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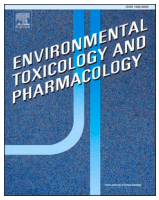

Can temperature influence the impacts induced in *Mytilus galloprovincialis* by neodymium? Comparison between exposure and recovery periods

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ABSTRACT

Climate change-associated factors and pollutants, such as rare earth elements (REEs), have been identified as contributors to environmental changes. However, the toxicity resulting from the combination of these stressors has received little attention. Neodymium (Nd) is a REE that has been widely used, and this study aimed to evaluate the responses of *Mytilus galloprovincialis* to Nd exposure (10 μg/L), under actual (17 °C) and predicted warming conditions (21 °C), after fourteen days of exposure followed by fourteen days of recovery (without Nd), analyzing Nd accumulation, histopathological and biochemical alterations. The results showed that increased temperature and Nd exposure caused histopathological injuries in the gills. Contaminated mussels at 17 °C showed cellular damage, while at 21 °C, mussels were able to avoid cellular damage. After the recovery period, no improvements in gill's *status* were found and cellular damage was still present, highlighting the impacts caused by previous exposure to Nd.

1. Introduction

Accompanying the population growth as well as the development of green and sustainable products by technological industries, the demand for rare earth elements (REEs) has also been increasing (USEPA, 2012). Rare earth elements are a group of emerging contaminants that include 15 lanthanides, scandium, and yttrium (Hirano and Suzuki, 1996; USEPA, 2012). Neodymium (Nd), a lanthanide, is included in the five most critical REEs until 2025 (U.S. Departament of Energy, 2011), considering the high risk associated with its supply and its importance in clean-energy technologies. Due to its highly valued magnetism and coercivity, Nd is useful in many emerging technological fields (Yao et al., 2021), such as the production of neodymium-iron-boron (NdFeB) magnets, that are widely used in small and large motors and generators (Charalampides et al., 2015), and it is also applied in magnetic resonance imaging and X-ray imaging (Rim et al., 2013). As a consequence of their wide use in the last decades, increasing amounts of REEs were found in aquatic systems (MacMillan et al., 2019; Merschel and Bau, 2015). In aquatic environments, as in the Berkeley Pit Lake (Montana, USA), the mean Nd concentration found was 495 μg/L (Gammons et al., 2003) while in the ex-mining pit lake in Malaysia was 10.8 μg/L (Adeel et al., 2019). The maximum Nd concentration recorded in the Terengganu River Basin (Malaysia) was 6.68 μg/L (Sultan and Shazili, 2009), while in an alluvial aquifer of the Guadiamar River (south-western Spain) the Nd concentration found ranged between 0.01 μg/L and 52.67 μg/L (Olías et al., 2005). He et al. (2010) recorded 0.095 μg/L of Nd in a section of the Yellow River (China) and Sneller et al. (2000) found concentrations between 0.0176 and 0.1466 μg/L in the Rhine estuary (North Sea). As REEs are poorly soluble, and only a fraction is available to enter the organisms (MacMillan et al., 2019), they have been neglected as xenobiotics until recent years (Rim, 2016). However, with the anthropogenic REEs enrichment, past studies showed an increase in the bioaccumulation of REEs in several species (e.g. Bustamante and Miramand, 2005; Censi et al., 2013). Still, information regarding the toxic effects of REEs in aquatic organisms is scarce (Pagano et al., 2015). In this regard, Freitas et al. (2020) demonstrated that Nd increased mussel's (*Mytilus galloprovincialis*) metabolic capacity to fuel up the defense mechanisms, decreasing their energy reserve

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content. However, the defenses were not enough to avoid cellular damage and loss of redox balance. In another study, Wang et al. (2011) assessed the effects of Nd on the cyanobacteria *Microcystis aeruginosa*. The results demonstrated that the lowest concentration stimulated the growth and increased the activity of antioxidant defenses, while the highest concentrations caused the decrease of catalase consequently leading to the increase of lipid peroxidation levels and to the inhibition of the growth.

