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Toxicological assessment of anthropogenic Gadolinium in seawater: biochemical effects in mussels *Mytilus galloprovincialis*

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ABSTRACT

Recently, anthropogenic enrichment of rare earth elements (REE) have been reported in natural environments, due to increasing use and discharges of hospital/industrial wastewaters. Gadolinium (Gd), which is mainly used as contrast agent for magnetic resonance imaging in medical exams, may reach concentrations in water up to two orders of magnitude larger than baseline levels. Nevertheless, in marine systems scarce information is available concerning the toxicity of REE towards inhabiting organisms. This study aimed to evaluate the biochemical impact of anthropogenic Gd in the Mediterranean mussel *Mytilus galloprovincialis*, which is a species of commercial interest and one of the most accepted pollution bioindicator. Organisms were exposed to different concentrations of Gd (0, 15, 30, 60, 120 µg/L) for 28 days. At the end of the experiments, biomarkers related to mussels' metabolic (electron transport system activity and energy reserves content), oxidative stress status (cellular damage and the activity of antioxidant and biotransformation enzymes) and neurotoxic effects (activity of the enzyme Acetylcholinesterase) were measured, as well as Gd bioconcentration in organisms. Results showed a high content of Gd (2.5±0.50 µg/g) in mussels exposed to the highest concentration, contrary to those at control condition and at 15 and 30 µg/L of Gd (levels below 0.38 µg/g). Although no mortality was observed during the experimental period, exposure to Gd strongly affected the biochemical performance of *M. galloprovincialis*, including the decrease on mussels' metabolism, induction of oxidative stress and neurotoxicity, particularly evidenced at intermediate concentrations. These results may indicate that up to certain stressful levels, although lowering their metabolism, organisms may be able to activate defense strategies to avoid cellular injuries which, on the other hand, may compromise mussels physiological performance such as growth and reproduction success. Nevertheless, our findings support that the widespread utilization of Gd may represent an environmental risk in the future.

Keywords: Rare earth elements; *Mytilus galloprovincialis*; Bioaccumulation; metabolisms; Oxidative stress; neurotoxicity.

1. INTRODUCTION

The rare earth elements (REEs) are distributed broadly in the Earth's crust in concentrations ranging on average between 150 and 220 parts per million (Kamenopoulos et al., 2016). These elements are called "rare" not because of their abundance, which is higher than that of gold or copper, but because REEs are typically dispersed in ores rather than in the native form of aggregates or nuggets (as in the case of gold or copper) (Goodenough et al., 2016; Zepf, 2015). Their unique characteristics, such as sharply defined energy states or ideal magnetic behaviour, made REE nowadays a strategic resource to high-technologies in different fields, from medicine to clean energy or electronics (Jacinto et al., 2018; Zepf, 2015). These elements can be identified in different environmental compartments (d'Aquino et al., 2009; Zhang and Shan, 2001), where they are persistent (Laveuf et al., 2012; Liang et al., 2005; Lu et al., 2003; Tang and Johannesson, 2006), with several studies reporting their accumulation in biota (d'Aquino et al., 2009; Dołęgowska and Migaszewski, 2013; Šmuc et al., 2012).

Gadolinium (Gd) is one of the metallic chemical elements known as the "Lanthanide Series", belonging to the REEs group. Gadolinium chelates are widely used as contrasting agent in magnetic resonance imaging (MRI) medical exams, due to the high magnetic moment of the paramagnetic Gd^{3+} ion (e.g., Kümmerer and Helmers, 2000; Migaszewski and Gałuszka, 2016; Möller et al., 2003). Since some Gd-based contrast agents are stable complexes and are not metabolized, after application they are excreted from the human body through urine and released to waste water treatment plants (WWTPs) and from here to aquatic environments almost unchanged (Knappe et al., 2005; Migaszewski and Gałuszka, 2016; Möller et al., 2000). Kümmerer and Helmers (2000) reported that the level of Gd in urine may reach 350 mg/L daily after the patient medical exam and 7 mg/L after 39 days. The use of Gd as contrast agent for MRI has been historically considered safe and well tolerated by humans when used at recommended dosing levels (Niendorf et al., 1991). However, for nearly a decade, Gd has been associated to nephrogenic systemic fibrosis (NSF) disease (Grobner, 2006), with recent reports demonstrating Gd accumulation in patients brain, bones and kidneys, despite normal renal functioning (Song et al., 2017; Vergauwen et al., 2018). The toxicity of Gd appears to be related with its action as a blocker of Ca^{2+} channels because its ionic radius is nearly equal to that of divalent Ca^{2+} (Sherry et al. 2009, as cited by Martino et al., 2017).

As a consequence of the increasingly exploitation and application of this element, Gd has been detected in a wide diversity of ecosystems (Migaszewski and Gałuszka, 2016; Rogowska et al., 2018) being recognized as an emergent micropollutant in aquatic environments (Rogowska et al., 2018). It has been reported Gd concentrations with natural background levels of 1-4 ng/L to values up to 200-1100 µg/L at

WWTPs effluent discharge points (Rogowska et al., 2018). Tepe et al. (2014) recorded increments in Gd concentrations in tap water sampled in Berlin, Germany, between 1.5 and 11.5-fold in just three years (2009-2012), while in San Francisco Bay, USA, Gd levels raised from *circa* 45 ρmol/kg to 180 ρmol/kg in less than 10 years (2004-2013) (Hatje et al., 2016). Positive Gd anomalies due to anthropogenic activities were also observed in river waters in Pennsylvania, USA (Bau et al., 2006), Poland (Migaszewski and Gałuszka, 2016) and South Korea (Song et al., 2017), in river and drinking water in Queensland, Australia (Lawrence and Bariel, 2010) and Prague, Czech Republic (Möller et al., 2002), in surface and ground waters in Italy (Möller et al., 2003), in Lake Paranoá, Brasil (Merschel et al., 2015) and in river and coastal waters of Southern France (Elbaz-Poulichet et al., 2002) and north western Germany (Kulaksiz and Bau, 2007), among other places over the world.

Although it is now recognized that Gd has negative consequences on human health (Rogosnitzky and Branch, 2016), the effects of Gd exposure on aquatic organisms are poorly understood. In 2010, Tai et al. studied the toxic effect of Gd and another 12 lanthanides on algae *Skeletonema costatum*, observing 50% reduction in their growth relative to control (72 hours) for a concentration of 29.04 ± 0.61 µmol/L, regardless of the lanthanide. In the work of González et al. (2015), using standard tests with *Aliivibrio fischeri*, *Pseudokirchneriella subcapitata*, *Daphnia magna*, *Heterocypris incongruens*, *Brachionus calyciflorus* and *Hydra attenuata*, it was concluded that crustaceans were less sensitive to $GdCl_3$ (along with cerium chloride and lutetium chloride) than rotifers and cnidarians. Recent studies developed by Martino et al. (2017) demonstrated that Gd exposure impaired the normal development of four sea urchin species: two from Europe, *Paracentrotus lividus* and *Arbacia lixula*, and two from Australia, *Heliocidaris tuberculata* and *Centrostephanus rodgersii*, with exposure to Gd resulting in inhibition or alteration of skeleton growth in the larvae.

