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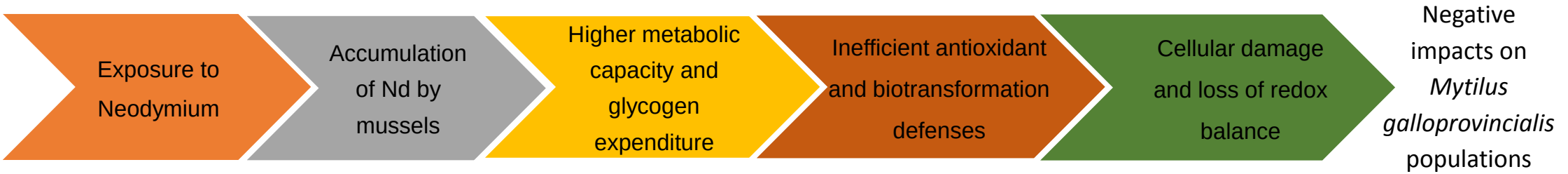
Exposure to Neodymium
Accumulation of Nd by mussels
Higher metabolic capacity and glycogen expenditure
Inefficient antioxidant and biotransformation defenses
Cellular damage and loss of redox balance
Negative impacts on *Mytilus galloprovincialis* populations

Toxicological effects of the rare earth element Neodymium in *Mytilus galloprovincialis*

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ABSTRACT

The wide range of applications of rare earth elements (REE) is leading to their occurrence in worldwide aquatic environments. Among the most popular REE is Neodymium (Nd), being widely used in permanent magnets, lasers, and glass additives. Neodymium–iron–boron (NdFeB) magnets is the main application of Nd since they are used in electric motors, hard disk drives, speakers and generators for wind turbines. Recent studies have already evaluated the toxic potential of different REE, but no information is available on the effects of Nd towards marine bivalves. Thus, the present study evaluated the biochemical alterations caused by Nd in the mussel *Mytilus galloprovincialis* exposed to this element for 28 days. The results obtained clearly demonstrated that Nd was accumulated by mussels, leading to mussel's metabolic capacity increase and GLY expenditure, in an attempt to fuel up defense mechanisms. Antioxidant and biotransformation defenses were insufficient in the elimination of ROS excess, resulting from the presence of Nd and increased electron transport system activity, which caused cellular damages (measured by lipid peroxidation) and loss of redox balance (assessed by the ratio between reduced and oxidized glutathione). The results obtained clearly highlight the potential toxicity of REEs, and in particular of Nd, with impacts at cellular level, which may have consequences in mussel's survival, growth and reproduction, affecting mussel's population.

Keywords: Rare earth elements, mussels, oxidative stress, metabolic capacity, bioconcentration.

1. INTRODUCTION

The extensive use of neodymium (Nd, Z = 60) by the industries has drawn attention from the scientific community in the last years. This light rare earth element is classified as one of the five most critical rare earth elements (REEs) until 2025 (U.S. Departament of Energy, 2011) due to its high economic importance and supply risk (Batinic et al., 2017; Critical Raw Materials - European Commission Report, 2018). Currently, Nd main application is related to permanent magnets (PMs) based on NdFeB, whose amount is increasing every year. Neodymium magnets are the strongest type of permanent magnet commercially available. It was announced that the annual use of these magnets increased from 20 thousand tons in 2006 to almost 55 thousand tons in 2017 (Liu et al., 2019), with three electric and electronic components containing PMs: hard disk drives, small electric motors and speakers. The results show that the weight percentage of the PMs varies from 4 to 6% in the speakers, 2.5 to 2.8% in the hard disks, and between 0.8 and 2% in some electric motors from hybrid electric vehicles (Menad and Seron, 2017). Despite these low representative numbers, there were generated, in 2018, 50 million tons of electric and electronic waste (UNEP et al., 2019). These numbers are concerning the governments and industry analysts regarding future prices and availabilities (Rabe et al., 2017) but also concerning environmental impact of this element in aquatic systems. Permanent magnets are also used in wind turbines, missiles, tanks, warplanes and submarines (Padhan et al., 2017). An advantage of using these magnets over alternative technologies in wind turbines is that they reduce the turbine´s size and decreases the overall weight (Rabe et al., 2017). Even so, typically wind turbines contain about 150–200 kg of Nd per megawatt of generating capacity (Hatch, 2012), which means that most offshore wind turbines may require two tons of this REE (Turra, 2018).

The increase application of Nd in high-tech processes and products leads to the release of this element into the environment, mainly in the rivers and coastal areas not only due to the disposal of e-waste (50 million tons in 2018) but also from the mining activities which is the primary source of REEs discharge into water systems (Adeel et al., 2019; UNEP et al., 2019). The concentration of Nd in waters depends on several factors such as climate, geology and vegetation and its most common oxidation state is Nd(III). A wide range of REEs concentrations has been detected in agricultural soils (< 15.9-249.1µg/g) and in groundwater (< 3.1-146.2 µg/L) at various sites worldwide (Adeel et al., 2019), with Nd being the third element most abundant (after Ce and La). The concentration of REEs in soils and sediment is higher than in water resources due to pH and cationic exchange capacity. This occurs because most REEs may adsorb to soils and sediments through their dissolution and surface complexation reactions with inorganic and organic ligands (Gwenzi et al., 2018). The concentration of Nd in the environment is in the order of ng/L: 2.8 ng/L in seawater (Tai et al., 2010), 0.76 – 15 ng/L in rain water, 16.9 ng/L in throughfall, 58 ng/L in aqueous phase of the soil and 84.9 ng/L in stream water (Kabata-Pendias and Mukherjee, 2007). Despite that, its concentration in surface waters is about a few µg/L, while in contaminated environments it increases until hundreds of µg/L. The Terengganu River Basin, in Malaysia, is an example of a surface water where concentrations of Nd between 0.0071 and 6.68 µg/L were detected (Sultan and Shazili, 2009). As for the contaminated environments, it was detected 771 µg/L in streams draining from acid sulphate soils during high-water flow events in autumn, in Finland (Åström, 2001). In an alluvial aquifer affected by acid mine drainage (Guadiamar, Spain) it was detected < 0.01 – 52.67 µg/L (Olías et al., 2005). It was also found a concentration of Nd of 10.8 µg/L (Khan et al., 2017) and 317 µg/L (Migaszewski et al., 2016) in the surface water of the

ex-mining pit lake (Malaysia) and in a mining pit in Wisniowka (Poland), respectively. In coastal areas the concentration of Nd found in the coast of Havaii (Kona) and the coast of Australia (Labrador beach), revealed concentrations of Nd in seawater of 24-32 μg/L (Adeel et al., 2019).

