacerbate the effects of pollutants such as the emergent rare earth elements. Therefore, the objective of this study was to assess the combined effects of rising temperature ($\Delta = +4$ °C) and decreasing pH ($\Delta = -0.4$ pH units) on the bioaccumulation and elimination of gadolinium (Gd) in the bioindicator bivalve species *Spisula solida* (Surf clam). We exposed surf clams to 10 μg L^{-1} of $GdCl_3$ for seven days, under warming, acidification, and their combination, followed by a depuration phase lasting for another 7 days and investigated the Gd bioaccumulation and oxidative stress-related responses after 1, 3 and 7 days of exposure and the elimination phase. Gadolinium accumulated after just one day with values reaching the highest after 7 days. Gadolinium was not eliminated after 7 days, and elimination is further hampered under climate change scenarios. Warming and acidification, and their interaction did not significantly impact Gd concentration. However, there was a significant interaction on clam's biochemical response. The augmented total antioxidant capacity and lipid peroxidation values show that the significant impacts of Gd on the oxidative stress response are enhanced under warming while the increased superoxide dismutase and catalase values demonstrate the combined impact of Gd, warming & acidification. Ultimately, lipid damage was greater in clams exposed to warming & Gd, which emphasizes the enhanced toxic effects of Gd in a changing ocean.

1. Introduction

Since the late 18th century, humans have systematically exploited natural resources, burned fossil fuels, and release pollutants into the environment, at continuously faster rates. A direct consequence of fossil fuel burn is a buildup of greenhouse gases (GHG), which in turn is responsible for the well-known Greenhouse Effect (Field and Barros, 2014). This effect is liable of absorbing and trapping heat from solar energy, increasing worldwide temperatures (Black and Weisel, 2010). In addition, the buildup of GHG increases CO_2 partial pressure ($pCO2$) oceanic uptake, which in turn leads to ocean acidification. In a worst-case scenario (i.e, SSP5-8.5), by the end of the 21st century, an

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increase of up to 3.3–3.9 °C and a decrease of 0.3–0.4 pH units is likely to take place in the Mediterranean Sea and North Atlantic Ocean (IPCC, 2021). Higher temperatures increase cell membrane fluidity, and it is well known that thermal stress imposes metabolic changes in aquatic biota (Anacleto et al., 2018) while acidification may cause malformation, alterations in buoyancy and spatial orientation of fish (Pimentel et al., 2014).

These climate abiotic changes may occur merged with other stressors, such as exacerbated levels of contaminants, which is likely to further challenge marine organisms' fitness (Maulvault et al., 2018). Furthermore, their joint effects on a wide array of emerging contaminants remains to be explored. The rare earth elements (REE) are a prime example of those emerging contaminants and are composed of the lanthanide group, plus scandium (Sc) and yttrium (Y) (Damhus et al., 2005). The escalating applications of REE and lack of regulation on usage and discharge has categorized these elements as contaminants of emergent concern (Dionisio et al., 2021). The REE are a crucial part in the functioning of the international markets and world's economy (Gonzalez et al., 2014) as several advanced and modern-day technologies are increasingly reliant on these elements for the manufacture of batteries, lasers, magnets, superconductors, and various others.

Among the elements used in medicine lies gadolinium (Gd). Gadolinium is extensively used as in magnetic resonance imaging (MRI) as an injected contract with paramagnetic features. Here, this element is required to be applied complexed to safeguard the recognized toxic effects of Gd^{3+} (Pagano et al., 2015). As a free ion, Gd^{3+} can impact Ca^{2+} channels, hindering biological processes (Caravan et al., 1999). The Gd-contrast shows a brief human body residence and is swiftly released through sewage. Gd-based contrast agents are regard as safe (Matsumura et al., 2013), however, Gd buildup in the human brain has been reported (Kanda et al., 2015), while its potential toxicological effects are yet largely unknown. Gadolinium is not efficiently eliminated in wastewater treatment plant processes (Rabiet et al., 2009), being hence discharged into the aquatic environment. Consequently, strong positive Gd anomalies in REE patterns has been found in surface water of populated and industrialized regions, with an established advanced medical system (Rogowska et al., 2018). While up to 200 ng L^{-1} of Gd are present in non-contaminated river waters (Birka et al., 2016; Hennebrüder et al., 2005; Raju et al., 2010), Gd levels can be up to 1100 mg L^{-1}, when wastewater treatment plants discharge into rivers (Bau and Dulski, 1996). Marine water shows natural Gd levels of up to 26.9 ng L^{-1}. Human caused positive Gd anomalies can disrupt the natural REE pattern in seawater bodies. In fact, up to 409 ng L^{-1} Gd has occurred in the vicinity of a submarine outfall (reviewed in Trapasso et al. (2021)).

Both the Gd-based contrast and the Gd^{3+} are prone to be accumulated by bivalves and other aquatic organisms and some toxic effects have been described (Lingott et al., 2016; Perrat et al., 2017). Seven days of Gd exposure (10 μg L^{-1}) rose the activity of Glutathione-S-Transferases (GST) in the freshwater bivalve *Dreissena rostriformis bugensis,* and increased lipid hydroperoxide and mitochondrial electron transfer in *Corbicula fluminea* (Perrat et al., 2017). Furthermore, 28-day exposure to 60 μg L^{-1} Gd decreased *Mytilus galloprovincialis* metabolism and instigated oxidative stress and neurotoxicity (Henriques et al., 2019). Hanana et al. (2017) showed that the same 28-day exposure period to 1250 μg L^{-1} $GdCl_3$ in zebra mussels triggered mitochondrial and anti-inflammatory pathways.

The limited literature on Gd induced environmental hazards shows how the Gd toxicity has been understudied by the scientific community, confirming the need for further exposure experiments, particularly on short-term exposures and on the combined effects of Gd and changing abiotic factors, such as warming, acidification, and their interaction.

The benthic filter-feeding bivalves are an essential part of coastal environments, while also being a valuable food source to numerous species, including humans. Bivalves are sessile and populate coastal regions that are particularly exposed to both acidification and thermal stress (Ferro-Azcona et a., 2019), and both are known to impose negative impacts on these commercially important species. Such impacts include slowed growth, enhanced metabolism, infectious transmissibility increase, geographical species distribution shift and ultimately mortality (Bramwell et al., 2021; Harvell et al., 2002; Poloczanska et al., 2013; Steeves et al., 2018; Stevens and Gobler, 2018).