The effects of pollutants could be amplified when in combination with another environmental stressor, such as ocean warming (Sokolova and Lannig, 2008). The ocean surface temperature increased by 0.88 °C between the periods 1850–1900 and 2011–2020 (IPCC, 2021). Furthermore, it is predicted that by the end of the 21st century the global surface temperature will increase between 1.4 °C (SSP1–1.9) and 4.4 °C (SSP5–8.5) compared to the values found in 1850–1900 (IPCC, 2021). It is also predicted that sea surface temperature increase will have a moderate to high risk of impacts on marine wildlife, while for bivalves the impacts are expected to be high to very high (IPCC, 2018), including alterations in bivalve's oxidative stress (Velez et al., 2017), metabolism (Anestis et al., 2007), growth and reproduction (Verdelhos et al., 2011). Furthermore, the seawater temperature rise may not only impact bivalves but can also change the behavior and effects of pollutants altering bioaccumulation and toxicity patterns and organism's responses (Banni et al., 2014; Boukadida et al., 2016; Leite et al., 2020; Morosetti et al., 2020).

Mussels are widely used as bioindicators of water quality due to their abundance, geographical distribution, sedentary habit, and capacity to tolerate and accumulate environmental contaminants (Banni et al., 2014; Wang et al., 1996). Thus, the purpose of this study was to evaluate the biological impacts of Nd (10 μg/L) in the mussel species *M. galloprovincialis* when exposed to two temperatures (control=17 °C and a higher value=21 °C) for fourteen days followed by a recovery period in non-contaminated seawater for extra fourteen days, evaluating mussels histopathological and metabolic alterations as well as oxidative *status*. Although recent studies already revealed the impacts of Nd in *M. galloprovincialis* after long-term exposure (Freitas et al., 2020), to our knowledge no information is available on the influence of temperature rise on the toxic effects of Nd on mussels and their recovery capacity after being exposed to this element.

2. Material and methods

2.1. Experimental conditions

In November of 2019 specimens of *Mytilus galloprovincialis* (length: 65.8 ± 3.1 mm; width: 37.0 ± 2.4 mm) were collected from the Ria de Aveiro coastal lagoon (Portugal), transported to the laboratory, and placed in aquaria for depuration and acclimation to the laboratory conditions (temperature 17.0 ± 1.0 °C; salinity 30 ± 1; pH 8.0 ± 0.1; natural photoperiod) during two weeks. Artificial seawater was prepared by mixing deionized water with a commercial salt (RED SEA SALT, Éilat, Israel). The artificial seawater (salinity 30) contains calcium (355–385 mg/L), magnesium (1060–1140 mg/L), potassium (320–350 mg/L), nitrate (≤ 0.3 mg/L), as well as phosphate (≤ 0.03). The water was continuously aerated and renewed every day for the first three days, during which mussels were not fed. After that, mussels were fed with Algamac protein plus (150.000 cells/animal/day).

After the depuration/acclimation period, mussels were distributed into different aquaria with 3 L of artificial seawater each (seven mussels per aquarium and three aquaria per treatment), and to simulate different water temperatures, aquaria were placed into two different climatic rooms: 17 ± 1 °C (control temperature, similar to the mean temperature of the sampling site in November of 2019 (16 °C) (Weather Spark, 2022)) and 21 ± 1 °C (increased temperature, resembling projected values by IPCC, 2021). The tested exposure treatments were: CTL (0 μg/L of Nd) and Nd (10 μg/L), both under 17 and 21 °C. Mussels were exposed to these treatments for fourteen days. The concentration of Nd was selected according to the values found in contaminated environments (Adeel et al., 2019; Olías et al., 2005) and concentrations used in previous studies, causing effects on mussels (Freitas et al., 2020).

During the exposure period, the aquaria were continuously aerated, and the salinity, pH, and photoperiod conditions were kept constant to match those from the acclimation period. Mussels were fed with Algamac protein plus (150.000 cells/animal/day) three times a week and seawater was totally renewed on the 8th day, after which the Nd concentration was re-established. During the first two weeks of the exposure period, water samples were collected from each exposure aquaria (one aquarium from CTL and two from contaminated aquaria) and blanks (two aquaria with 10 μg/L of Nd without organisms) immediately after spiking, to obtain the real exposure concentration, and immediately before water renewal (only in blanks), to verify the Nd stability in solution after one week of spiking.