The increase concentration of Nd in aquatic systems may have significant impacts in the organisms inhabiting these systems. Asian continent, namely in China, shows the most critical risk of REEs pollution level followed by Europe, Africa, USA and Australia (Adeel et al., 2019). However, there are only few papers published regarding this thematic. Wang et al. (2011) evaluated the effect of Nd on a freshwater cyanobacteria, *Microcystis aeruginosa*, specifically on its growth and biochemical changes. The results showed that Nd(III) concentration ≤1 mg/L can stimulate the growth of *M. aeruginosa*; also, the content of chlorophyll a (Chl-a), soluble protein and the activity of antioxidant defences increased when compared with the control. However, with high concentration of Nd(III) (5.00–10.00 mg/L), the growth of *M. aeruginosa* was inhibited while increasing the content of malondiadehyde (MDA) and decreased the activity of the enzyme catalase (CAT). It was also studied the Nd effects on rat (liver), and the results showed an accumulation in hepatocyte nuclei and mitochondria, a decrease of superoxide dismutase (SOD) and CAT, and an increase of the activity of glutathione peroxidase (GPx) and lipid peroxidation levels (Adeel et al., 2019; Rim et al., 2013). Regardless of the increasing presence of Nd in oceans and consequently bioaccumulation in aquatic organisms, there are only a few toxicological studies on organisms published in the literature. Also, those studies evaluate the effects on freshwater organisms, but Nd discharged by the industries will reach the oceans.

Therefore, the present study aimed to evaluate the biological effects induced by Nd in the mussel species *Mytilus galloprovincialis*, through the exposure of the

organisms to five different concentrations (2.5, 5, 10, 20 and 40 µg/L of Nd), during twenty-eight days. For this, biomarkers related with oxidative stress, redox balance and metabolic status were measured in *M. galloprovincialis*, trying to identify potential impacts of Nd on this species.

2. MATERIALS AND METHODS

2.1 Experimental conditions

The Mediterranean mussel *Mytilus galloprovincialis* was selected as biological model for the present study. Mussels with similar size (5.7±0.7 cm length; 3.0±0.4 cm width) were collected in September 2018, at the Ria de Aveiro lagoon (Portugal). After sampling mussels were placed in aquaria for depuration and acclimation to laboratory conditions for 2 weeks, during which animals were maintained under constant aeration in different tanks with in artificial seawater (made by reverse osmosis water and artificial salt - Tropic Marin® SEA SALT) at temperature, pH and salinity values resembling the sampling site conditions (18.0 ± 1.0 ºC; 8.0 ± 0.1, 30 ± 1, respectively). Seawater was renewed daily during the first week and then every three days until the end of the acclimation period. After the three first days of acclimation, mussels were fed with Algamac protein plus (150,000 cells/animal) after each water renewal.

After acclimation organisms were distributed in different aquaria, with four aquaria (containing 3L of artificial seawater each) per treatment: control (CTL, 0 µg/L of Nd), 2.5, 5.0, 10, 20 and 40 µg/L of Nd (6 treatments). A total of 20 mussels were used per tested concentration, with 5 mussels per replicate/aquarium (total of 120 mussels in total). Concentrations of Nd identified in low to highly contaminated environments (see references above) were considered to select test treatments.

To guarantee the presence of Nd in the water medium, the stability of Nd in the seawater was evaluated running a preliminary experiment in the absence of mussels. For this, glass containers with 500 mL of artificial seawater were spiked with 2.5 and 40 µg/L of Nd (10 containers per concentration) and, during seven days (corresponding to the period between water renewals along the twenty-eight days experimental assay), aliquots of 5 mL were daily collected to assess concentrations of Nd in the water.

Results concerning the stability of Nd in seawater medium showed that, in the absence of mussels, concentrations were maintained along seven days' exposure period, with results showing that the values after exposure to 2.5 and 40 µg/L of Nd were, respectively, 3.5±0.2 and 54±4.4 µg/L of Dy. These results clearly demonstrate the stability of Nd during the seven days' exposure period, the interval used between water renewal along the experimental assay, allowing to perform a twenty-eight days exposure period with water renewal ever week.

During the experimental period (twenty-eight days), water medium was renewed weekly and exposure conditions re-established, including Nd concentrations and seawater characteristics (temperature 17 ± 1 ºC, pH 8.0± 0.1 and salinity 30 ± 1). Every week, immediately after seawater renewal, water samples were collected from each aquarium for Nd quantification, to compare nominal and real exposure concentrations. During the exposure period, organisms were fed with Algamac protein plus (150,000 cells/animal) three times per week. Mortality was also daily checked, with 100% of survival recorded during the experimental period. During the exposure period water medium in each aquarium was continuously aerated with a photoperiod of 12h light:12h dark.

At the end of the exposure period, organisms were frozen individually with liquid nitrogen and stored at -80ºC, until homogenization of each individual soft tissue using a mortar and a pestle under liquid nitrogen. Each homogenized organism was divided into aliquots (each with 0.5 g fresh weight, FW) for biomarkers analyses and Nd quantification.

2.2 Neodymium quantification in water and tissue samples

To guarantee that nominal and real concentrations were similar, Nd concentrations in water samples, collected every week from each aquaria immediately after water contamination, were quantified using inductively coupled plasma mass spectrometry (ICP-MS), on a Thermo ICP-MS X Series equipped with a Burgener nebulizer after adequate sample dilution and acification to pH <2. Water samples collected daily to evaluate the stability of Nd in seawater (in the absence of mussels), along seven days experimental period, were analysed following the same procedure.

Total Nd concentrations in *M. galloprovincialis* whole soft tissues (2 individuals per replicate, 8 individuals per condition) were also quantified by ICP-MS, after microwave assisted acid digestion. After freeze-drying, mussel samples with 100–200 mg were digested in a CEM MARS 5 microwave, firstly with 2 mL of HNO_3 (70%) at 170 °C for 15 min, followed by a second identical microwave cycle with 0.5 mL of H_2O_2 (30%). After addition of H_2O_2, the mixture was allowed to stand for 15 min so that the microwave reaction was not as violent. The obtained digests were transferred into 25 mL polyethylene vessels and the volume made up with ultrapure water.