The surf clam (*Spisula solida*) is found in the continental shelf of the Eastern North Atlantic coastal waters, from southern Iceland to Portugal, Morocco and the Mediterranean, and was chosen as the biological model due to its bioaccumulation ability of REE (Figueiredo et al., 2022a) and susceptibility to climate change (Mesquita et al., 2011; Pousse et al., 2020). *S. solida* is harvested along the entire Portuguese continental shelf (Gaspar et al., 2003; Baptista and Leitão, 2014) and this is influenced by climatic conditions of the North Atlantic and the Mediterranean Sea (Cunha, 2001) we have selected the most severe predicted climate change predictions to the end of the century (i.e., SSP5-8.5, IPCC 2021), for both water masses. Therefore, the objective of this study was to assess the combined effects of rising temperature ($\Delta = +4$ °C) and decreasing pH ($\Delta = -0.4$ pH units) on the bioaccumulation and elimination of Gd in the bioindicator bivalve species *S. solida.* Other Gd exposure trials, with marine bivalve species, used wide exposure concentration gradients (10, 50, 250 and 1250 μg L^{-1} and 0, 15, 30, 60, 120 μg L^{-1}), hence, to achieve an environmental realistic exposure, the selected concentration mimicks the first point of the gradients used in similar ecotoxicology studies (Hanana et al., 2017; Henriques et al., 2019) and was according to Gd levels present at contaminated aquatic systems (González et al., 2015; Rogowska et al., 2018). Here, we exposed surf clams to 10 μg L^{-1} of $GdCl_3$ for seven days, under warming, acidification, and their combination, followed by an elimination phase that lasted another seven days. Furthermore, we investigated the Gd bioaccumulation and oxidative stress-related responses after 1, 3 and 7 days of exposure and 7 days of elimination. Specifically, we measured lipid peroxidation (LIPO), total antioxidant capacity (TAC), oxidative stress enzymes - superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), GST, and chaperoning - heat shock proteins (HSP) and ubiquitin-proteasome system mechanisms -total ubiquitin (Ub).

2. Materials and methods

2.1. Specimen collection and culturing

A total of two hundred and eighty-eight adult clams (mean ± standard deviation of total weight: 6.3 ± 0.55 g; shell length: 28 ± 2.5 mm; shell width: 23 ± 0.63 mm) were collected in a single sampling event by dredging in Comporta (Setubal, Portugal) in April 2021 and transported to *Aquarium Vasco da Gama*, in Lisbon. Specimens were kept in aerated water and in stable pH, temperature, and salinity, mimicking their environmental conditions. Organisms were acclimated and the physicochemical parameters of their habitat, were chosen as control abiotic conditions for the upcoming experiment: 12 h light/12 h dark photoperiod, salinity = 35 ± 0.1 psu (V2 refractometer, TMC Iberia), water temperature = 15 ± 0.1 °C (TFX 430 Precision Thermometer, WTW GmbH) and pH 8.0 ± 0.050 (SG8 e SevenGo pro™ pH/Ion meter, Mettler-Toledo International Inc), for seven days. Specimens consumed green and brown marine phytoplankton (Reef Phytoplankton™, Seachem), *ad libitum,* one hour before water change. Mortality was checked daily throughout the experiment.

2.2. Warming, Gd and CO_2 exposure

Eight individuals per treatment and sampling time were allocated at random in 8 experimental 14 L glass tanks representative of 8 treatments (Fig. 1).

For the clam's acclimation to the selected climate change scenarios, the temperature was increased 1 °C, and the pH was reduced by 0.1 daily.

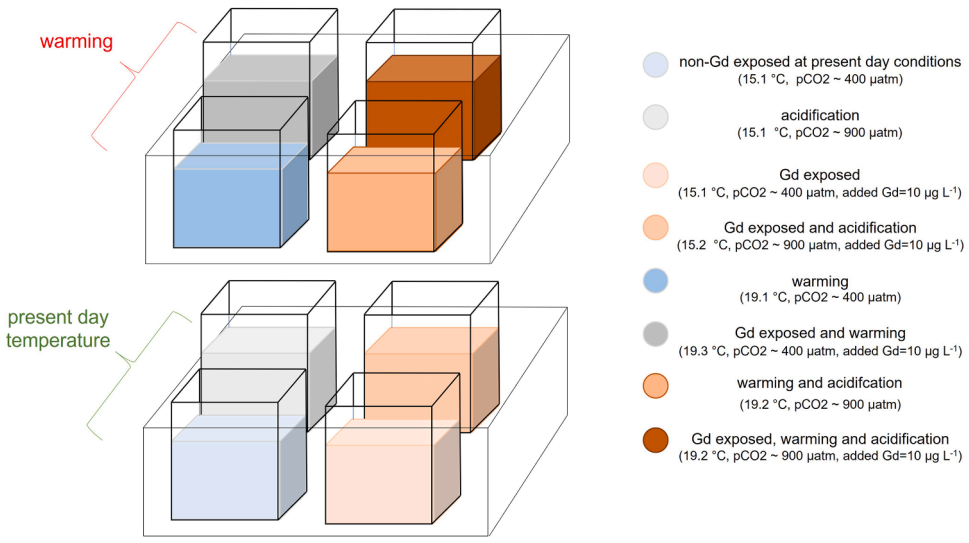

Fig. 1. Experimental design and treatments. The experimental tanks were randomly distributed to avoid bias.

As a previous work showed that Gd is stable in seawater, under present-day and climate change scenarios, for more than 24 h (Figueiredo et al., 2022b), during the experiment, the water in every experimental 14 L glass tank was changed every other day. During the 7-day Gd-exposure phase, a Gd standard solution (10 mg L^{-1}, prepared with high purity $GdCl_3$, Merck), prepared before the beginning of the trial in filtered ultra-pure water (18.2 MΩ, Sartorius) and acidified 2% (HNO_3) to assure stability, was added after water change, in the four Gd exposure treatments, to achieve 10 µg L^{-1} Gd exposure level. Water aliquots were collected after 24 h of the first spiking event to validate dissolved Gd levels. These aliquots were filtered for particle removal (0.45 µm Millipore) and acidified (20% ultrapure HNO_3).

2.3. Water chemistry

The temperature was maintained with access to a bath monitored by heaters (V_2Therm 200 W aquarium heater, TMC) and chillers (Hailea, HC-250 A). The $pH_{total\ scale}$ was routinely monitored by a Profilux 3.1T (GHL) system and pH probes (GHL, precision ± 0.01 units). The probes were calibrated with TRIS-HCl (TRIS) and 2-aminopyridineHCl (AMP) (Mare, Belgium) seawater buffers. The pH was upregulated with filtered air, via an air compressor (Hailea, ACO 328) and downregulated with CO_2, via electric solenoid valves (Etopi). To obtain the most stable pH values, hysteresis was set at the lowest possible (0.05 units of pH).

Water alkalinity was determined every sampling time (Alkalinity checkers, Hanna® Instruments) and the carbonate system speciation was calculated (CO2SYS software, Pierrot et al. (2006)), with dissociation constants from Mehrbach et al. (1973) as refitted by Dickson and Millero (1987). For the alkalinity determination water samples were collected directly to the provided 10 ml glass cuvettes (Hanna® Instruments).

2.4. Sampling

Clams were sampled immediately at the start of the experiment, before the Gd spike, (T0) and after 1 (T1), 3 (T3), and 7 (T7) days. Afterwards, clams were kept in renewed water and the elimination phase began, for 7 days (T14).