From the scarce literature on the toxicity of Gd to aquatic organisms, it is verified that less data is available for marine organism comparatively to freshwater ones, and that more experiments, with longer exposure periods are needed (Gonzalez et al., 2014; Rogowska et al., 2018). Considering the importance of marine and estuarine environments, which provide various resources and services, and that they are the final recipients of most Gd continental inputs, it is crucial to study the potential impacts of this contaminant on inhabiting biota.

Considering the above mentioned, in the present study we investigated the effects of Gd on the biochemical performance of the mussel species *Mytilus galloprovincialis*. For this, mussels' metabolic capacity, oxidative stress and neurotoxic status were assessed by measuring several biochemical markers

after exposure of organisms to different concentrations of Gd. Due to their sedentary and filtration behavior, as well as their capacity to respond to environmental alterations, *M. galloprovincialis* mussels are widely used as bioindicators of a vast diversity of pollutants, including classical elements such as metals (among others, (Coppola et al., 2018; Maanan, 2007; Mejdoub et al., 2018; Regoli and Principato, 1995) and compounds considered of emerging concern including pharmaceuticals (Balbi et al., 2018; Maria et al., 2016), and nanoparticles (Andrade et al., 2018; Auguste et al., 2018; Taze et al., 2016).

2. MATERIALS AND METHODS

2.1 Experimental conditions

Mytilus galloprovincialis specimens were collected in October 2017 during low tide in the Ria de Aveiro estuary (Portugal). The mean body weight of the specimens was 21.3 ± 6.60 g, fresh weight (FW).

Organisms were transported from the field to the laboratory where they were placed in different aquaria for depuration and acclimation during two weeks. Conditions in the laboratory were: temperature 17.0±1.0 ºC; pH 8.0±0.1, 12 hours light and 12 hours dark as a photoperiod and continuous aeration, in artificial seawater (salinity 30±1) (Tropic Marin® SEA SALT from Tropic Marine Center). Seawater was renewed every day during the first three days and every three days until the end of this period. During the first week animals were not fed while after this initial period animals were fed with Algamac protein plus (150.000 cells/animal) two-three times per week

After, organisms were distributed into different aquaria, at temperature 17.0±1.0 ºC and salinity 30±1, with the following range of Gd concentrations: **CTL**) 0 µg/L; **C1**) 15 µg/L; **C2**) 30 µg/L; **C3**) 60 µg/L and **C4**) 120 µg/L. Per condition 3 replicates were used with 4 mussels per aquarium. These Gd concentrations were selected accordingly with values reported in pristine and contaminated aquatic systems by recent works (González et al., 2015; Rogowska et al., 2018; Tepe et al., 2014). A stock solution of 50 mg/L Gd, prepared by dilution of commercial Gd standard (Alfa Aesar Specpure® plasma standard solution 1000 mg/L) in ultrapure water, was used to fortify seawater. The control temperature (17 ºC) was selected considering the mean temperature of the sampling site (IPMA, 2018).

During the entire experimental period (28 days) aquaria were continuously aerated and maintained under a 12 hours light: 12 hours dark photoperiod. Temperature (17±1.0 ºC), pH (8.0±0.1) and salinity (30±1) were daily checked and adjusted if necessary. Mortality was also daily checked. During this period organisms were fed with Algamac protein plus (150.000 cells/animal) twice a week and seawater was renewed weekly, after which the respective Gd concentration was re-established. Immediately after the seawater renewal and Gd spiking into the water, samples of seawater were collected form each aquarium for further quantification of Gd, aiming to obtain the real exposure concentrations.

At the end of the exposure, organisms were frozen individually with liquid nitrogen and stored at -80ºC, until they were manually homogenized with a mortar and a pestle under liquid nitrogen. Each homogenized organism was divided into aliquots for biomarkers analyses and Gd quantification.

2.2 Gadolinium quantification in seawater and mussels tissues

The Gd concentration in water samples (one per replicate, three per condition), collected every week immediately after spiking, was obtained by inductively coupled plasma mass spectroscopy (ICP-MS), on a Thermo ICP-MS XSeries equipped with a Burgener nebuliser. The limits of detection and quantification of the method were 0.03 µg/L and 0.08 µg/L, respectively, with an acceptable coefficient of variation among replicates (n≥2) of 5%. Calibration curve was made with standards in the range of 2 to 100 µg/L.

Total Gd concentration in *M. galloprovincialis* (three per replicate, nine per condition) was also determined by ICP-MS, after microwave-assisted acid digestion. Freeze-dried samples (100–200 mg) were digested in a CEM MARS 5 microwave, first with 2 mL HNO_3 (70%) for 15 min at 170 °C, followed by a second microwave cycle with 0.5 mL H_2O_2 (30%) for 15 min at 170 °C. After addition of H_2O_2, the mixture was left to sit for 15 min to allow any gas to vent, before the reaction vessels were tightened and placed in the microwave. The obtained digests were transferred into 25 mL polyethylene vessels and the volume made up with ultrapure water. The quality control was assured by running procedural blanks (reaction vessels with only HNO_3 and H_2O_2) and certified reference material BCR-668 (Mussel tissue; 13.0±0.6 mg/Kg of Gd). Quantification of Gd in blanks gave values that were always below the detection limit of the methodology and obtained and certified values in reference material were in the range 77 to 102%, showing a good performance of the digestion and quantification method. The limit of quantification of Gd in mussels was 0.38 mg/Kg.

2.3 Biological responses

Biological responses were assessed using biochemical markers, determined in organism whole soft tissues. For each biochemical determination, 0.5 g fresh weight (FW) soft tissue per organism was used (three individuals per replicate, nine per condition). For each condition, metabolic capacity (electron transport system activity, ETS), energy-related biomarkers (glycogen content, GLY; total protein content, PROT), oxidative stress indicators (superoxide dismutase activity, SOD; catalase activity, CAT; glutathione peroxidase activity, GPx; glutathione S-transferases activity, GSTs; lipid peroxidation levels, LPO and glutathione content ratio, GSH/GSSG) and neurotoxicity (Acetylcholinesterase activity, AChE) were assessed. All biochemical parameters were performed at least in duplicate. All measurements were done using a microplate reader (BioTek, Synergy HT). The extraction for each biomarker was performed with specific buffers (see for example, Almeida et al., 2014; Coppola et al., 2017). These samples were sonicated

for 15 sec at 4 ºC and centrifuged for 10 min at 10 000 *g* (or 3 000 *g* for ETS). Supernatants were stored at -80 ºC or immediately used.