2.3 Biochemical markers

The whole tissue of mussels was used for biomarkers determination. For each parameter, 0.5 g FW of tissue per organism was used, with 2 individuals per replicate (8 individuals per condition). For each condition, metabolic capacity (electron transport system activity, ETS), energy reserves (glycogen content, GLY; total protein content, PROT), antioxidant and biotransformation defences (activities of superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx; glutathione S-transferases, GSTs), cellular damage (lipid peroxidation levels, LPO) and redox balance (ratio between

reduced glutathione and oxidized glutathione, GSH/GSSG) markers were assessed. Each sample was performed at least in duplicate, i.e., from each 0.5 g samples two sub-samples were measured for each biomarker to guarantee the quality of the data. All measurements were done using a microplate reader (BioTek, Synergy HT). The extraction for each biomarker was performed with specific buffers: phosphate buffer for SOD, CAT, GSTs, PROT and GLY; magnesium sulphate buffer for ETS; trichloroacetic acid buffer for LPO and KPE buffer for GSH/GSSG. Each sample was sonicated for 15 s at 4 ºC and centrifuged for 25 min (or 15 min for GSH/GSSG) at 10,000 g (or 3,000 g for ETS) (Andrade et al., 2019; Coppola et al., 2019; De Marchi et al., 2018; Freitas et al., 2019). Supernatants were stored at -80 ºC.

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). Absorbance was measured during 10 min at 490 nm with intervals of 25 s and the extinction coefficient ε = 15,900 M^{-1} cm^{-1} was used to calculate the amount of formazan formed. Results were expressed in nmol per min per g of FW.

For GLY quantification the sulphuric acid method was used, as described by Dubois et al. (1956). Glucose standards were used (0–10 mg/ mL) to produce a calibration curve. Absorbance was measured at 492 nm after incubation during 30 min at room temperature. Results were expressed in mg per g FW.

The PROT content was determined according to the spectrophotometric Biuret method (Robinson and Hogden, 1940). Bovine serum albumin (BSA) was used as standard calibration curve (0–40 mg/mL). Absorbance was read at 540 nm. The results were expressed in mg per g FW.

226

227 *Antioxidant defences*

228 SOD activity was determined by the Beauchamp and Fridovich (1971) method
229 after adaptations performed by Carregosa et al. (2014). The standard curve was formed
230 using SOD standards (0.25-60 U/mL). Samples' absorbance was read at 560 nm after 20
231 min of incubation at room temperature. Results were expressed in U per g FW where
232 one unit (U) represents the quantity of the enzyme that catalyzes the conversion of 1
233 μmol of substrate per min.

234 CAT activity was quantified according to the Johansson and Borg (1988) method
235 and the modifications performed by Carregosa et al. (2014). The standard curve was
236 determined using formaldehyde standards (0–150 μmol/L). Absorbance was measured
237 at 540 nm. The enzymatic activity was expressed in U per g of FW, where U represents
238 the amount of enzyme that caused the formation of 1.0 nmol formaldehyde per min at
239 25 °C.

240 GPx activity was quantified following Paglia and Valentine (1967). The
241 absorbance was measured at 340 nm in 10 sec intervals during 5 min and the enzymatic
242 activity was determined using the extinction coefficient $\varepsilon = 6.22\ mM^{-1}cm^{-1}$. The results
243 were expressed as U per g FW, where U represents the amount of enzyme that caused
244 the formation of 1.0 μmol NADPH oxidized per min.

245

246 *Biotransformation defences*

247 GSTs activity was quantified following Habig et al. (1974) protocol with some
248 adaptations performed by Carregosa et al. (2014). The absorbance was measured at
249 340nm and the activity of GSTs was determined using the extinction coefficient $\varepsilon = 9.6$
250 $mM^{-1}cm^{-1}$. The enzymatic activity was expressed in U per g of FW where U is defined

as the amount of enzyme that catalysis the formation of 1 μmol of dinitrophenyl thioether per min.

Cellular damage

LPO determination was done following the method described by Ohkawa et al. (1979). LPO levels were measured trough the quantification of malondialdehyde (MDA), a by-product of lipid peroxidation. Absorbance was measured at 535 nm and the extinction coefficient ε = 156 mM^{-1} cm^{-1} was used to calculate LPO levels, expressed in nmol of MDA formed per g of FW.

Redox balance

GSH and GSSG glutathione contents were measured at 412 nm (Rahman et al., 2014) and used as standards (0–60 μmol/L) to obtain a calibration curve. Absorbance was measured at 412 nm, for both assays. The results were expressed as nmol per g of FW. The ratio GSH/GSSG was determined taking in account the number of thiol equivalents (GSH / 2*GSSG).

2.5 Data analyses

Bioaccumulation factor (BCF) was calculated dividing the mean Nd concentration found in mussel's tissues at the end of the experimental period by the mean value of Nd found in seawater immediately after spiking (corresponding to the real exposure concentration).

All the biochemical results (ETS, GLY, PROT, SOD, CAT, GPx, GSTs, LPO and GSH/GSSG) and Nd concentrations in mussel's tissues, obtained from each tested treatment, were submitted to 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 and when significant (p<0.05) differences were observed pairwise comparisons were performed among conditions. Significant differences were identified in the figures with different lowercase letters.

3. RESULTS AND DISCUSSION

With the technological advances and economic development, the multiplicity and wide variety of applications of electrical and electronic equipment have extremely increased over the last decades. Consequently, the quantity of end-of-life products is also growing, resulting into increasing amounts of hazardous substances, including REEs. As a consequence, different authors have reported the presence of these elements in aquatic systems and inhabiting organisms (Akagi and Edanami, 2017; Adeel et al., 2019; Rim, 2016; Rim et al., 2013). Considering this, recent studies have evaluated the impacts of REE in different aquatic species, trying to identify harmful effects caused by these substances (Oral et al., 2010; Adeel et al., 2019; Henriques et al., 2019; Pinto et al., 2019; Rim, 2016; Rim et al., 2013).

The present study aimed to evaluate the accumulation and effects of Nd exposure in the mussel species *Mytilus galloprovincialis*, after a chronic exposure period.