After the fourteen days of exposure, three mussels per aquarium (nine per treatment) were collected: two of them were immediately frozen in liquid nitrogen and preserved at − 80 °C for biochemical analysis and Nd quantification; the other one was used for the histopathological analyses. All the remaining mussels were maintained, for another fourteen days, in clean seawater (without Nd) to evaluate the mussel's recovery capacity. Water and food conditions were maintained to match the exposure period. Water was renewed after 7 days, with the re-establishment of the medium conditions (pH, temperature, and salinity).

At the end of the recovery period, one mussel per aquarium (three per treatment) was used for the histopathological analyses and the remaining ones were frozen with liquid nitrogen and stored at − 80 °C. Two frozen organisms per aquarium, from the exposure and recovery periods, were manually homogenized with a mortar and pestle under liquid nitrogen. Each homogenized organism was divided into five aliquots of 0.5 g fresh weight (FW) each for biochemical analyses after which the remaining tissue of one of them was used for Nd quantification.

All methods related to Nd quantification, histopathological and biochemical analyses are described in the Supplementary material. Histopathological analyses were conducted in the gills and digestive tubules of three mussels per treatment and histopathological indexes (I_h) were measured (see Supplementary material for details). Biochemical analyses were conducted in six organisms per treatment and included the quantification of electron transport system (ETS) activity, total protein (PROT) content, superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GRed), glutathione S-transferases (GSTs) activities, lipid peroxidation (LPO) and protein carbonylation (PC) levels (see Supplementary material for details).

2.2. Statistical analyses

Neodymium concentrations, histopathological indexes ($I_{h\ G}$, $I_{h\ DT}$), and biochemical markers (ETS, PROT, SOD, CAT, GRed, GSTs, LPO, PC) were submitted to a non-parametric permutational analysis of variance (PERMANOVA + add-on in PRIMER v6) (Anderson et al., 2008). Values lower than 0.05 ($p < 0.05$) were considered significantly different. When significant differences were observed in the main test pairwise comparisons were performed. The null hypotheses tested were: i) for each temperature and biological response (Nd accumulation (n = 3), histopathological (n = 36 per organ) and biochemical (n = 6) markers), no significant differences existed between control (CTL) and contaminated mussels (Nd 10 μg/L) for exposure and recovery periods. Significant differences are represented in figures with different uppercase letters for 17 °C and lowercase letters for 21 °C; ii) for each temperature and biological response (Nd accumulation, histopathological and biochemical markers), no significant differences existed between exposure and recovery for control (CTL) and contaminated mussels (Nd 10 μg/L). Significant differences are represented in figures with different

uppercase letters for 17 °C and lowercase letters for 21 °C; iii) for a given experimental period, for control (CTL) and contaminated mussels (Nd 10 μg/L) and each biological response (Nd accumulation, histopathological and biochemical markers), no significant differences existed between temperatures (17 and 21 °C). Significant differences are represented in figures with an asterisk.

3. Results

3.1. Neodymium concentration in water and mussel's soft tissues

The concentration of Nd found in water samples collected from non-contaminated treatments was below the quantification limit (< 0.4 μg/L with dilution) (Table 1). The mean concentration of Nd in water collected immediately after spiking from contaminated treatments was 13.6 ± 1.7 and 12.8 ± 1.3 μg/L at 17 °C and 21 °C, respectively. The results concerning the Nd concentration measured in artificial seawater from blanks immediately after spiking and before water renewal (Table 1) showed losses of Nd of 17 % for 17 °C and 11 % for 21 °C, although after spiking concentrations in water were slightly higher than nominal ones.

Accumulated Nd concentrations in mussels after 14 days of exposure, followed by 14 days of recovery are presented in Fig. 1. The results showed that contaminated mussels (identified as Nd) under both temperatures (17 and 21 °C) presented higher Nd concentrations than non-contaminated ones (identified as CTL), regardless of the experimental period (exposure and recovery). In contaminated mussels, comparing Nd concentrations between experimental periods (exposure vs recovery), at 17 °C no significant differences were observed while at 21 °C the concentration of Nd decreased significantly after recovery in comparison to values recorded after exposure. In contaminated mussels, comparing temperatures (17 and 21 °C) after exposure, mussels maintained at 21 °C showed higher Nd concentration than mussels at 17 °C; while after recovery period an opposite pattern was observed, with higher Nd concentration in mussels maintained at 17 °C compared to mussels under warming conditions.