Metabolic capacity and energy related biomarkers

The activity of ETS was measured based on the method of King and Packard (1975) with modifications performed by Coen and Janssen (1997). The absorbance was measured at 490 nm during 10 min with intervals of 25 s. The amount of formazan formed was calculated using the extinction coefficient (Ɛ) 15,900 M^{-1} cm^{-1}. The results were expressed in nmol/min per g fresh weight (FW).

The GLY content was quantified following the sulfuric acid method (Dubois et al. 1956), using 8 glucose standards in the concentration range of 0 to 10 mg/mL in order to obtain a calibration curve. Absorbance was measured at 492 nm after being incubated for 30 min at room temperature. The results were expressed in mg per g FW.

The PROT content was determined according to the Biuret method described by Robinson and Hogden (1940). A stock solution of bovine serum albumin (BSA) was used to prepare 5 standards (0–40 mg/mL) to obtain a calibration curve. After 10 minutes of incubation at 30 ºC, absorbance was measured at 540 nm. The results were expressed in mg per g FW.

Oxidative stress: enzymatic markers

The activity of SOD was determined according to the method of Beauchamp and Fridovich (1971). For the calibration curve 7 SOD standards (0.25 - 60 U/mL) were used. After 20 min of incubation at room temperature, the absorbance was measured at 560 nm. The activity was expressed in U per g FW, where U corresponds to a reduction of 50% of nitroblue tetrazolium (NBT).

The activity of CAT was quantified according to Johansson and Borg (1988). For the calibration curve 9 formaldehyde standards (0 - 150 µmol/L) were used. The absorbance was measured at 540 nm and activity expressed in U per g F, where U represents the amount of enzyme that caused the formation of 1.0 nmol formaldehyde per min.

The activity of GPx was determined following the method of Paglia and Valentine (1967). Absorbance measurements were performed at 340 nm during 5 min in 10 s intervals and the activity was determined using the extinction coefficient (Ɛ) 6.22 $mM^{-1}cm^{-1}$. Results were expressed in U per g FW, where

U corresponds to the quantity of enzyme which catalyzes the conversion of 1 µmol nicotinamide adenine dinucleotide phosphate (NADPH) per min.

The activity of GSTs was quantified based on the method of Habig et al. (1974) with modifications performed by Carregosa et al. (2014). Absorbance was read at 340 nm during 5 min in 10 s intervals and the amount of thioether formed was calculated using the extinction coefficient (Ɛ) 9.6 mM^{-1} cm^{-1}. The results were expressed in U per g FW, where U corresponds to the quantity of enzyme that causes the formation of 1 µmol of dinitrophenyl thioether per min.

Oxidative stress: non-enzymatic markers

LPO levels were determined by the quantification of malondialdehyde (MDA), a by-product of lipid peroxidation according to the method described in Ohkawa et al. (1979). Absorbance was measured at 535 nm and the amount of MDA formed was calculated using the extinction coefficient (Ɛ) 156 mM^{-1} cm^{-1}. The results were expressed in nmol per g FW.

The quantification of GSH and GSSG was performed following the method described in Rahman et al. (2007), using GSH and GSSG as standards (0–90 µmol/L). Absorbance was read at 412 nm during 2 min in 30 s intervals and the results expressed in µmol per g FW. The GSH/GSSG ratio was determined [GSH / GSSG = GSH / (2 ∗ GSSG)].

Neurotoxicity

The activity of AChE was determined using Acetylthiocholine iodide (ATChI, 470 µM) substrates, according to the methods of Ellman et al. (1961) with modification performed by Mennillo et al. (2017). The activity was measured at 412 nm during 5 min and expressed in nmol/min per g FW using the extinction coefficient (Ɛ) $13.6x10^{3}$ $M^{-1}cm^{-1}$.

2.4 Statistical analyses

All the biochemical results (ETS, PROT, GLY, CAT, SOD, GPx, GSTs, LPO, GSH/GSSG, and AChE) and Gd concentrations obtained from each exposure condition were submitted to a statistical hypothesis testing using permutational analysis of variance, employing the PERMANOVA+add-on in PRIMER v6 (Anderson et al., 2008). The pseudo-F p-values in the PERMANOVA main tests were evaluated in terms of

significance. When significant differences were observed in the main test, pairwise comparisons were performed. Values lower than 0.05 ($p \leq 0.05$) were considered as significantly different. The null hypothesis tested was: for each biomarker, no significant differences existed among exposure concentrations (0, 15, 30, 60 and 120 µg/L). For each biomarker, significant differences among conditions were represented in figures with different letters (lowercase letters).

3. RESULTS AND DISCUSSION

3.1 Gadolinium concentrations in seawater and mussels tissues

Uncontaminated seawater (control condition) showed Gd levels always lower than 0.50 µg/L, assuring the low value of this element in the artificial seawater used in the experiment. In Gd exposure conditions, concentrations in water samples collected immediately after spiking ranged between 11±0.70 and 14±0.60 µg/L for C1 (nominal concentration 15 µg/L), 25±1.2 and 30±1.7 µg/L for C2 (nominal concentration 30 µg/L), 50±0.90 and 59±2.7 µg/L for C3 (nominal concentration 60 µg/L), and 102±1.6 and 114±5.0 µg/L for C4 (nominal concentration 120 µg/L). Thus, the levels of Gd measured in water agreed well with expected ones, with maximum deviations to the nominal concentrations along the exposure period not higher than 10% (Table 1). These differences may be related to the uncertainty associated with fortification either in the preparation of the 50 mg/L stock solution or in pipetting to the aquaria. Uncertainties in quantification should also be considered. Studies conducted by González et al. (2015) and by Braun et al. (2018) also demonstrated the stability of Gd after spiking process.