3.1 Accumulation of Neodymium in mussel's tissues

In terms of accumulation, the results obtained showed that when exposed to environmentally relevant concentrations, Nd levels in mussel's tissues increased along the increasing exposure gradient, with significant differences among all tested conditions (Table 1). However, bioconcentration factor (BCF) showed similar values among tested conditions, indicating the efforts of mussels to prevent Nd accumulation with the increasing exposure concentrations (Table 1). The present results are in accordance with previous studies, conducted under laboratory conditions, which revealed similar responses, with accumulation of different REE by marine mussels (*M. galloprovincialis*, Henriques et al., 2019; Pinto et al., 2019), freshwater mussels (*Dreissena polymorpha*, Hanana et al., 2018), and freshwater clams (*Corbicula*

fluminea, Bonnail et al., 2017). Such findings highlight the capacity of bivalves to accumulate REE, which may impair their physiological and biochemical performances. Nevertheless, the present study further revealed the capacity of mussels to limit the accumulation of Nd, as similar accumulation rate was observed in all tested treatments, regardless the concentration of exposure. These results may indicate that, along the increasing exposure gradient, mussels were able to limit the Nd accumulation by reducing filtration and respiration capacity and/or were able to increase the detoxification of this element. Since, higher metabolic capacity was observed in contaminated mussels and no differences were observed among these treatments (except for the lowest Nd concentration), the results obtained may indicate that respiration and filtration rates were not decreased under the exposure of Nd. Therefore, the efforts of mussels to limit Nd accumulation may result from mussel's detoxification capacity. Similarly, Oliveira et al. (2017) demonstrated that *M. galloprovincialis* decreased bioconcentration factor (BCF) along the increasing exposure gradient of carbamazepine (CBZ). It was already showed that in the presence of pollutants bivalves may limit their filtration rate to avoid their accumulation, an effort that may increase with increasing exposure concentration (Almeida et al., 2015; Chen et al., 2014). In particular, Chen et al. (2014) reported a decrease in the filtration rate of the clam *Corbicula fluminea* after exposure to CBZ by comparison with non-contaminated clams. Also Almeida et al. (2015) observed lower BCF values at the highest CBZ exposure concentration in *R. philippinarum*.

3.2 Metabolic capacity and energy reserves

Except for the lowest tested concentration (2.5 µg/L), mussels exposed to Nd significantly increased their metabolic capacity compared to non-contaminated

organisms under control condition (Figure 1A). However, except for the lowest Nd concentration, higher electron transport system (ETS) values compared to control organisms did not vary among tested treatments, which may explain similar BCF values in concentrations higher than 2.5 µg/L. As mentioned above, as a result of similar metabolic capacity among different exposure concentrations, mussels may have presented similar filtration rates which led to a similar accumulation rate among different tested conditions. It was already demonstrated that ETS activity may be used as an indication of metabolic activity in marine macrofauna (Cammen et al., 1990), and an increase in bivalves ETS activity was already identified as a protective behavior, associated with the activation of defense mechanisms under pollution exposure, including the increase of antioxidant and biotransformation enzymes. In particular, different authors already demonstrated that marine bivalves (*Ruditapes philippinarum* and *M. galloprovincialis*) increased their metabolic capacity, measured by ETS activity, when in the presence of nanoparticles (multi-walled carbon nanotubes) and drugs (salicylic acid) (De Marchi et al., 2018; Freitas et al., 2019). Nevertheless, several other studies addressing the metabolic capacity of bivalves under pollution stress evidenced a decrease on ETS activity, which was associated to a decrease in the filtration rate to prevent pollutants accumulation (among others, Almeida et al., 2015; Oliveira et al., 2017). Also, a previous study with other REE (Gadolinium, Gd) but testing a similar concentration range (between 15 and 60 µg/L) and the same exposure period (twenty-eight days), showed that the ETS activity in *M. galloprovincialis* decreased significantly after the experimental period (Henriques et al., 2019). Such findings may indicate higher toxicity of Nd in comparison to other pollutants, as mussels under higher stress conditions may increase their metabolic capacity to fight against the stressful condition. Therefore, considering the results obtained and previous studies with bivalves exposed

to pollutants we can hypothesize that up to certain stress levels bivalves are able to decrease their metabolism for short periods of time, to avoid accumulation of pollutants and reduce their toxic impacts. This strategy can seriously affect bivalves physiological and biochemical performance, being tolerable for a limited period of time. Therefore, it seems that at higher stressful conditions this strategy is no longer valid and organisms increase their ETS activity to activate defense mechanisms, which results into higher production of reactive oxygen species (ROS) by mitochondrial electron transport system, with negative impacts on organism's cellular oxidative status.

As a consequence of higher metabolic activity, the results obtained showed that associated with higher ETS activity contaminated organisms presented significantly lower glycogen (GLY) content in comparison to control mussels (Figure 1B). These findings evidence the need of mussels to use their energy reserves to fuel up defense mechanisms. Previous studies conducted by Lagadic et al. (1994) already suggested that energy reserves could be considered as biomarkers reflecting sublethal changes from a stressful xenobiotic exposure. Also Pellerin-Massicotte et al. (1994) highlighted that GLY reserves may be depleted in the presence of contaminants. Other authors identified the possible use of GLY for the synthesis of lipids and/or proteins for gametogenesis (Bayne et al., 1975; Parra et al., 2005). In the present study we may hypothesize that GLY reserves were used in the activation of defense mechanisms. A similar response was also observed by other authors, assessing the effects carbon nanotubes in the clam *R. philippinarum* (De Marchi et al., 2018).

The results obtained further demonstrated that although the GLY content decreased in contaminated mussels compared to control ones, the protein (PROT)

content was maintained regardless the Nd concentration of exposure, with no significant differences among tested conditions (Figure 1C). Such results demonstrated that mussels were neither using PROT as energy reserves to fuel up defense mechanisms nor increasing the production of enzymes to fight against the stress caused by Nd. Nevertheless, previous studies with REE showed that *M. galloprovincilais* were able to increase the PROT content in the presence of an increasing concentration gradient of Gd and Lanthanum (La) (Henriques et al., 2019; Pinto et al., 2019), which could be related to the capacity of mussels to increase production of enzymes to fight against the stress induced, indicating also that in this case mussels were experiencing a mild stress condition with no need to use PROT as energy source while being able to enhance the production of enzymes.

Overall, the results obtained evidenced that under Nd exposure mussels increased their metabolic capacity, probably to fuel up defense mechanisms (namely antioxidant enzymes activity), which was accompanied by expenditure of GLY reserves but not a decrease in PROT content. It was already described that up to certain stress levels stored GLY is the first source of energy used, while energy stored in lipid and PROT being used at higher stress levels (Sonawane and Sonawane, 2018).