3.2. Histopathological parameters

The exposure of mussels to Nd and higher temperature led to an increase of damage severity in mussel's gills and digestive tubules (Figs. 2 and 3). In terms of I_h for gills, mussels after the exposure period showed significantly higher values when exposed to Nd at 17 °C compared to mussels at CTL at the same temperature (Fig. 2A), presenting an increase of enlargement of the central vessel (both ways arrows in Fig. 3) as well as loss of cilia, while at 21 °C no significant differences were observed between non-contaminated mussels and contaminated ones (Fig. 2A). Comparing temperatures, the only significant difference was observed in non-contaminated mussels after the exposure period, with significantly higher values in the gills of mussels at 21 °C than the ones at 17 °C (Fig. 2A). In this case, mussels revealed increased enlargement of the central vessel and hemocyte infiltration (one-way arrows in Fig. 3). After the recovery period, no significant differences were observed between non-contaminated mussels and contaminated ones, at both temperatures. Also, no significant differences were detected between experimental periods at both temperatures (Fig. 2A).

Regarding the I_h for mussel's digestive tubules after the Nd exposure period, no significant differences were found between non-contaminated mussels and contaminated ones at 17 °C (Fig. 2B); nevertheless, contaminated mussels showed higher atrophy ("at" in Fig. 3) and the presence of necrosis ("n" in Fig. 3). At 21 °C, contaminated mussels revealed higher I_h values (not statistically significant) than non-contaminated mussels (Fig. 2B), with an increase in atrophy and hemocyte infiltration (one-way arrows in Fig. 3). No significant differences were detected between temperatures after the exposure period (Fig. 2B). After the recovery period, significantly higher I_h was obtained in mussels previously exposed to Nd at 17 °C in comparison to mussels at CTL at the same temperature (Fig. 2B), with an increase of atrophy (Fig. 3). Mussels at 21 °C presented higher I_h values (not statistically significant) when exposed to Nd compared to the ones at CTL (Fig. 2B), with increasing lipofuscin aggregates and the presence of necrosis ("n" in Fig. 3). Comparing temperatures, significant differences were only observed in mussels at CTL after a recovery period, where mussels at 21 °C showed higher I_h values than mussels at 17 °C (Fig. 2B), with increased atrophy ("at" in Fig. 3).

Table 1
Neodymium concentration (μg/L) in the artificial seawater collected immediately after spiking from CTL, contaminated aquaria with mussels and blanks (aquaria without mussels), and before water renewal (blanks). Results are the mean with standard deviation.

Treatments		After spiking	Before water renewal
17 °C	CTL	< 0.4	–
	Nd	13.6 ± 1.7	–
	Blanks	12.7 ± 0.8	10.6 ± 1.3
21 °C	CTL	< 0.4	–
	Nd	12.8 ± 1.3	–
	Blanks	12.8 ± 1.3	11.4 ± 2.7

3.3. Biochemical parameters

3.3.1. Metabolic capacity and energy reserves content

After both experimental periods and at both temperatures, no significant differences were detected between non-contaminated mussels and Nd contaminated ones regarding the ETS activity. Also, no significant differences were observed between temperatures for each of the tested treatments. Comparing both experimental periods, overall, lower ETS values were observed in mussels after the recovery period compared to the ones collected after the exposure period, regardless of the temperature tested (Fig. 4A).

After the exposure period, significantly higher PROT content was found in contaminated mussels at 17 °C compared to non-contaminated ones at the same temperature, while no differences were observed at 21 °C. Comparing temperatures in mussels collected after exposure, no significant differences were observed between non-contaminated mussels, but significantly lower content was noticed in contaminated mussels under 21 °C in comparison to contaminated ones at 17 °C. After the recovery period, mussels previously exposed to Nd at 17 °C showed significantly lower PROT content than the non-contaminated ones at the same temperature, while no differences were observed in mussels under 21 °C. Comparing mussels at different temperatures after the recovery period, no significant differences were observed in non-contaminated mussels, while previously contaminated mussels under 21 °C revealed significantly higher PROT content than contaminated ones maintained at 17 °C. No significant differences were noticed between the experimental periods in non-contaminated mussels at both temperatures. In contaminated mussels at 17 °C, significantly lower PROT content was found in mussels collected after the recovery period compared to the ones collected after the exposure period, while no significant differences were found between experimental periods at 21 °C (Fig. 4B).