The concentration of Gd determined in mussels' soft tissues after the experimental period (28 days) at the CTL and at the lowest exposure concentration (C1) were below the quantification limit (LOQ = 0.38 µg/g). Determinations in the mussels exposed to higher concentrations (C2, C3 and C4) revealed the presence of this element, with contents ranging from 0.44±0.10 µg/g at the lowest exposure concentration to 2.5±0.50 µg/g in mussels exposed to the highest Gd concentration. The bioconcentration factors (BCFs), defined as the ratio between Gd concentration in mussel tissue at the end of the exposure and the initial concentration of this REE in water, were shown to be independent of the exposure condition, with values being very close among conditions (16 for C2; 15 for C3 and 23 for C4). Indeed, the concentration of Gd measured in the organisms exposed to C2, C3 and C4 conditions corresponded always to about 10% of the theoretical maximum concentration (C_{max}, µg/g) hypothetically present in the organism considering the uptake of all Gd available from seawater along the experimental period, which can be calculated from of the following equation:

$$C_{\max} = \left(\left(C_0 \times V \right) \times t \right) / \left(m \times n \right) \qquad (1)$$

where C_0 (µg/L) is the initial concentration of Gd measured in seawater, *V* the volume of water in the aquarium (L), *t* the number of weekly water renewals (4), *m* the mean mussel body weight (µg/g; DW) and *n* the number of mussels in each aquarium (n=4).

These results evidence the capacity of mussels to accumulate Gd, with a direct relationship between accumulated and exposure concentration. Despite the scarcity of research data on this topic, these findings are similar to previously published ones, with a limited number of studies revealing a concentration-dependent uptake of REE by organisms (Carpenter et al., 2015; Hanana et al., 2017), particularly Gd with recent studies showing the accumulation of this element by freshwater bivalve species (the mussel *Dreissena rostriformis bugensis* and the clam *Corbicula fluminea*) (Perrat et al., 2017) and aquatic plants (Braun et al., 2018, Lingott et al., 2016). Furthermore, the BFCs observed in the present study were within the range of 3.2-86.4 already reported for different aquatic plants and organisms (*Trichoderma strains*, *Daphnia magna*, *Lemna minor*, *Lepidium sativum*, *Cyprinus carpio*) (Rogowska et al., 2018).

3.2 Biological responses

3.2.1. Metabolic capacity and energy related biomarkers

Mussels exposed to Gd significantly decreased their electron system (ETS) activity in comparison to non-contaminated mussels (CTL), with the lowest values in organisms exposed to C2, C3 and C4 concentrations (Figure 1A). On the contrary, contaminated mussels significantly increased their energy reserves (glycogen content, GLY and protein content, PROT) in comparison to CTL organisms, with the highest concentrations in organisms exposed to concentration C3 (Figures 1B and C). These results clearly revealed that in response to Gd exposure mussels strongly decreased their metabolic capacity, probably as a result of mussels filtration rate reduction to avoid the accumulation of Gd. Nevertheless, Gd concentrations in mussels tissues revealed that the element accumulation rate was independent of the concentration of exposure, i.e., the percentage of Gd incorporated compared to the amount available in the seawater was always the same and about 10%. Furthermore, the decrease of ETS was not proportional to the increase of Gd concentrations, revealing that above certain limits of stress (here represented by concentrations higher than C2) mussels were not able to continue to further decrease their metabolic rate. The present work also demonstrated that the decrease on mussels metabolism was accompanied by an increase of mussels energy reserves; i.e., by lowering their metabolic capacity mussels were able to prevent the expenditure of their energy reserves. Nevertheless, at the highest exposure concentration (C4) mussels evidenced that

even with their metabolic capacity reduced they started the expenditure of their energy reserves, indicating that at higher stress levels GLY and PROT were probably necessary to fuel up defence mechanisms.

Metabolic depression, associated with reduction of bivalve's filtration rate, with decrease of energy reserves expenditure was already reported by other authors when exposing bivalves to pollutants, probably to limit the accumulation of such substances. Although to the best of our knowledge no information is available on the impacts of REE in bivalves metabolism, studies conducted with *M. galloprovincialis* also demonstrated a metabolic depression and storage of energy reserves as a response to the exposure to carbamazepine (Anderson et al., 2008), titanium (Monteiro et al., 2019) and mercury (Coppola et al., 2017).

3.3.2. Oxidative stress: enzymatic markers

The activity of superoxide dismutase (SOD) was significantly higher in mussels exposed to Gd in comparison to CTL organisms, with the highest values in organisms exposed to concentration C2. No significant differences in terms of SOD activity were observed between organisms exposed to C4 and C3 concentrations, as also between organisms exposed to C4 and the two lowest concentrations (C1 and C2) (Figure 2A). The activity of catalase (CAT) increased in mussels exposed to Gd comparatively to uncontaminated mussels (CTL), with significantly higher values recorded in organisms exposed to concentrations C1, C2 and C3 (Figure 2B). The activity of glutathione peroxidase (GPx) was significantly higher in organisms exposed to concentrations C2 and C3 in relation to the remaining conditions (CTL, C1 and C4), with the highest values observed in organisms exposed to C2 (Figure 2C).

It is well established that when organisms are exposed to pollutants an overproduction of reactive oxygen species (ROS) can occur with an associated antioxidant defence response, including the increase of antioxidant enzymes activity such as SOD, CAT and GPx (among other, Regoli and Giuliani, 2014). The results here presented indicate that mussels increased their antioxidant defence capacity in the presence of Gd, but this response was only effective up to certain limits since the highest enzymes activities were observed at intermediate concentrations. These results evidence that at higher stress levels, namely highest Gd concentration (C3 and C4), mussels were not able to proportionally increase their antioxidant capacity, showing enzymes activities similar to control levels. Such behaviour may result from the low metabolic capacity evidenced by organisms, which was not enough to activate enzymes at these conditions; or may indicate that the over production of ROS may inhibited the activity of these enzymes; or may also indicate that organisms were capable of developing other defence mechanisms that prevent toxicity by Gd and there was no need for higher antioxidant defence. A similar response was observed by Sureda et al. (2018)

studying the impacts of a sunscreen with TiO_2 in its composition in *M. galloprovincialis* after an exposure of 24 hours. These authors revealed that the activities of the antioxidant enzymes and the detoxification GSTs evidenced a hormetic shape response with increased activities at lower sunscreen concentrations, a response that was abolished at the highest concentration. Hanana et al. (2017) showed that in the freshwater mussel *Dreissena polymorpha* La caused an antioxidant and prooxidant effects depending on the concentration and the duration of exposure.