3.3 Antioxidant defenses

In terms of superoxide dismutase (SOD) activity, the results obtained showed no significant differences among conditions except for the lowest Nd concentration where the activity of this enzyme was significantly higher compared to the remaining conditions (Figure 2A). In the case of glutathione peroxidase (GPx), at the highest exposure concentration no significant differences were observed to control organisms,

while at the remaining exposure concentrations significantly lower activity was recorded compared to control and the highest concentration (Figure 2B). On the contrary, mussels exposed to Nd tended to increase their catalase (CAT) activity, which was significantly higher at concentrations 5.0, 10 and 40 µg/L compared to the remaining conditions (Figure 2C). It is well known that when in the presence of a stressful condition, including the presence of pollutants, organisms may increase the production of ROS. To avoid damages caused by ROS (including lipid peroxidation, protein carbonilation and DNA damage), organisms may increase the activity of antioxidant enzymes. Among these enzymes are SOD, GPx and CAT that have the capacity to eliminate ROS (namely, superoxide anion, hydroxyl radical, and hydrogen peroxide), preventing organisms from cellular damages. Nevertheless, this response normally occurs when oxidative stress is not very high or very long-during. On the other hand, when exposed to extremely high stressful conditions or if the stress is persisting, the proteins damage became profound and a decrease of these enzymes activity may occur (either via direct oxidative damage of the enzymes molecules, or via oxidative stress-altered enzymes gene expression, or both). Among others, Manduzio et al. (2004) hypothesized that the over production of ROS inhibited the SOD activity in *Mytilus edulis* collected from a polluted area. Studies conducted by Matozzo et al. (2001) highlighted that the significant inhibition of SOD activity in Cu-exposed *R. philippinarum* clams observed might be due to the oxidation of the enzyme SH groups mediated by ROS, which production is increased by Cu (Halliwell and Gutteridge, 1984). In accordance to this, the present study may indicate that mussels were exposed to high toxic conditions since the results obtained showed that *M. galloprovincialis* exposed to Nd were not able to significantly increase their antioxidant defenses, namely in terms of SOD and GPx activity, which was especially noticed at higher exposure

concentrations. This behaviour may have limited mussel's capacity to eliminate the excess of ROS generated by the presence of this element. Nevertheless, one of the three antioxidant enzymes analyzed seemed to be sensitive to some of the treatments, which can suggest not only a complex mode of action of this element but also that not all the mechanisms involved in the onset of oxidative stress due to Nd have been investigated. Furthermore, the results obtained may also evidence that increased metabolic capacity was not enough to significantly activate antioxidant enzymes and increased ETS activity also contributed to the generation of higher ROS amount. Previous studies also showed that in the presence of Gd *M. galloprovincialis* presented limited capacity to activate their antioxidant enzymes, but in this case only at higher exposure concentrations (60 and 120 µg/L) mussels were not able to continue to increase antioxidant enzymes activity (Henriques et al., 2019). Such results can, once again, corroborate the hypothesis that Nd may be more toxic than Gd towards *M. galloprovincialis*, exposed to a similar concentration range.

3.4 Biotransformation defenses

Concerning biotransformation capacity, mussels exposed to lower concentrations (2.5, 5.0 and 10 µg/L) tended to increase glutathione S-transferases (GSTs) activity, with significantly higher values at 5.0 and 10 µg/L compared to the remaining conditions (Figure 3). As for the antioxidant enzymes, mussels were not able to increase the activity of biotransformation enzymes along the increasing exposure concentration, showing limited capacity to increase GSTs activity at higher Nd concentrations (20 and 40 µg/L). GSTs are a superfamily of Phase II detoxification enzymes involved in the detoxification of ROS and toxic xenobiotics. These enzymes are able to catalyze the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for the

458 purpose of detoxification and, therefore, in the presence of pollutants, GSTs activity is
459 induced to achieve efficient cell protection. Thus, from the results obtained, it is
460 possible to identify mussel's efforts to detoxify cells from Nd under intermediate
461 concentrations, while exposure to the highest concentrations (20 and 40 µg/L) mussels
462 were no longer able to continue to activate this defense mechanism. The decrease of
463 GSTs activity at higher concentrations may be related to diminished levels of GSH
464 susceptible of being conjugated. A similar response was observed when *M.*
465 *galloprovincialis* mussels were exposed to La and Gd, with higher activity at lower
466 concentrations and lower activity at higher concentrations (Henriques et al., 2019; Pinto
467 et al., 2019). Therefore, the results obtained in the present study as well as in previous
468 studies showed the capacity of GSTs to detoxify REE, with greater detoxification
469 capacity at lower exposure concentrations.
470
471 Overall, in what regards to defense mechanisms, the results obtained indicate high
472 toxicity of Nd, especially at higher concentrations, which may have resulted into i)
473 increase of SOD activity only at the lowest tested concentration, with inactivation of
474 this enzyme at higher concentrations; ii) inhibition of GPx in contaminated mussels; iii)
475 decrease of GSTs at higher exposure concentrations; iv) only the activation of CAT in
476 contaminated organisms.
477

478 ## 3.4 Cellular damage

479 Levels of lipid peroxidation (LPO) significantly increased in organisms exposed
480 to Nd in comparison to control organisms, with no significant differences among 5.0,
481 10, 20 and 40 µg/L exposure concentrations (Figure 4A). These results may result from
482 the fact that mussels were not able to efficiently activate antioxidant enzymes, resulting

into cellular damages in the presence of Nd. Previous studies already demonstrated that the presence of pollutants results into an overproduction of ROS that, if not eliminated by antioxidant enzymes, can react with lipids of the cellular membrane, causing lipid peroxidation that corresponds the oxidative degradation of lipids (see for example, Regoli and Giuliani, 2014). Although a high diversity of studies already showed that the presence of metals, nanoparticles and drugs may originate increased LPO levels in bivalves even if antioxidant enzymes were activated (see for example, Freitas et al., 2019b, 2019a; McCarthy et al., 2013; Monteiro et al., 2019; Vlahogianni and Valavanidis, 2007), less studies demonstrated the occurrence of LPO when bivalves are exposed REE. In particular, Henriques et al. (2019) demonstrated that when *M. galloprovincialis* were exposed to Gd LPO significantly increased in comparison to control values, although antioxidant enzymes were increased, especially at intermediate concentrations (30 and 60 µg/L). Hanana et al. (2017) revealed a significant increase of LPO in the freshwater mussel *Dreissena polymorpha* after 28 days of exposure but only when exposed to the highest concentration of La (1250 µg/L), with no significant differences evidenced among the other tested concentrations (10, 50, 250 µg/L). With other aquatic invertebrates, it was also demonstrated the capacity of REE to enhance the production of ROS, such was in the freshwater crustacean *Daphnia magna* exposed to Cerium and Erbium. LPO levels also increased in the sea urchin *Paracentrotus lividus* larvae exposed to Dysprosium (Oral et al., 2017). Also Wang et al. (2011) showed that the significant increasing activities of antioxidant enzyme observed in the freshwater cyanobacteria *Microcystis aeruginosa* may result from overproduction of ROS due to the exposure to Nd concentrations. Altogether, these findings clearly demonstrate the capacity of REE to induce cellular damages.