2.5. Gadolinium levels assessment

Samples ($n = 5$ individuals per treatment and sampling times, run in technical triplicates) were freeze-dried, and homogenized before being digested with HNO_3 (distilled, 65% v/v) and H_2O_2 (suprapur, 30% v/v) in labware that had been formerly decontaminated with HNO_3 (20%) and rinsed with ultra-pure water (Milli-Q water – 18.2 MΩ).

Gadolinium concentrations in clams were determined in a quadrupole ICP-MS (NexION 2000) equipped with a concentric Meinhard nebulizer, a cyclonic spray chamber and a dual detector (Figueiredo et al., 2020). A 6-point calibration curve and an internal standard (Indium, Alfa Aesar, Plasma Standard Solution, Specpure®, In 1000 µg·mL^{-1}) was used. Water samples were diluted to salinity ~3 with Mili-Q water to reduce matrix interference and allow ICP-MS quantification. Three procedural blanks, random sample duplicates, a multi-element Quality Control (QC) solution, and the certified reference material BCR 668 (muscle of *Mytilus edulis*) were always run after 20 samples to monitor the results accuracy and precision. Reagent blanks accounted <1% of samples concentrations. Gadolinium concentrations are shown in microgram per gram of tissue dry weight (µg g^{-1}, dw).

Water samples were preconcentrated with a known isotopic ratios standard, using an automated Elemental Scientific Inc. SeaFAST system (SeaFASTpico™) before being analyzed by ICP-MS (NexION 2000). The methods described by Hatje et al. (2014) were followed. Water samples were run with blanks, seawater quality controls and certified reference materials (CASS-6 and NASS-7). A six-point calibration curve was used, and the detection limit was determined through the method blanks. The Gd detection limit for the water samples was 0.040 µg L^{-1}.

2.6. Bioaccumulation factor and elimination coefficient

The Gd bioaccumulation factor and elimination coefficient were assessed to evaluate the effectiveness in Gd accumulation and clam's Gd recover ability, respectively, as in Figueiredo et al. (2018). Briefly, the bioaccumulation factor corresponds to the quotient of the mean values of Gd levels in exposed clams and the Gd exposure concentration, for each sampling time and treatment. The elimination coefficient corresponds to the quotient of Gd levels after the elimination period (T14) and the Gd levels at the end of the exposure period (T7), for each of the Gd exposure treatments.

2.7. Biomarkers

Three clams per treatment and sampling time (run in technical duplicates) were homogenized (Ultra-Turrax, Staufen), in 3 mL of PBS (phosphate-buffered saline solution: 0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.47 mM KH_2PO_4, pH 7.4), centrifuged (10.000 x g for 15

min at 4 °C) and frozen (-80 °C). Results were normalized to total protein concentration (Bradford, 1976). All biomarkers were quantified as detailed in Figueiredo et al. (2022a).

As an indicator of cellular damage, LIPO was ascertained by the quantification of malondialdehyde (MDA) according to Uchiyama and Mihara (1978). For the assessment of LIPO, a calibration curve of 0 – 0.3 μM Malondialdehyde (MDA) was used.

Total antioxidant capacity was ascertained agreeing to Kambayashi et al. (2009). The absorbance was read at 410 nm (Biotek Synergy HTX multi-mode reader, USA) and the TAC was computed from a calibration curve, based on a series of Trolox (0–0.3 mM).

Superoxide dismutase inhibition was established through an adjustment of the method defined by McCord and Fridovich (1969). The absorbance was read at 550 nm (Biotek Synergy HTX multi-mode reader, USA).

Catalase activity was established based on Johansson and Borg (1988). The absorbance was read at 540 nm (Biotek Synergy HTX multi-mode reader, USA). As one unit of catalase is the sum that will trigger the creation of 1.0 nmol of formaldehyde per minute at 25 °C, formaldehyde concentration was assessed through a calibration curve (from 0 to 75 μM formaldehyde).

Glutathione peroxidase was measured corresponding to Lawrence and Burk (1976). The GPx activity was determined using a β-NADPH coefficient extinction.

The GST activity was read spectrophotometrically at 340 nm, per minute, 6 times (Biotek Synergy HTX multi-mode reader, USA). A CDNB extinction coefficient (εμM) of 0.0053 (μM^{-1} cm^{-1}) was used to assess the reaction rate.

Heat Shock Protein 70 was measured by ELISA. The selected antibodies were anti-HSP70/HSC70 in 1% BSA (OriGene, USA) and alkaline phosphatase-conjugated anti-mouse IgG (Fc specific, Sigma-Aldrich, USA). The HSP levels were assessed through purified HSP70 active protein dilutions (0–2.000 μg mL^{-1}, OriGene Technology, USA).

Ubiquitin levels were assessed by ELISA, agreeing to Lopes et al. (2019). The selected antibody was P4D1, sc-8017, HRP conjugate (Santa Cruz, USA). The Ub levels were assessed by means of dilutions of purified ubiquitin (0–1 μg $mL^{-1,}$ UbpBio, E-1100, USA).

2.8. Statistical analyses

Normality and homogeneity of variance were checked using Kolmogorov-Smirnov and Levene's tests, respectively. Data was tested Two- (for T0) and Three-way (for T1, T3, T7 and T14) ANOVAs with Temperature, pCO_2, and Contamination as factors were utilized to explore significant variations in Gd concentration and biomarkers activity. To investigate differences of Gd accumulation and biomarkers values between sampling times, for each Gd exposure treatment, a one-way ANOVA was applied. Whenever an ANOVA table proved to be significant, all pairwise comparisons using Tukey's procedure (Braun, 1995) were carried out. The biomarker values were Log_{10} transformed to conform with the normality assumptions. Statistical evaluations were achieved in *In Vivo* Stat, 4.3 at a significance level of 0.05.

3. Results

3.1. Water chemistry

The water chemistry was stable (Table 1). The Gd levels in spiked seawater treatments showed maximum variation between 10 and 12% from the desired nominal exposure concentration (10 μg L^{-1} Gd).

3.2. Gadolinium accumulation

Concentrations of Gd (μg g^{-1}, dry weight) in spiked treatments are represented in Fig. 2. The highest bioaccumulation values occurred after 7 days of exposure, being highest in the Acidification & Gd treatment, followed by the Warming & Gd, Warming, Acidification & Gd and finally by the Gd exposure treatment, although we did not observe significant differences between the 4 Gd-exposed treatments (Supplemental Table 1) (Fig. 3).

Just before the beginning of the trial, Gd concentrations varied between 0.070 μg g^{-1} dw (dry weight) in the control temperature and pCO_2 treatment and 0.080 μg g^{-1} dw in the warming and acidification treatment. These represent baseline concentrations of Gd in non-exposed clams and, in fact, throughout the experiment, the Gd concentrations in clams from non-exposed treatments remained stable and did not show significant differences among them (Tukey's pairwise comparisons, $p > 0.05$, Supplemental Table 1, Supplemental Table 3a). At one day of exposure (T1), Gd accumulation occurred in all four Gd exposure treatments, as these showed significantly higher values than the non-Gd exposed treatments (Tukey's pairwise comparisons, Supplemental Table 3a).