The activity of glutathione-S-transferases (GSTs) enzymes was significantly higher in organisms exposed to Gd in comparison to control values. The highest GSTs values were obtained in mussels exposed to C2, with significant differences to mussels exposed to the remaining concentrations (Figure 3). When exposed to pollutants organisms develop mechanisms of defence that, associated with antioxidant responses, are responsible for lowering the stress induced. Such mechanisms involve the detoxification of xenobiotics as the case of GSTs that main function is to catalyse the conjugation of a diverse array of electrophilic compounds with glutathione. In the present study mussels exposed to Gd increased the activity of GSTs enzymes, evidencing that higher exposure concentrations were not accompanied by higher activity levels. As for the antioxidant enzymes, the results here presented indicate that at higher Gd exposure concentrations mussels were no longer able to continue to increase the activity of GSTs enzymes along with the increase of Gd. An explanation for this response may be related to the fact that, as demonstrated for rats, Gd ions via blocking Ca channels inhibit GSTs and shift the dose-inhibitory response curves for protein kinase C inhibitors, which are also known as suppressors of drug metabolizing enzymes (Kim et al., 1998). In the mussels *D. polymorpha* La caused a decrease in GSTs activity after 14 days but not after 28 days of exposure (Hanana et al., 2017). A similar behaviour was observed in *M. galloprovincialis* exposed to the drug cetirizine (Teixeira et al., 2017) and in the clams *Ruditapes philippinarum* exposed to functionalized multi-walled carbon nanotubes (De Marchi et al., 2018). Nevertheless, Perrat et al. (2017) studying the effects of Gd in the freshwater bivalves *D. rostriformis bugensis* and *C. fluminea,* demonstrated that GSTs were an efficient defence mechanism, detoxifying cells from Gd, which allowed to minimize cell toxicity and therefore biochemical responses. The role of this group of enzymes in the detoxification of other pollutants in *M. galloprovincialis* mussels was already demonstrated by several authors, revealing the increase of GSTs activity when in the presence of trace elements (Coppola et al., 2017; Freitas et al., 2017; Monteiro et al., 2019) and nanoparticles (Canesi et al., 2010; Della Torre et al., 2015).

3.3.3. Oxidative stress: non-enzymatic markers

Lipid peroxidation (LPO) levels were significantly higher in mussels exposed to concentrations C2, C3 and C4 in comparison to mussels under CTL condition and the lowest Gd concentration (C1). Significantly higher LPO values were observed at C2 concentration in comparison to the remaining conditions. No significant differences were observed in LPO levels in mussels exposed to CTL and the lowest concentration (Figure 4A). Organisms exposed to Gd showed significantly lower GSH/GSSG values comparatively to control values, with no significant differences among Gd exposure conditions (C1, C2, C3 and C4) (Figure 4B).

These findings clearly demonstrated that Gd induced cellular damage in mussels exposed to this element, which were accompanied by an oxidative status evidenced by low GSH/GSSG values. Furthermore, although antioxidant and biotransformation enzymes activities were higher at C2, higher LPO levels were observed at this condition, evidencing the high stress level induced and the insufficient response of defence mechanisms. On the other hand, lower LPO levels at higher concentrations (C3 and C4) were not explained by higher increased antioxidant defences since at both conditions mussels' activities also decreased. These results may indicate that the stress induced by Gd at the highest concentrations (C3 and C4) was at a certain point restricted with limited antioxidant and biotransformation responses. Since concentrations of Gd accumulated by mussels were higher at C3 and C4 conditions in comparison to C2 we may hypothesize that the toxicity of Gd was at a certain extent limited by the performance of other defence mechanisms, including metallothioneins. This last hypothesis is not supported by Hanana et al. (2017) investigation that demonstrated that when the *D. polymorpha* mussels were exposed to La this element was bioaccumulated but mussels did not trigger metallothionein induction. Pagano et al. (2016) showed an increase of LPO levels in *Paracentrotus lividus* sea urchin pluteus larvae (48 hours post-fertilization) exposed to Gd.

3.3.4. Neurotoxicity

The activity of Acetylcholinesterase (AChE) was decreased in the presence of Gd, with a significant inhibition at concentrations C2, C3 and C4 (Figure 5). These results clearly revealed the neurotoxic capacity of Gd, which may be related to the fact that Gd has the capacity to block K-type Ca^{2+} channels (Palasz and Czekaj, 2000) a similar behaviour also observed for La that inhibit Ca binding to brain synaptosomal membrane, with a marked depression in the activities of neural Ca^{2+}-ATPase, Mg^{2+}-ATPase, and cholinesterase after acute exposure to this element (Basu et al., 1982).

Similar neurotoxic response pattern was already demonstrated by *M. galloprovincialis* exposed to trace elements (Lionetto et al., 2003) or inhabiting areas contaminated by pesticides and discharges of domestic/industrial effluents (Moreira and Guilhermino, 2005), and by other bivalves such as the clam *R. philippinarum* exposed to metalloids and nanoparticles (Freitas et al., 2018).

Conclusions

The present study revealed for the first time the toxic effects of seawater contaminated with Gd in the mussel *M. galloprovincialis*, evidencing the capacity of this REE to induce mussels oxidative stress and neurotoxicity as well as to reduce their metabolic capacity. The activities of SOD, CAT and GSTs were always higher in the organisms exposed to Gd, while the ratio between reduced and oxidized forms of glutathione, as well as ETS activity decreased significantly in the presence of Gd. Furthermore, mussels were shown to accumulate Gd along with an increasing exposure gradient. Thus, our findings confirm that the increasing use of Gd, which is expected to continue in the future, may represent a significant environmental and human health risk.

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Figure captions

Figure 1: A) Electron transport system activity (ETS); B) Glycogen content (GLY); C) Protein content (PROT), in *Mytilus galloprovincialis* after 28 days-exposure to Gadolinium at concentration: CTL) 0 µg/L; C1) 15 µg/L; C2) 30 µg/L; C3) 60 µg/L and C4) 120 µg/L. Results are expressed as mean + standard deviation. Significant differences among exposure concentrations are represented with lowercase letters. n=9

Figure 2: A) Superoxide dismutase activity (SOD); B) Catalase activity (CAT); C) Glutathione peroxidase activity (GPx), in *Mytilus galloprovincialis* after 28 days-exposure to Gadolinium at concentration: CTL) 0 µg/L; C1) 15 µg/L; C2) 30 µg/L; C3) 60 µg/L and C4) 120 µg/L. Results are expressed as mean + standard deviation. Significant differences among exposure concentrations are represented with lowercase letters. n=9

Figure 3: Glutathione S-transferases activity (GSTs), in *Mytilus galloprovincialis* after 28 days-exposure to Gadolinium at concentration: CTL) 0 µg/L; C1) 15 µg/L; C2) 30 µg/L; C3) 60 µg/L and C4) 120 µg/L. Results are expressed as mean + standard deviation. Significant differences among exposure concentrations are represented with lowercase letters. n=9

Figure 4: A) Lipid peroxidation levels (LPO); B) GSH/GSSG, in *Mytilus galloprovincialis* after 28 days-exposure to Gadolinium at concentration: CTL) 0 µg/L; C1) 15 µg/L; C2) 30 µg/L; C3) 60 µg/L and C4) 120 µg/L. Results are expressed as mean + standard deviation. Significant differences among exposure concentrations are represented with lowercase letters. n=9