3.5 Redox balance

The ratio between reduced (GSH) and oxidized (GSSG) glutathione was significantly lower in mussels exposed to Nd in comparison to non-exposed ones, with no significant differences among contaminated mussels (Figure 4B). Such results clearly reveal that GSH content decreased while GSSG increased in contaminated mussels, indicating loss of redox homeostasis in organisms exposed to Nd and the high demand for GSTs. To eliminate the excess of ROS generated by a stress condition, besides antioxidant enzymatic defenses, organisms further present low molecular scavengers that are also to neutralize ROS by direct reaction with them, being GSH the most abundant. In its reduced form, GSH, glutathione is capable of scavenging reactive oxygen and nitrogen species, thereby contributing to the control of redox homoeostasis. Therefore, the glutathione system acts as the major redox buffer in the majority of cells (Couto et al., 2016). In the presence of ROS, GSH can be oxidized into GSSG and, thus, under stressful conditions the ratio GSH/GSSG tends to decrease as a result of GSSG increase. Organisms which use glutathione for redox homoeostasis are able to synthesize reduced glutathione, but they are also characterized by their ability to recycle glutathione. Glutathione reductase (GRed) is an essential enzyme that recycles oxidised glutathione back to the reduced form (Couto et al., 2016). Thus, the increased content of GSSG observed by lower GSH/GSSG levels in contaminated mussels indicates that GRed failed to oxidize glutathione into its reduced form. The ratio GSH/GSSG is often used to assess the oxidative stress of organisms exposed to pollutants (e.g. Peña-Llopis et al., 2002; Almeida et al., 2015; Sellami et al., 2015; Freitas et al., 2018; 2019). Similarly, to the present results, recent studies also demonstrated that in the presence of REEs mussels significantly decreased in mussels exposed to La and Gd (Henriques et al., 2019; Pinto et al., 2019).

4. CONCLUSIONS

The present findings revealed high toxicity of Nd towards *M. galloprovincialis*, which showed low capacity to prevent injuries caused by this REE. After exposure, mussels accumulated Nd with higher concentrations at higher exposure levels. Accumulation of Nd revealed to be costly to mussels, which revealed higher metabolic activity and increased expenditure of GLY content when in the presence of this element. Also, after exposure to Nd, mussels showed inefficient antioxidant and biotransformation strategies, leading to cellular damage and loss of redox balance provoked by the excess of ROS, namely as a result of higher electron transport system activity. Considering that tested concentrations resemble low to highly polluted areas, the results here presented highlight the hazardous capacity of Nd towards *M. galloprovincialis*. Toxic effects observed at individual level may result into negative impacts to mussel's population as changes observed at cellular level may result into impairments on organism's survival, growth, abundance and reproduction capacity.

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FIGURE CAPTIONS

Figure 1. A: Electron transport system activity (ETS), B: Glycogen content (GLY); and C: Protein content (PROT), in *Mytilus galloprovincialis* exposed to different Neodymium concentrations (CTL-0, 2.5, 5.0, 10, 20 and 40 µg/L of Nd). Values are mean + standard deviation. Significant differences among concentrations are represented with different letters.

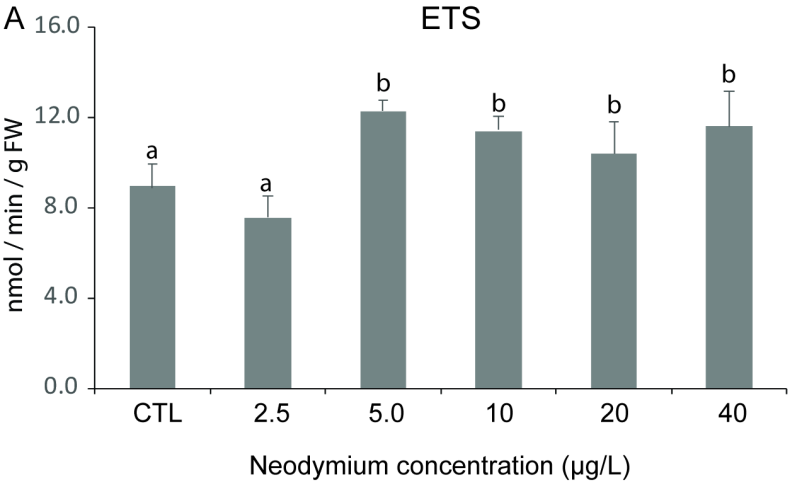
Figure 2. A: Superoxide dismutase activity (SOD); B: Glutathione peroxidase activity (GPx); and C: Catalase activity (CAT), in *Mytilus galloprovincialis* exposed to different Neodymium concentrations (CTL-0, 2.5, 5.0, 10, 20 and 40 µg/L of Nd). Values are mean + standard deviation. Significant differences among concentrations are represented with different letters.

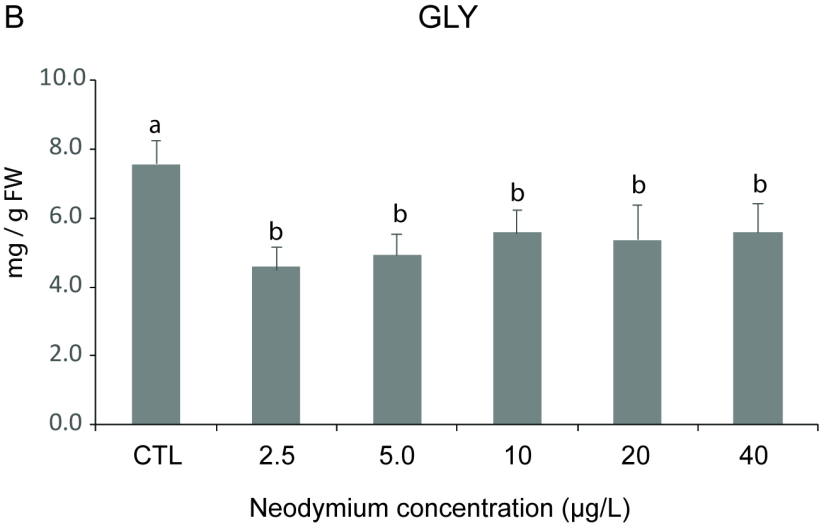
Figure 3. Glutathione S-transferases activity (GSTs), in *Mytilus galloprovincialis* exposed to different Neodymium concentrations (CTL-0, 2.5, 5.0, 10, 20 and 40 µg/L of Nd). Values are mean + standard deviation. Significant differences among concentrations are represented with different letters.