Overall, we did not observe significant differences between the Gd exposed treatments, at each sampling time (Tukey's pairwise comparisons, $p > 0.05$, Supplemental Table 3a). For the Gd exposed clams, at control temperature and pCO_2, the Gd concentrations were significantly different between T1 and T7 (Tukey's pairwise comparison, $p = 0.047$, 95% C.I.= [-0.72, -0.12], Supplemental Table 3b). In the warming & Gd exposed treatment, the Gd concentrations were significantly different

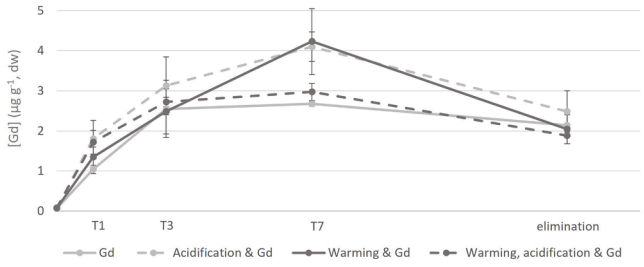

Fig. 2. Concentrations of Gadolinium (μg g^{-1}, dry weight) in the clams' whole soft body exposed to Gd; acidification & Gd; warming & Gd and warming, acidification & Gd in the different sampling times (T1, T3, T7 and T14). Values correspond to medians ± SE.

Table 1
Summary of physicochemical water parameters and carbonate system specifics. Temperature, pH and total alkalinity measured values were used to calculate pCO_2 (carbon dioxide partial pressure). N correspond to the total number of individuals per tank. Values represent mean ± standard deviation.

Treatment	N	Temperature (°C)	Salinity	$pH_{Total\ scale}$	Total Alkalinity (μmol/kg SW)	*p*CO2 (μatm)
Control	40	15 ± 0.20	35 ± 0.10	8.0 ± 0.10	1928 ± 109	367 ± 67
Acidification	40	15 ± 0.30	35 ± 0.10	7.6 ± 0.10	1643 ± 64	893 ± 73
Gd exposed	32	15 ± 0.20	35 ± 0.10	8.1 ± 0.04	2536 ± 43	372 ± 28
Acidification & Gd	32	15 ± 0.10	35 ± 0.10	7.6 ± 0.09	1769 ± 108	894 ± 35
Warming	40	19 ± 0.20	35 ± 0.10	8.0 ± 0.06	2307 ± 102	442 ± 65
Warming & acidification	40	19 ± 0.30	35 ± 0.10	7.6 ± 0.14	1550 ± 83	812 ± 22
Warming & Gd	32	19 ± 0.10	35 ± 0.10	8.1 ± 0.04	2206 ± 69	338 ± 38
Warming, acidification & Gd	32	19 ± 0.20	35 ± 0.10	7.6 ± 0.10	1671 ± 95	834 ± 28

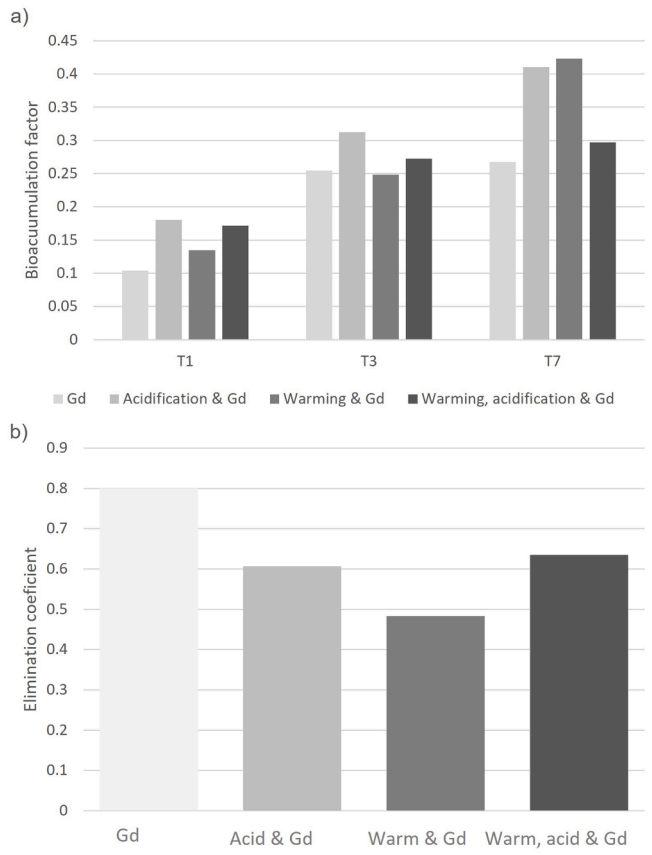

Fig. 3. (a) Bioaccumulation factor for the four Gd exposure treatments in the three exposed sampling times (T1, T3 and T7); (b) Elimination coefficient for the four Gd exposure treatments.

between T1 and T7 (Tukey's pairwise comparisons, $p = 0.041$, 95% C.I.= [-0.64, -0.11]), but also T7 and T14 (Tukey's pairwise comparison, $p = 0.042$, 95% C.I.= [-0.61, -0.10]). The Gd concentrations in the clams exposed to warming, acidification & Gd were only different between T7 and T14 (Tukey's pairwise comparison, $p = 0.041$, 95% C.I.= [-0.35, -0.06]). In the acidification & Gd treatment the Gd concentration did not vary significantly through time (Tukey's pairwise comparisons, $p > 0.05$, Supplemental Table 3b).

Gadolinium accumulation did not prompt death as no mortality was observed throughout the experiment.

3.3. Gadolinium clearance

The highest elimination factor occurred in the Gd exposure treatment at present-day temperature and pH, followed by the acidification & Gd. The lowest elimination occurred in the warming & Gd treatment, followed by the warming, acidification & Gd. After a 7-day elimination period (T14), the previously accumulated Gd values did not diminish to control-like values. All the Gd exposure treatments remained statistically different from their control counterparts (Tukey's pairwise comparisons, $p < 0.0001$) and presented values similar to the ones sampled at T3. At T14, the highest Gd concentration was exhibited by clams previously exposed to acidification & Gd (2.1 ± 0.30 μg g^{-1} dw).

3.4. Biochemical responses

Biochemical outputs in *Spisula solida'* soft body are reported as mean ± standard deviation (Table 2).

3.4.1. Lipid peroxidation

Before the 7th day of exposure, no significant differences between the LIPO levels of clams exposed to the different experimental treatments were found (Fig. 4a). At T7 the effects of temperature on lipid peroxidation levels were highlighted as the control and the acidification treatments proved to be significantly different than the warming and warming & Gd and warming & acidification (Supplemental Table 4b). However, there were no significant differences between treatments at T14.