Figure 5: Acetylcholinesterase activity (AChE), in *Mytilus galloprovincialis* after 28 days-exposure to Gadolinium at concentration: CTL) 0 µg/L; C1) 15 µg/L; C2) 30 µg/L; C3) 60 µg/L and C4) 120 µg/L. Results are expressed as mean + standard deviation. Significant differences among exposure concentrations are represented with lowercase letters. n=9

Table 1. Gadolinium (Gd) concentrations (µg/L) in seawater samples collected every week immediately after spiking with 15, 30, 60 and 120 µg/L of Gd. n=3

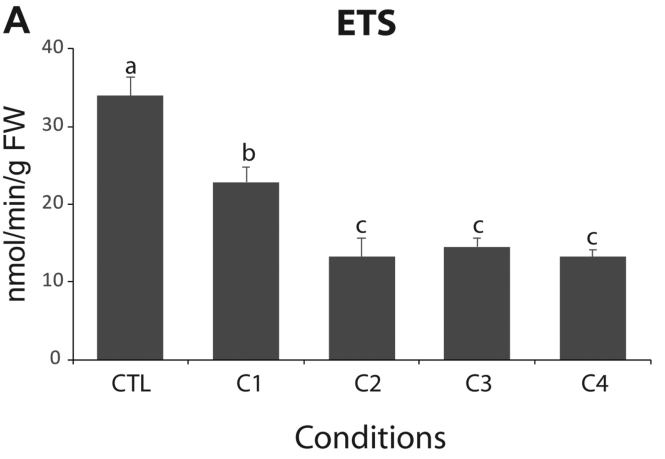
	1st week	2nd week	3rd week	4th week
CTL	0.30±0.10	0.44±0.10	0.34±0.07	0.50±0.60
C1	14±0.60	11±0.70	12±0.60	14±0.30
C2	27±1.9	26±1.1	25±1.2	30±1.7
C3	50±1.0	57±2.8	50±0.90	59±2.7
C4	102±1.6	109±2.5	103±5.8	114±5.0

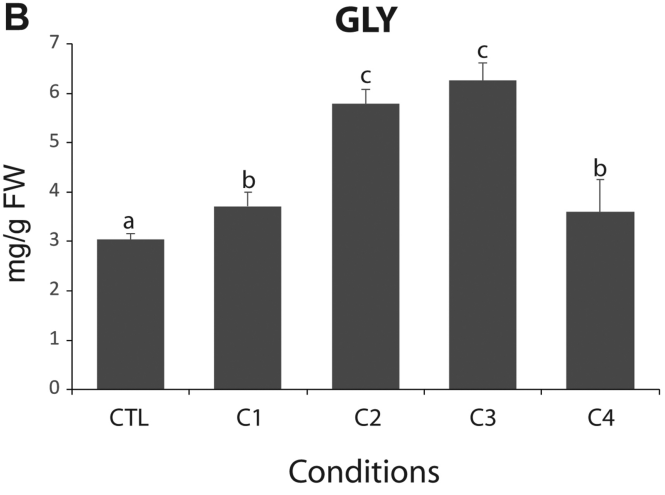
Table 2. Gadolinium (Gd) concentrations (µg/g) in mussels soft tissues after 28 days of exposure to seawater spiked with 15, 30, 60 and 120 µg/L of Gd. Limit of quantification (LOQ) was 0.38 µg/g dry weight. Different letters represent significant differences among tested concentrations. n=9

	Gd concentrations (µg/g)
CTL	< 0.38
C1	< 0.38
C2	0.44 ± 0.10^{a}
C3	0.81 ± 0.070^{b}
C4	2.5 ± 0.50^{c}

Highlights

- *Mytilus galloprovincialis* bioaccumulated Gadolinium after a 28 days exposure period
- Contaminated mussels decreased their metabolic capacity
- Mussels exposed to Gd activated their antioxidant and biotransformation defences
- Contaminated mussels showed increased lipid peroxidation and lower GSH/GSSG ratio
- Neurotoxicity was induced in contaminated mussels


A
ETS
nmol/min/g FW
40
30
20
10
0
a
b
c
c
c
CTL
C1
C2
C3
C4
Conditions


B
GLY
mg/g FW
7
6
5
4
3
2
1
0
a
b
c
c
b
CTL
C1
C2
C3
C4
Conditions

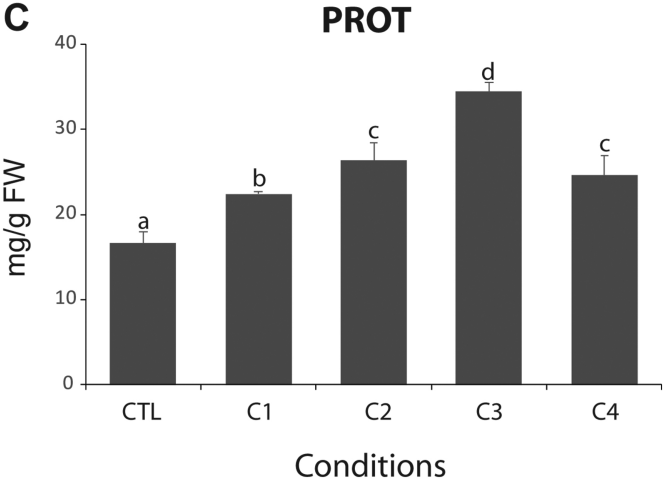
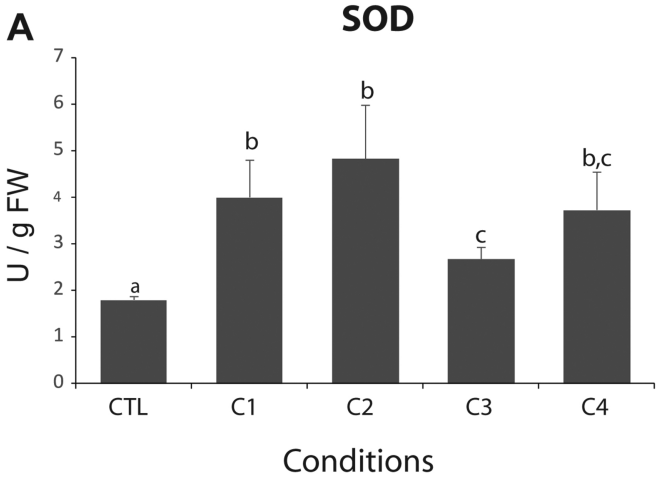
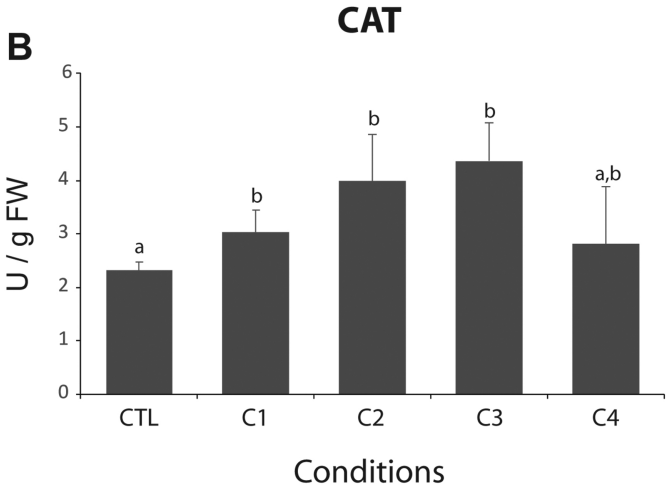