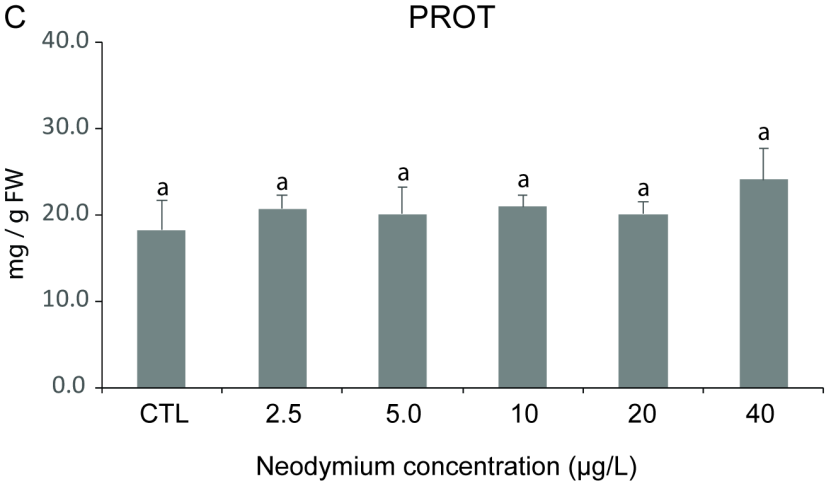
Figure 4. Lipid peroxidation levels (LPO); and B: Ratio between reduced (GSH) and oxidized (GSSG) glutathione (GSH/GSSG), in *Mytilus galloprovincialis* exposed to different Neodymium concentrations (CTL-0, 2.5, 5.0, 10, 20 and 40 µg/L of Nd). Values are mean + standard deviation. Significant differences among concentrations are represented with different letters.

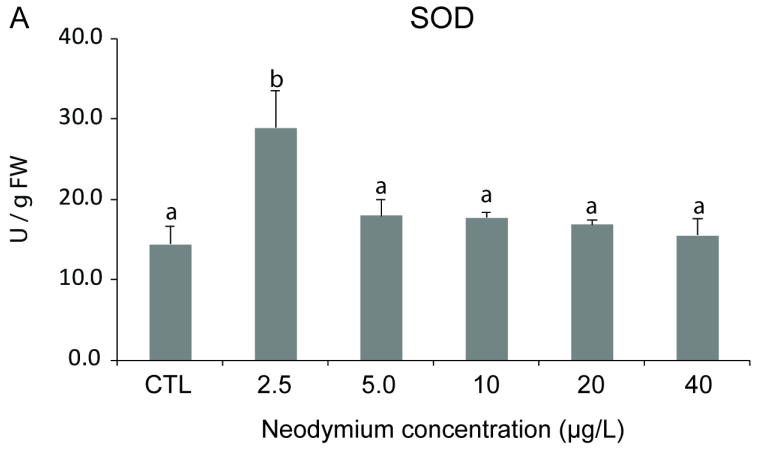
Table 1- Neodymium (Nd) mean concentrations, in water (µg/L), collected immediately after spiking at the 1st, 2nd, 3rd and 4th weeks of exposure, and in mussels tissues (µg/g dry weight) at the end of the experimental period (28 days) from each condition (0-control, 2.5, 5.0, 10, 20, 40 µg/L of Nd). Different letters among exposure concentrations denote statistical significance. LOQ for water samples 10 ng/L; LOQ for tissue samples 0.0025 µg/g. Bioconcentration factor (BCF) corresponds to the concentration of Nd in mussel's tissues divided by the mean values for the real exposure concentration during the four weeks of exposure.

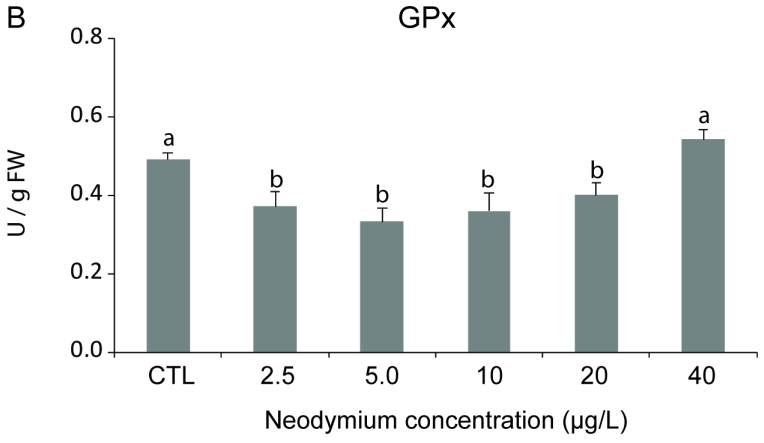
Nd concentrations (µg/L)	Water	Mussels tissues (µg/g)	BCF
	During the four weeks	In the 4th week	
CTL	<LOQ	0.095±0.006	---
2.5	2.6±0.32	0.136±0.016[a]	0.05
5.0	5.3±0.30	0.26±0.013[b]	0.05
10	10±0.3	0.435±0.003[c]	0.04
20	22±0.8	0.982±0.008[d]	0.05
40	43±3.0	1.72±0.03[e]	0.04

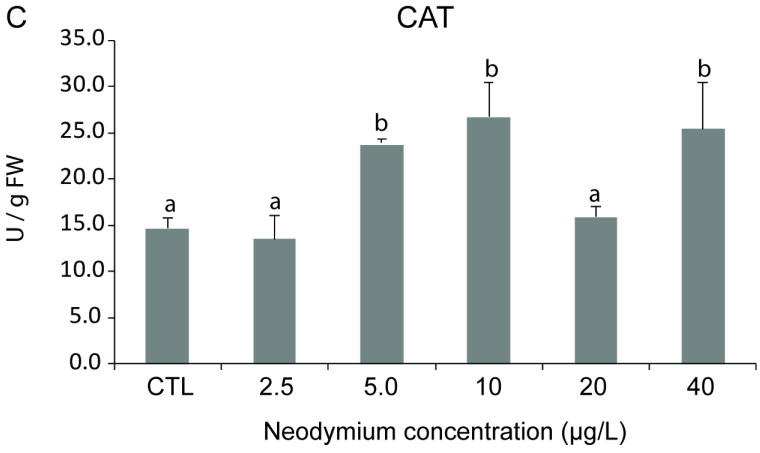

A
ETS
16.0
12.0
8.0
4.0
0.0
nmol / min / g FW
a
a
b
b
b
b
CTL
2.5
5.0
10
20
40
Neodymium concentration (µg/L)