Lipid peroxidation values on the Gd exposed treatment decreased from T1 to T3 (Tukey's pairwise comparison, $p = 0.0401$, 95% C.I.= [0.10, 0.53]), increased from T3 to T7 (Tukey's pairwise comparison, $p = 0.0041$, 95% C.I.= [-0.79, -0.31]), and decreased again from T7 to T14 (Tukey's pairwise comparison, $p = 0.0284$, 95% C.I.= [-0.62, -0.14], Supplemental Table 4b). From T3 to T7, LIPO values were raised in all four Gd exposure treatments. From T1 to T7 the LIPO values varied significantly in the warming & Gd and warming, acidification & Gd treatment (Tukey's pairwise comparisons, $p = 0.0452$ and $p = <0.0001$, 95% C.I.= [-0.99, -0.18] and 95% C.I.= [-0.59, -0.43], respectively). Finally, from the end of the exposure phase (T7) until the end of the elimination phase (T14), LIPO varied in all Gd exposure treatments, except for the warming & Gd (Tukey's pairwise comparison, p=0.113, 95% C.I.= [-0.87, -0.05]).

3.4.2. Total antioxidant capacity

One day of exposure (T1) was enough to trigger significant differences between TAC levels of clams from the warming treatment and the warming & Gd (Tukey's pairwise comparison, $p = 0.0455$, 95% C.I.= [-0.67, -0.18], Supplemental Table 4a, Fig. 4b). Total antioxidant capacity levels were also higher in the warming & Gd treatment in comparison to the warming & acidification (Tukey's pairwise comparison, $p = 0.0302$, 95% C.I.= [0.19, 0.65]). Overall, the treatments exposed to Gd exhibited higher TAC levels. On T3, although a global effect was shown (ANOVA, $F(7,9) = 3.71$, $p = 0.358$, Supplemental Table 4a), non-significant post-hoc effects occurred. At T7, only clams exposed to the warming treatment showed significantly higher TAC levels than the control (Tukey's pairwise comparison, $p = 0.0375$, 95% C.I.= [-0.57, -0.17]). After the elimination phase (T14), no significant differences were found between treatments.

Total antioxidant capacity changed through time in the acidification & Gd and warming, acidification & Gd treatments (Supplemental Table 4b). The first varied from T1 to T3 (Tukey's pairwise comparison, p=0.0077, 95% C.I.= [-0.57, -0.25]), T1 to T7 (Tukey's pairwise comparison, $p = 0.0054$, 95% C.I.= [-0.61, -0.29]) and from T1 to T14 (Tukey's pairwise comparison, $p = 0.0052$, 95% C.I.= [-0.61, -0.29]). The second treatment varied from T1 to T3 ($p = 0.0082$) and from T1 to T7 (Tukey's pairwise comparison, $p = 0.0099$, 95% C.I.= [-0.26, -0.09]).

3.4.3. Oxidative stress enzymes

Superoxide dismutase (% inhibition). It took 7 days of exposure to induce significant differences in SOD levels between treatments (Fig. 5a, Supplemental Table 4a).

Superoxide dismutase levels varied in clams exposed to Gd and climate change (i.e., ocean warming, acidification, and their combination) from T1 to T14 (Supplemental Table 4b). Additionally, the SOD values in T1 were significantly lower than the ones in T7, for the warming & Gd (Tukey's pairwise comparison, $p = 0.0389$, 95% C.I.= [-0.32, -0.05]) and warming, acidification & Gd treatments (Tukey's pairwise comparison, $p = 0.0121$, 95% C.I.= [-0.19, -0.05]). In the warming, acidification & Gd the SOD levels were also different between T3 and T7 (Tukey's pairwise comparison, $p = 0.0109$, 95% C.I.= [-0.22, -0.06]) and between T3 and T14 (Tukey's pairwise comparison, $p = 0.0295$, 95% C.I.= [0.04, 0.20]). Superoxide dismutase values also varied between T3 and T14, and T7 and T14 in clams exposed to Gd and

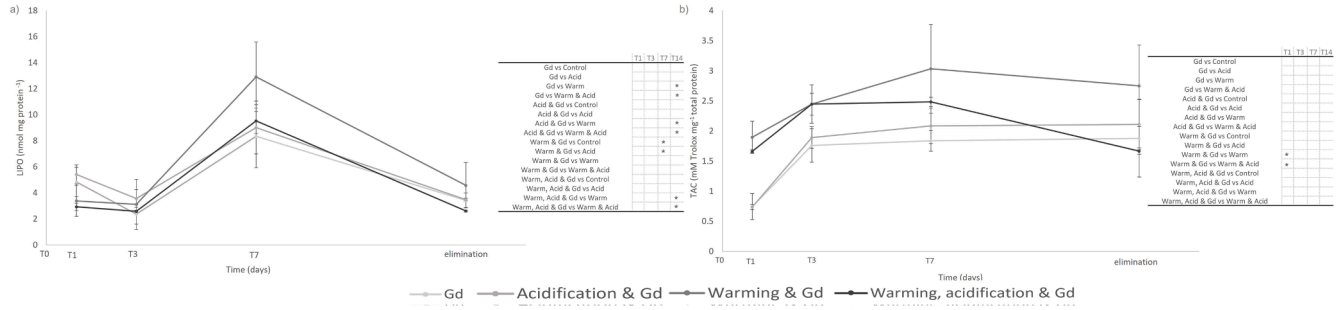

Fig. 4. Mean ± SD values of (a) Lipid peroxidation (LIPO, nmol mg protein $^{-1}$); (b) total antioxidant capacity (TAC, mM Trolox mg^{-1} total protein) in *Spisula solida*' soft body exposed to Gd; Acidification & Gd; Warming & Gd; Warming, acidification & Gd. Letters represent significant differences between exposed treatments, for each sampling time. Asterisks represent significant differences between the Gd-exposed and non-Gd-exposed treatments (See Supplemental Table 3 a and b).