C
PROT
mg/g FW
40
30
20
10
0
a
b
c
d
c
CTL
C1
C2
C3
C4
Conditions

Figure 1


A
SOD
U / g FW
0
1
2
3
4
5
6
7
a
b
b
c
b,c
CTL
C1
C2
C3
C4
Conditions


B
CAT
U / g FW
0
1
2
3
4
5
6
a
b
b
b
a,b
CTL
C1
C2
C3
C4
Conditions

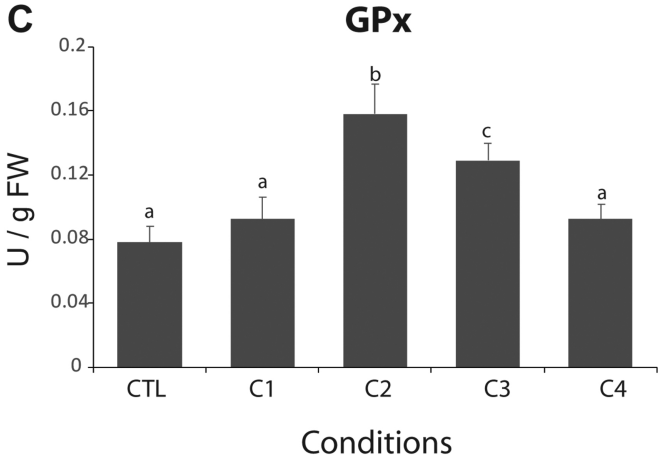

C
GPx
U / g FW
0
0.04
0.08
0.12
0.16
0.2
a
a
b
c
a
CTL
C1
C2
C3
C4
Conditions

Figure 2

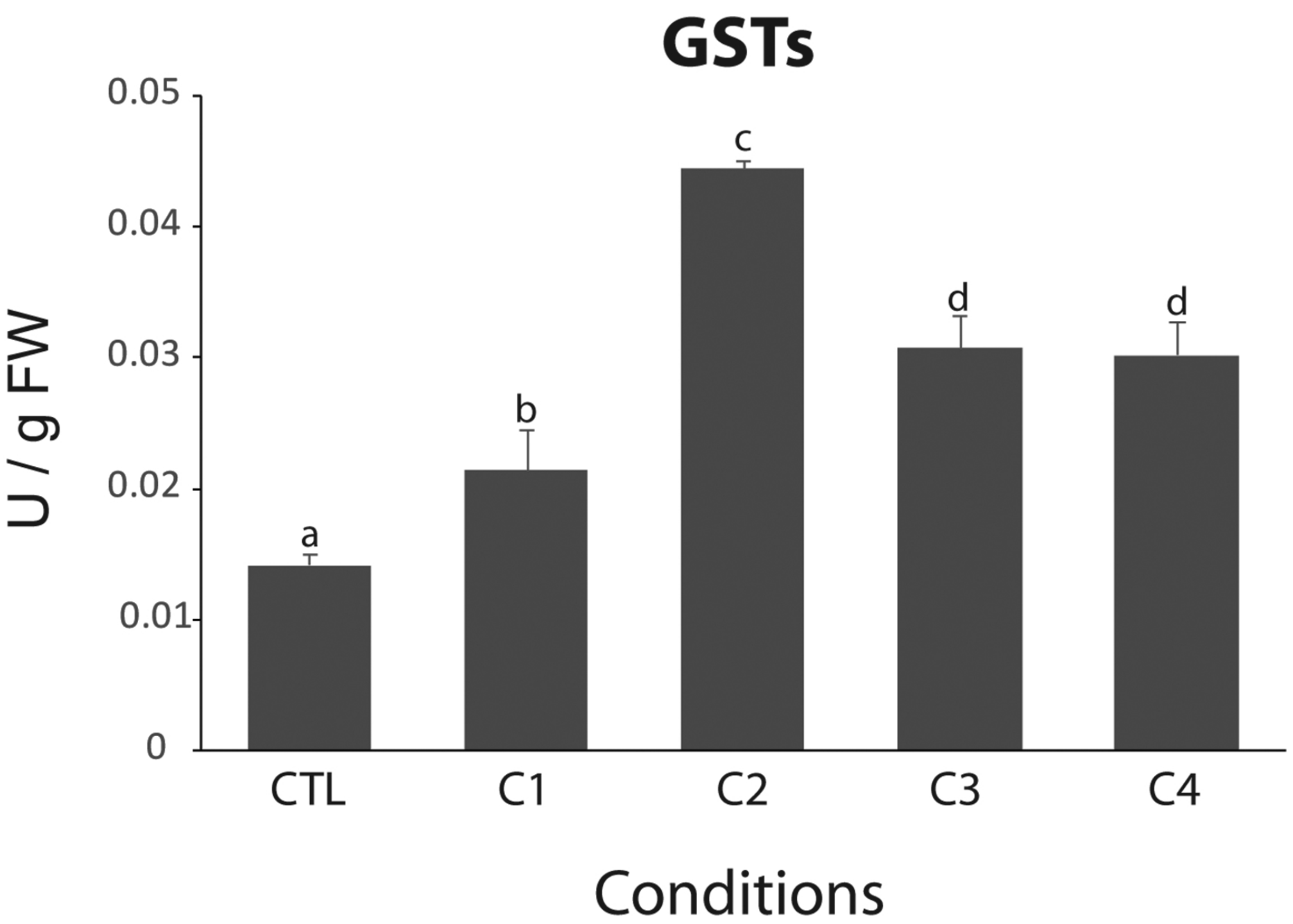
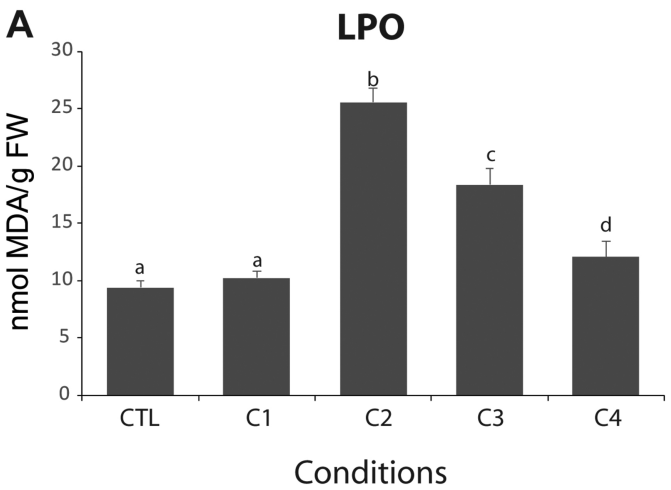