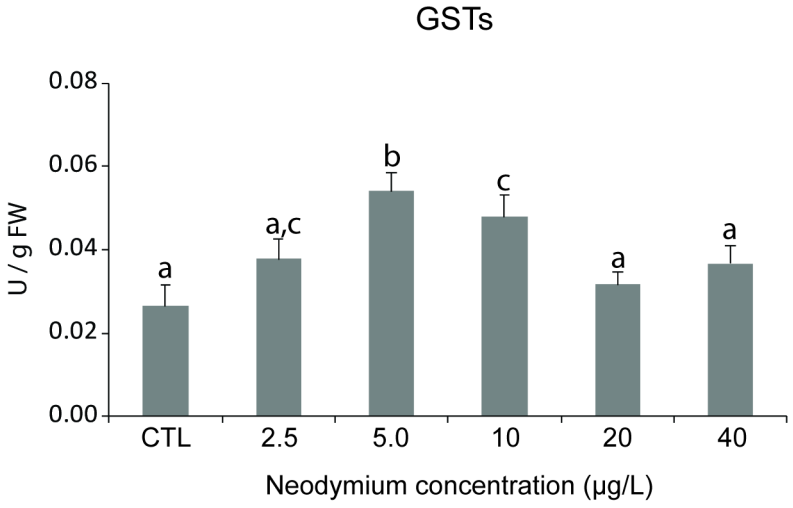

B
GLY
10.0
8.0
6.0
4.0
2.0
0.0
mg / g FW
a
b
b
b
b
b
CTL
2.5
5.0
10
20
40
Neodymium concentration (µg/L)

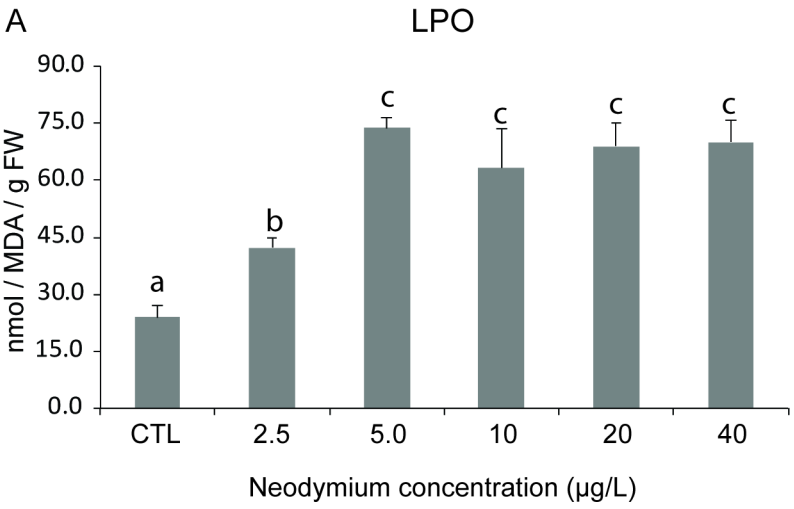

C
PROT
40.0
30.0
20.0
10.0
0.0
mg / g FW
a
a
a
a
a
a
CTL
2.5
5.0
10
20
40
Neodymium concentration (µg/L)

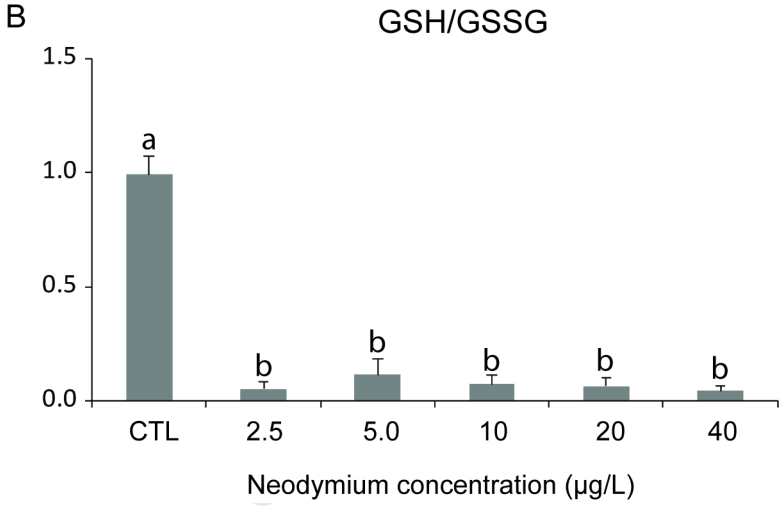

A
SOD
U / g FW
40.0
30.0
20.0
10.0
0.0
a
b
a
a
a
a
CTL
2.5
5.0
10
20
40
Neodymium concentration (µg/L)


B
GPx
U / g FW
0.8
0.6
0.4
0.2
0.0
a
b
b
b
b
a
CTL
2.5
5.0
10
20
40
Neodymium concentration (µg/L)


C
CAT
U / g FW
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
a
a
b
b
a
b
CTL
2.5
5.0
10
20
40
Neodymium concentration (µg/L)




A
LPO
nmol / MDA / g FW
90.0
75.0
60.0
45.0
30.0
15.0
0.0
a
b
c
c
c
c
CTL
2.5
5.0
10
20
40
Neodymium concentration (µg/L)


B
GSH/GSSG
1.5
1.0
0.5
0.0
a
b
b
b
b
b
CTL
2.5
5.0
10
20
40
Neodymium concentration (µg/L)

- *Mytilus galloprovincialis* bioaccumulated Neodymium (Nd)
- Mussels exposed to Nd increased their metabolic capacity, with glycogen expenditure
- Limited antioxidant and biotransformation capacity in contaminated mussels
- Lipid peroxidation occurred in Nd contaminated mussels
- Loss of redox balance in mussels exposed to Nd

Rosa Freitas and Eduarda Pereira are supervisors of the students that co-authored this ms(Silvana Costa, Celso Cardoso, Tiago Morais, Pedro Moleiro, Ana C. Matias, Ana F. Pereira, Joana Machado, Beatriz Correia, Diana Pinheiro, Adriana Rodrigues, João Colónia). Students did the exposure assay (for 28 days under controlled conditions), performed all methods and analyses for Nd quantification and biomarkers determination.

Rosa Freitas and Eduarda Pereira gave the idea of this study to the students that accepted this challenge and performed all the analyses during their last year of their bachelor degree. Eduarda Pereira is the responsible for the laboratory where Nd quantification was done. Rosa Freitas and Amadeu Soares are the responsible persons for the labs where biomarkers were determined. Eduarda Pereira, Rosa Freitas and Amadeu Soares funded this study.

Conflict of Interest

The Authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affi liations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.