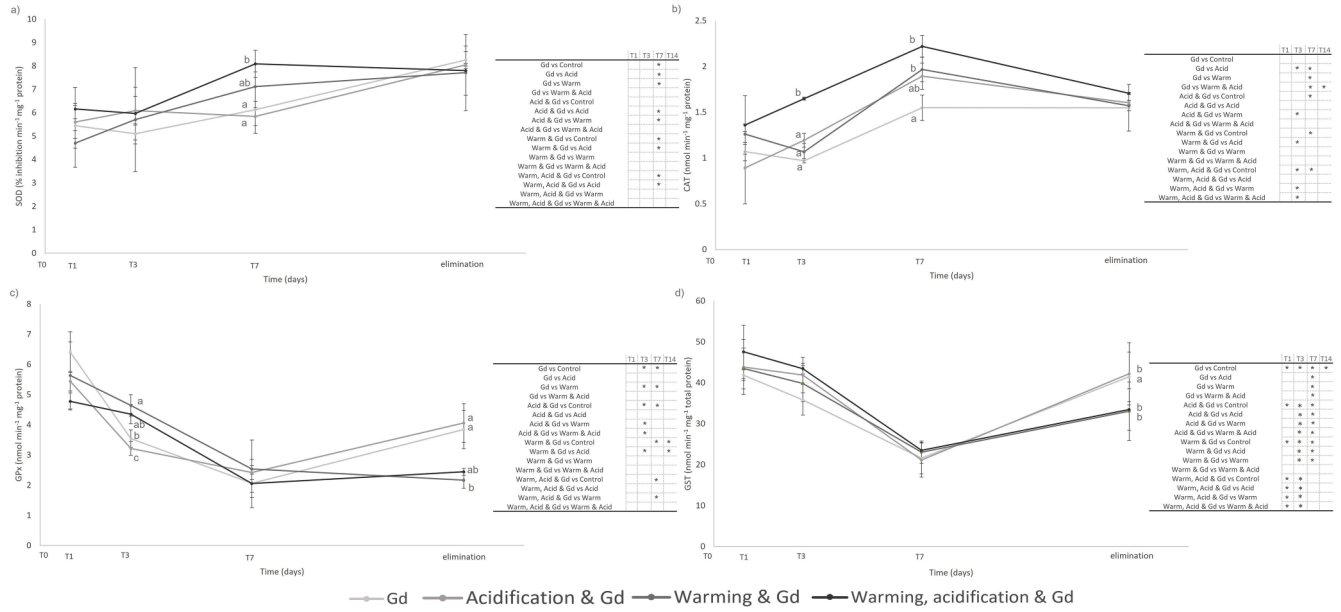

Fig. 5. Mean ± SD values of (a) Superoxide dismutase (SOD, % inhibition min^{-1} mg^{-1} protein); (b) Catalase (CAT, nmol min^{-1} mg^{-1} protein); (c) Glutathione peroxidase (GPx, nmol min^{-1} mg^{-1} protein); (d) Glutathione S-transferase (GST, nmol min^{-1} mg^{-1} total protein) in *Spisula solida*' soft body exposed to Gd; Acidification & Gd; Warming & Gd; Warming, acidification & Gd. Letters represent significant differences between exposed treatments, for each sampling time. Asterisks represent significant differences between the Gd-exposed and non-Gd-exposed treatments (See Supplemental Table 3 a and b).

acidification & Gd.

Catalase. At T3, several post-hoc differences of CAT activity between treatments were shown (Fig. 5b, Supplemental Table 4a). Noteworthily, the CAT activity was highest in the warming, acidification & Gd treatment, being significantly different than the activity shown in clams of the warming & Gd treatment (Tukey's pairwise comparison, $p = 0.0007$, 95% C.I.= [-0.26, -0.12], Supplemental Table 4a) and the acidification & Gd (Tukey's pairwise comparison, $p = 0.0089$, 95% C.I.= [-0.21, -0.08]).

From T1 to T7 and from T3 to T7 CAT activity was significantly enhanced in clams exposed to Gd, acidification & Gd and warming & Gd. From T1 to T14, this activity was significantly different in the clams exposed to Gd and acidification & Gd. Finally, CAT concentrations exhibited at T3 were different than that of T14 in clams from the Gd and warming & Gd exposure treatments (Supplemental Table 4b).

Glutathione peroxidase. Overall, glutathione peroxidase decreased with time (Fig. 5c). At T3 clams exposed to Gd presented significantly lower GPx levels than the ones kept in control conditions (Tukey's pairwise comparison, $p = 0.0038$, 95% C.I.= [0.06, 0.17], Supplemental Table 4a). At this time, clams exposed to acidification & Gd exhibited the overall lowest GPx values and these were significantly lower than the levels of clams exposed to warming, acidification & Gd (Tukey's pairwise comparison, $p = 0.0024$, 95% C.I.= [-0.19, -0.07]). At T7, the control clams showed significantly higher GPx levels than the clams from the four treatments exposed to Gd (i.e. Gd; Acidification & Gd; Warming & Gd; Warming, acidification & Gd).

Glutathione peroxidase levels of T1 and T3 were significantly higher than the ones of T7 and T14 for all four Gd exposure treatments, apart from T3 vs T14 in the Gd exposed one (Supplemental Table 4b). Furthermore, GPx levels of the Gd treatment and the acidification & Gd displayed significant differences between T1 and T3, and T7 and T14.

Glutathione S- transferase. After just one day of exposure (T1), the four treatments exposed to Gd showed higher GST levels than the control (Fig. 5d, Supplemental Table 4a). Additionally, clams exposed to warming & acidification presented significantly lower GST levels than those exposed to warming, acidification & Gd (Tukey's pairwise comparison, $p = 0.0491$, 95% C.I.= [0.05, 0.23]).

An overall decrease of GST levels in the Gd exposed treatments occurred from T3 to T7. In fact, unlike T1 and T3, at T7 the Gd exposed, acidification & Gd, and warming & Gd treatments were significantly

lower than the control (Tukey's pairwise comparisons, $p = 0.0028$, $p = 0.0012$, and $p = 0.0173$, 95% C.I.= [0.08, 0.23], [0.10, 0.24] and [0.06, 0.20], respectively).

Glutathione S-transferase levels were significantly lower from T1 to T7, from T3 to T7 and increased from T7 to T14 in all Gd exposure treatments (Supplemental Table 4b). Furthermore, GST levels registered at T3 were significantly higher than the ones at T7, in the warming & Gd and warming, acidification & Gd treatments. Finally, exposure to warming, acidification & Gd triggered significantly lower GST levels from T3 to T14.

3.4.4. Chaperoning and ubiquitin-proteasome system mechanism

Heat shock protein (HSP70). Heat shock proteins expression during the experiment was not linear (Fig. 6a). A significant lower HSP concentration in clams exposed to Gd than clams exposed to warming was observed at T7 (Tukey's pairwise comparison, $p = 0.0117$, 95% C.I.= [-0.73, -0.25], Supplemental Table 4a). At T14 the effects of climate change on HSP expression were evident.

Regarding the acidification & Gd treatment, it was only between T1 and T7 that different HSP expression occurred (Tukey's pairwise comparison, $p = 0.0155$, 95% C.I.= [0.18, 0.61], Supplemental Table 4b).

Total ubiquitin. At T7, the Gd exposed clams showed lower Ub levels than the four non-exposed treatments (i.e., Control; acidification; warming; warming & acidification; Fig. 6b). Interestingly, the Ub levels of the previously exposed clams to Gd at present-day conditions did not recover during the clearance phase and the Ub levels were kept as the lowest in T14, and as significantly lower from all the other experimental treatments (Supplemental Table 4a).