GSTs
0.05
0.04
0.03
0.02
0.01
0
U / g FW
a
b
c
d
d
CTL
C1
C2
C3
C4
Conditions

Figure 3


A
LPO
nmol MDA/g FW
30
25
20
15
10
5
0
a
a
b
c
d
CTL
C1
C2
C3
C4
Conditions

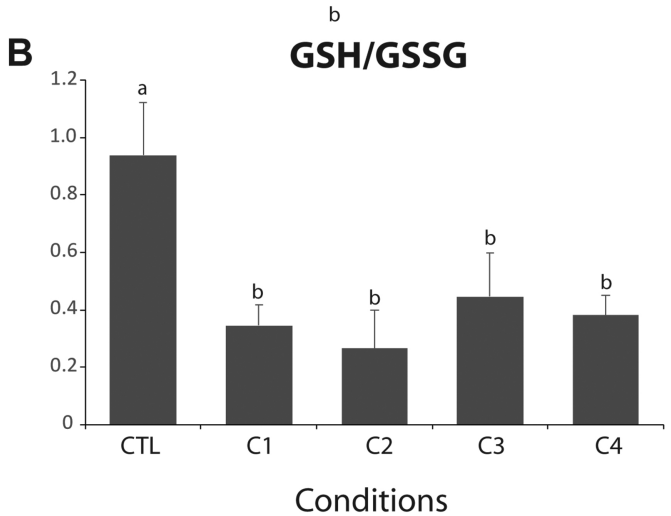

B
GSH/GSSG
1.2
1.0
0.8
0.6
0.4
0.2
0
a
b
b
b
b
CTL
C1
C2
C3
C4
Conditions

Figure 4

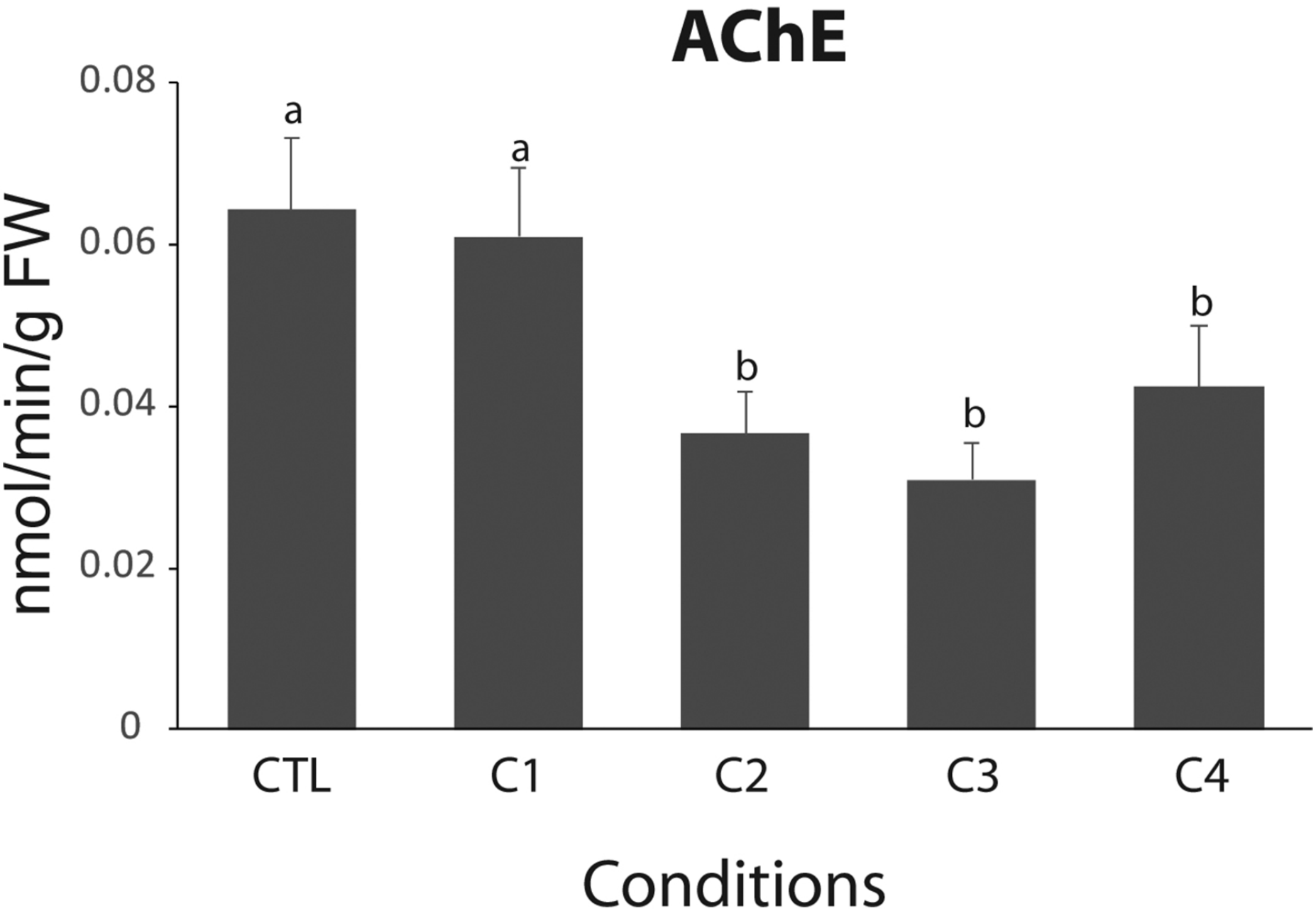

AChE
0.08
0.06
0.04
0.02
0
nmol/min/g FW
a
a
b
b
b
CTL
C1
C2
C3
C4
Conditions

Figure 5