3.3.2. Oxidative stress levels

Concerning SOD activity, no significant differences were found between non-contaminated and Nd contaminated mussels after the exposure period at both temperatures, although a slight increase was observed in contaminated mussels under 21 °C. After the exposure period, no significant differences were noticed between temperatures for CTL and Nd-contaminated mussels. Similarly, after the recovery period, no significant differences were noticed between mussels at CTL and exposed to Nd at both temperatures, but a slight increase in SOD was observed in previously contaminated mussels at 17 °C compared to non-

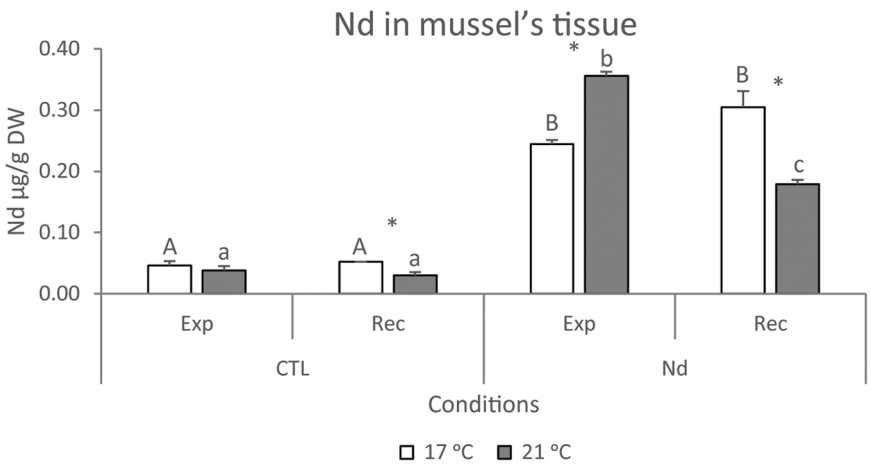
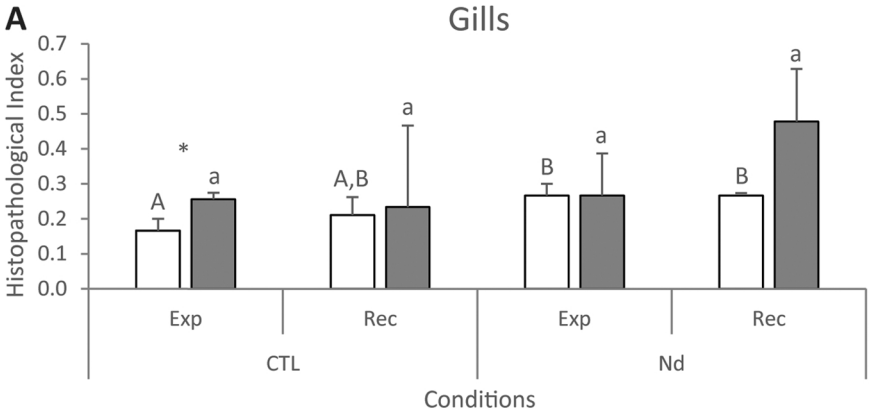


Fig. 1. Neodymium (Nd) concentrations (µg/g) in mussel's whole soft dried tissue exposed to different temperatures (17 and 21 °C) in the absence (CTL) and presence of neodymium (Nd) for 14 days (Exp) followed by 14 days or recovery period without Nd (Rec). Results are means with standard deviations. Significant differences ($p < 0.05$) among treatments and experimental periods are identified by different letters (uppercase letters for 17 °C and lowercase letters for 21 °C). Significant differences ($p < 0.05$) between the two temperatures are represented with an asterisk (n = 3).