Clams exposed to Gd showed Ub levels significantly greater from T1 to T7 (Tukey's pairwise comparison, $p = 0.0075$, 95% C.I.= [0.34, 0.94]), from T1 to T14 (Tukey's pairwise comparison, $p = 0.0031$, 95% C.I.= [0.46, 1.1]), from T3 to T7 (Tukey's pairwise comparison, $p = 0.0007$, 95% C.I.= [0.60, 1.2]) and from T7 to T14 (Tukey's pairwise comparison, $p = 0.0013$, 95% C.I.= [-0.45, 0.21]), with the lowest levels being registered at T14. The values of Ub were also significantly greater between T3 and T7 in the acidification & Gd and warming, acidification & Gd treatments (Tukey's pairwise comparisons, $p = 0.0234$ and $p = 0.0219$, 95% C.I.= [0.27, 1.1] and [0.23, 0.77] Supplemental Table 4b).

4. Discussion

The present work integrated the possible effects of multiple abiotic stressors and a rare earth element of interest on an economically pertinent bivalve species. Furthermore, new insights into the elimination of Gd in bivalves were delivered. Hence, this work help bridge the knowledge gap around the potential impacts of climate change and emergent rare earth elements on bivalve species.

The effects of ocean warming and acidification on clams have previously been studied (Hornstein et al., 2018; Stevens and Gobler, 2018). Warmer temperatures are known to enhance energy metabolism and reduce filtration rates in clams, while the combination of ocean warming, and acidification is known to trigger unforeseen effects with species-specific responses. Above this puzzling information, the assessment of warming and acidification impacts on Gd bioaccumulation, elimination, and ecotoxicity was, before this study, never achieved, which obstructs comparison with the literature.

Bivalves are known to accumulate a diverse array of contaminants, including Gd, as their food sources are suctioned across their siphons, in a non-selective filter-feeding manner, in both their soft tissues and shells (Akagi and Edanami, 2017; Figueiredo et al., 2022c). Furthermore, marine and freshwater mussels are known to accumulate Gd under laboratory exposure experiments (Hanana et al., 2017; Henriques et al., 2019; Perrat et al., 2017). Hanana et al. (2017) exposed the freshwater mussel (*Dreissena polymorpha*), for 28 days, to 10 μg L^{-1} Gd, while Henriques et al. (2019) exposed the mussel *M. galloprovincialis* for the same period to increasing concentrations of Gd (0, 15, 30, 60, 120 μg L^{-1}), nonetheless, these studies only evaluated the accumulation at the end of the exposure period. Perrat et al. (2017) exposed the freshwater bivalves *D. rostriformis bugensis* and *C.fluminea* for 7 and 21 days to 1 and 10 μg L^{-1} of Gd, respectively. However, again, information on the first day of exposure to Gd in bivalve species was not available. Our data showed that Gd accumulation occurred just after one day of exposure (T1), in all Gd exposed treatments. Overall, the accumulation increased with exposure time and was highest in T7, as illustrated by the bioaccumulation factor. Our accumulation results are not in agreement with the ones described by Henriques et al. (2019), as the mussels *M. galloprovincialis* exposed to 15 and 30 μg L^{-1} of Gd revealed levels below the ICP-MS detection limit (0.38 μg g^{-1} Gd), on the 28th day of exposure. This could be related to an unidentified Gd detoxifying method. Accordingly, Figueiredo et al. (2018) described that *Anguilla anguilla* exposed to 120 ng L^{-1} Lanthanum (La) for 7 days, peaked accumulation of this REE on the third day of exposure, and then concentrations diminished, from the 3rd to 7th exposure day. This highlights the importance of understanding the REE accumulation pattern by employing more frequent sampling events within the exposure period. On another hand, the discrepancy in these results could be related to intrinsic species-specific responses to REE. Despite the absence of effects of warming and acidification on Gd accumulation and elimination in this study, increased temperature improved bioaccumulation of another rare earth element (Lanthanum) in *Anguilla anguilla* glass eels (Figueiredo et al., 2020).

In our study, the elimination coefficient was lower under the three climate change scenarios. Data also showed that during the 7-day elimination period the Gd concentrations did not diminish to control like values. This may be related to the fact that Gd^{3+} is insoluble at physiologic pH, which prompts delayed systemic excretion (reviewed in

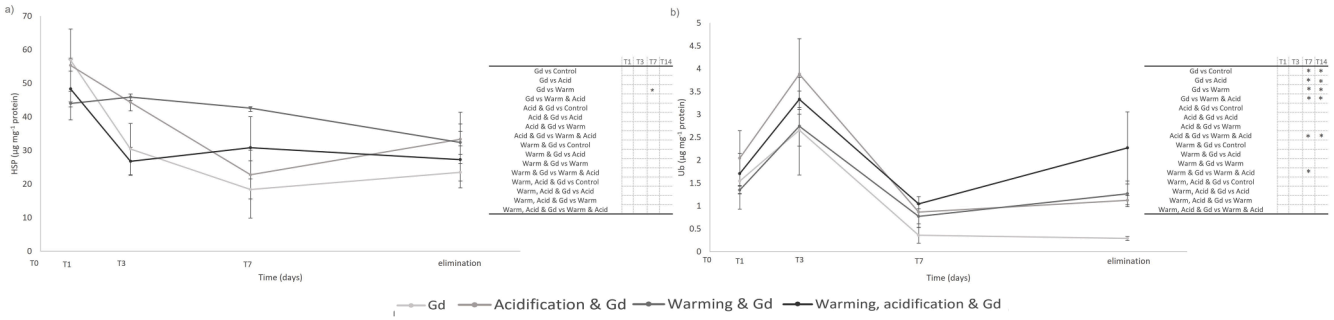

Fig. 6. (a) Heat Shock Protein 70 (HSP, μg mg^{-1} protein) and (b) Ubiquitin (Ub, μg mg^{-1} protein) in *Spisula solida*' soft body exposed to Gd; Acidification & Gd; Warming & Gd; Warming, acidification & Gd. Letters represent significant differences between exposed treatments, for each sampling time. Asterisks represent significant differences between the Gd-exposed and non-Gd-exposed treatments (See Supplemental Table 3 a and b).

Ramalho et al. (2016)). Figueiredo et al. (2022a) exposed surf clams to 15 μg L^{-1} to another rare earth element, Lanthanum, for a period of 7 days, following an elimination phase with the same duration, under present-day conditions and ocean warming and acidification, and showed that La was not eliminated, however, elimination was more efficient under increased temperatures. This result was not observed in this study which highpoints to a rare earth element specific response. In the previously discussed articles that studied Gd bioaccumulation on bivalve species, the Gd elimination was not assessed which highpoints the knowledge gap on organisms' capacity to withhold pollution events and thus we suggest that the elimination phase should be further investigated.

Even though no significant differences in Gd concentration were observed between Gd exposed in present-day, warming, acidification, and warming & acidification conditions, their interaction could nonetheless impact the organism response to the observed bioaccumulation. The biomarkers have long been accepted as early-warning signals of a host response to external levels of exposure and/or internal levels of tissue contamination (Van der Oost et al., 2003). Hence, the observed biochemical response could correspond to a sensitive index of environmental hazards such as pollutant bioavailability and climate change related abiotic alterations.

The current knowledge on the ecotoxicological outcomes of REE in aquatic biota is very limited and far from being considered conclusive. The variability in bioindicators of oxidative stress in bivalve and gastropod mollusks depends on several factors, such as feeding, size-age, and temperature (Grilo et al., 2018; Juhel et al., 2017). In order to diminish the confounding factors, we used roughly same size clams to guaranty ontogeny similarity, and from the same location and sampling event. Furthermore, the clams were fed *ad libidum* the same mixture of algae, at the same time, for the same period.

Our results showed that Gd is the main driver of this oxidative stress response, however, their impacts may be exacerbated by warming and/or acidification. Bivalves are known to overproduce reactive oxygen species (ROS) in the presence of contaminants (Renault, 2015). Under control circumstances, the ROS buildup consequences are prevented through a collection of antioxidant defense mechanisms, that include SOD, CAT, and GPx. Superoxide dismutase eliminates O_2 whilst producing H_2O_2 that is then reduced to H_20 by CAT and GPx (Regoli and Giuliani, 2014). Exposure to the three stressors, Warming, acidification & Gd caused the greatest damage, as highlighted by the significantly enhanced SOD and CAT levels, in comparison to Gd exposure alone. A significant decrease in GPx values in mussels *M. galloprovincialis* in the presence of Neodynium has been previously reported (Freitas et al., 2020b). However, this was not observed when the same species was exposed for the same period (28 days) to similar concentrations of Dysprosium (Freitas et al., 2020a), which suggests that bivalve species may display an REE specific oxidative stress response. Accordingly, Andrade et al. (2022) described that mussels, *M. galloprovincialis*, show distinct tactics to regulate oxidative stress, when exposed to 10 μg L^{-1} Gd at salinity 20, 30 and 40 for 28 days.

Glutathione S-transferases (GST) are essential in a phase II response by clearing ROS and toxic xenobiotics. This enzyme is naturally activated under stress conditions to avert damaging effects caused by the toxic substances' buildup. Our results are not in agreement with Henriques et al. (2019) that described enhanced SOD, CAT, and GST values in mussels *M. galloprovinciallis* exposed to Gd for 28 days. On one hand, this may be due to the previously discussed lack of sampling points during the exposure phase, to understand the enzymatic behavior, on another hand may be related to a species-specific strategy to cope with Gd accumulation or to the different exposure durations as our study focused on a short-term response. Martino et al. (2017) studied Gd effects on the embryonic development of European sea urchin species *Paracentrotus lividus* and *Arbacia lixula* and Australian ones, *Heliocidaris uberculate* and *Centrostephanus rodgersii* and described different sensitivities to Gd, while arguing that Gd may display distinct toxicity levels on marine organisms, even within the same taxonomic group. The previously discussed studies on Gd toxicology towards bivalves opted for a longer exposure time, while the present study delivered multiple sampling times within a short 7-day exposure timeframe.

The ROS buildup that occurs when the antioxidant defense system fails, leads to the reaction of free radicals with membrane lipids, altering membrane permeability with detrimental consequences on a cellular level. Lipid peroxidation (oxidation of polyunsaturated fatty acids, LIPO) indicates oxidative degradation of cell membrane lipids. At T7 the climate change impacts on LIPO were evident through the visible synergistic effects of warming & Gd, as clams exposed to this scenario showed higher LIPO values than the controls. Similarly, Hanana et al. (2017) found increased SOD levels upon exposing freshwater zebra mussels to $GdCl_3$ for 28 days, while CAT and GST were downregulated and no effects on LIPO were observed, at a control temperature.

A heat shock reaction mediated by HSPs' is the first cell response to thermal stress. The damaged structure of thermally unfolded proteins is reestablished through this response (Somero, 2020). In our study, we observed reduced HSP levels in Gd exposed clams, in comparison to the control, at T7, and the effects of increasing temperature were only observed on T14. Martino et al. (2021) investigated the impacts of both thermal stress and Gd in the embryos and larvae of the sea urchin *Paracentrotus lividus* and described that elevated temperatures reduced the Gd effects as the abnormality percentage was reduced, the skeleton growth increased, and the HSP expression was regulated. This is not underlined by data derived in the present study. If HSP fails to reestablish the unfolded proteins, Ub target these proteins for degradation. In the present study, at T7, the level of this biomarker was lower for all the four Gd-exposed treatments, in comparison to their control counterparts. Furthermore, at T14, previously Gd-exposed clams exhibited lower Ub levels, in comparison to the remaining treatments. Hence, we propose that the heat shock response was incapable of guaranteeing ideal protein function and ubiquitin, in the presence of Gd, could not target those proteins to be eliminated.

Although we did not observe mortality during the experimental period, exposure to 10 μg L^{-1} Gd, which may already match realistic concentrations reported in impacted environments (reviewed in Rogowska et al. (2018)) may cause serious deleterious outcomes on organisms' physiological function with consequences on feeding, growth, reproduction, and ultimately resilience and survival. About the non-enzymatic antioxidant reaction, we observed increased TAC values on clams exposed to warming, and warming & Gd, in comparison to the control. The observed resilience of the surf clam to climate change variables was expected as this species inhabits shallow coastal habitats that are subject to great everyday abiotic variations. However, clams were not able to proficiently regulate the oxidative stress response in the presence of multiple stressors and the combination of warming & Gd triggered lipid damage, which emphasizes the enhanced toxic effects of Gd in a changing ocean, particularly in a warmer, and warmer & acidified scenario.

5. Conclusion

The results derived from this study showcased the rapid ability of the surf clam *S. solida* to accumulate Gd. This accumulation increased steadily in the first 7 days of exposure and was independent of temperature and pCO_2. Furthermore, we showed that Gd was not eliminated to reach non-contaminated like values, derived from a 7-day elimination period, and the elimination coefficient was lower under climate change. Nevertheless, Gd accumulation in a warming, acidification, and warming & acidification scenario further impacted the clams' biochemical response. The results herein illustrate the enhanced toxic effects of Gd in a changing ocean which suggest that deleterious impacts to the population of surf clams are likely to be exacerbated in the near future.

CRediT authorship contribution statement

Cátia Figueiredo: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Funding acquisition. **Tiago F. Grilo:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Funding acquisition. **Rui Oliveira:** Conceptualization, Validation, Investigation, Writing – review & editing, Funding acquisition. **Inês João Ferreira:** Methodology, Validation, Writing – review & editing. **Fátima Gil:** Validation, Writing – review & editing. **Clara Lopes:** Validation, Writing – review & editing. **Pedro Brito:** Conceptualization, Validation, Resources, Writing – review & editing. **Pedro Ré:** Validation, Writing – review & editing. **Miguel Caetano:** Conceptualization, Validation, Investigation, Resources, Writing – review & editing, Funding acquisition. **Mário Diniz:** Conceptualization, Validation, Resources, Writing – review & editing, Funding acquisition. **Joana Raimundo:** Conceptualization, Validation, Resources, Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

The authors have no relevant financial or non-financial interests to disclose.

Data Availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2022.106346.

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