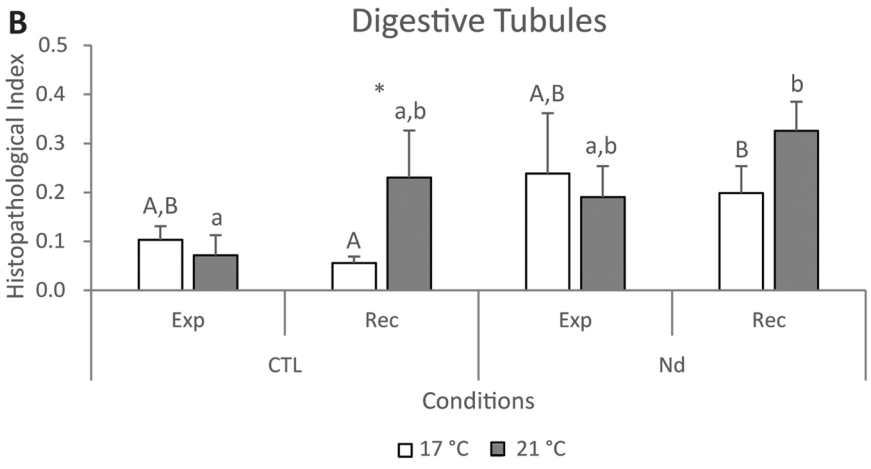


Fig. 2. A Histopathological index in gills ($I_{h\ G}$); B: Histopathological index in digestive tubules ($I_{h\ DT}$), in *Mytilus galloprovincialis* exposed to different temperatures (17 and 21 °C) in the absence (CTL) and presence of neodymium (Nd) for 14 days (Exp) followed by 14 days or recovery period without Nd (Rec). Results are means with standard deviations. Significant differences ($p < 0.05$) among treatments and experimental periods are identified by different letters (uppercase letters for 17 °C and lowercase letters for 21 °C). Significant differences ($p < 0.05$) between the two temperatures are represented with an asterisk (n = 36 per organ).

contaminated ones at the same temperature and the opposite pattern was noticed at 21 °C. After recovery CTL and Nd contaminated mussels showed no significant differences between temperatures. Comparing experimental periods, non-contaminated mussels at 17 °C showed significantly lower SOD activity after the recovery period in comparison to the ones collected after exposure, while at 21 °C no significant differences were noticed between non-contaminated mussels after both experimental periods. Mussels exposed to Nd and temperature control (17 °C) showed no significant differences between experimental periods, while at 21 °C significantly lower SOD activity was observed in mussels collected after recovery (Fig. 5A).

In terms of CAT activity, no significant differences were observed between the tested treatments both after exposure and recovery. Regarding temperatures, it was only observed a significant difference in Nd-contaminated mussels after the recovery period, with significantly higher CAT activity in mussels maintained at 21 °C in comparison to the ones at 17 °C (Fig. 5B).

After the exposure period, significantly higher GRed activity was observed in mussels exposed to Nd at 17 °C compared to non-contaminated mussels at the same temperature, while at 21 °C no significant differences were observed between contaminated mussels and non-contaminated ones. Comparing mussels at different temperatures

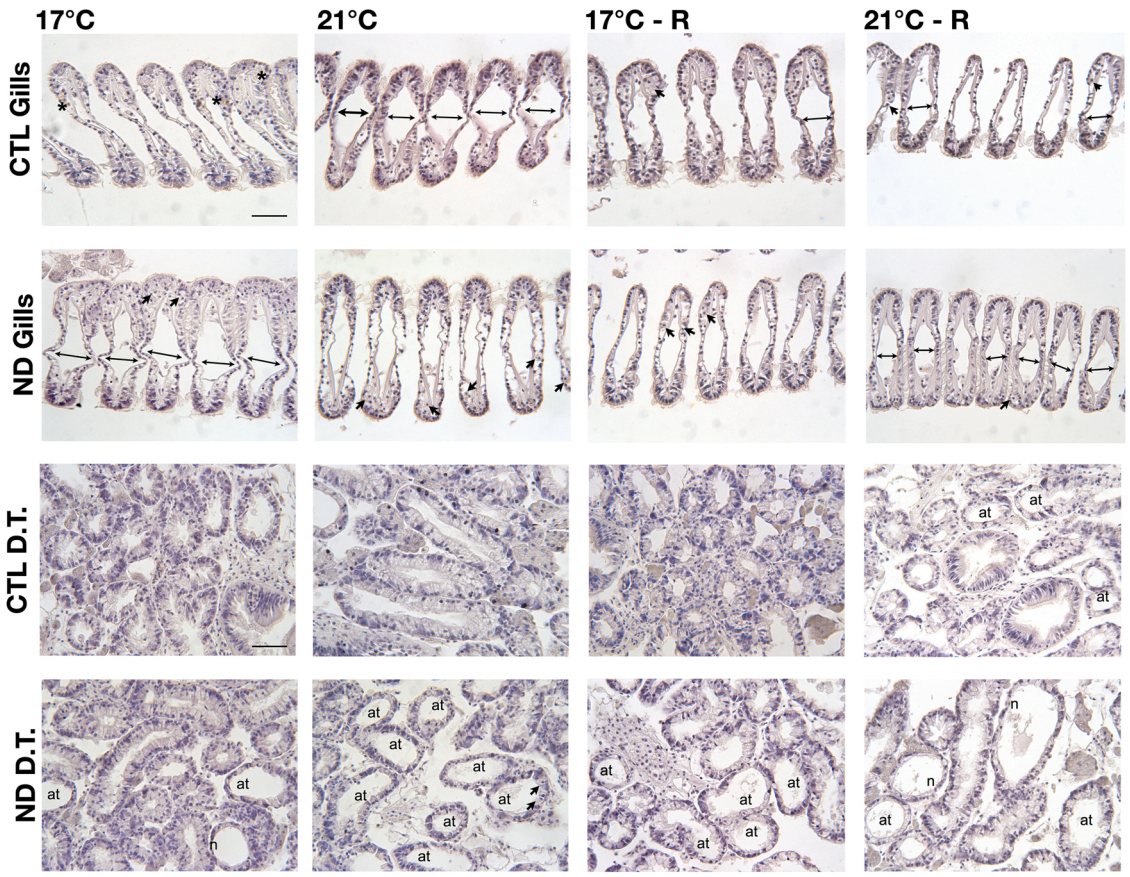


Fig. 3. Micrographs of histopathological alterations observed in different tissues of *Mytilus galloprovincialis* exposed to different temperatures (17 and 21 °C) in the absence (CTL) and presence of neodymium (Nd) for 14 days followed by 14 days or recovery period without Nd (R) stained with hematoxylin. (i) Gills: lipofuscin aggregates (*); enlargement of the central vessel (both ways arrows); hemocytes infiltration (one-way arrows); (ii) Digestive tubules: hemocytes infiltration (one-way arrows); atrophied digestive tubule (at) and necrosis (n). Scale bar 50 μm.

after the exposure period, no significant differences were observed at CTL, but a significantly lower GRed activity was found in mussels exposed to Nd under 21 °C compared to the ones at 17 °C. After the recovery period, no significant differences were observed between contaminated mussels and non-contaminated ones at both temperatures. Regarding the differences between temperatures, after the recovery period no significant differences were observed in CTL mussels, while a significantly lower GRed activity was found in mussels exposed to Nd under 21 °C compared to the ones at 17 °C. Overall, mussels collected after recovery presented lower GRed activity than the ones collected after the exposure period (Fig. 5C).

Regarding GSTs activity, no significant differences were observed between CTL and Nd contaminated mussels for a given temperature and experimental period. Comparing temperatures, significant differences were only observed in contaminated mussels after the recovery period, where mussels at 21 °C showed significantly higher GSTs activity than mussels at 17 °C. The comparison between experimental periods revealed that non-contaminated and Nd-contaminated mussels at 17 °C presented significantly lower GSTs activity after the recovery period. No significant differences were observed between experimental periods at 21 °C, both for CTL and Nd-contaminated mussels (Fig. 6).

Levels of LPO were significantly higher in contaminated mussels at 17 °C after exposure in comparison to non-contaminated ones collected at the same experimental period and temperature. At 21 °C no significant differences were noticed between contaminated mussels and non-contaminated ones after the exposure period. After the recovery period and at both temperatures, mussels previously exposed to Nd showed higher LPO levels than mussels at CTL. Comparing experimental periods, for CTL mussels no significant differences were found at both temperatures. In contaminated mussels at 17 °C, no significant differences were observed between experimental periods, while significantly higher LPO levels were found in contaminated mussels under 21 °C collected after the recovery period in comparison with mussels collected after exposure at the same temperature. No significant differences were observed between temperatures for exposure and recovery periods, both in CTL and Nd-contaminated mussels (Fig. 7A).

After the exposure period, PC levels showed no significant differences between contaminated mussels at 17 °C and non-contaminated ones at the same temperature, while significantly lower values were found in contaminated mussels at 21 °C compared with non-contaminated ones. In terms of the recovery period, previously contaminated mussels at 17 °C presented higher PC levels than non-contaminated ones at the same temperature, while at 21 °C no differences were detected. Between experimental periods, in CTL and contaminated mussels significantly lower PC values were found after recovery at 17 °C, while at 21 °C no significant differences were observed between experimental periods. No significant differences were observed between temperatures for exposure and recovery periods, both in CTL and Nd-contaminated mussels (Fig. 7B).

4. Discussion

4.1. Effects after the exposure period

The present results showed that when exposed to Nd mussels were able to accumulate this element, regardless of the temperature. Similarly, a previous study conducted by Freitas et al. (2020) demonstrated the ability of *Mytilus galloprovincialis* to accumulate Nd after 28-days of

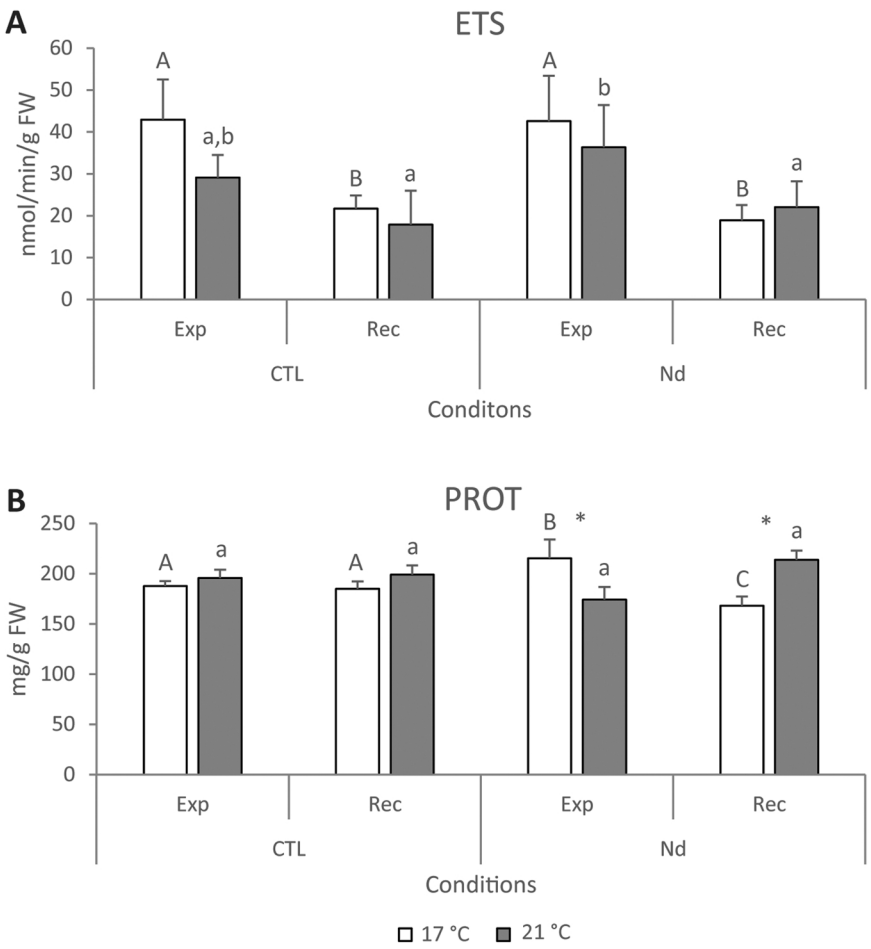


Fig. 4. A: Electron transport system (ETS); B: Protein (PROT) content in *Mytilus galloprovincialis* exposed to different temperatures (17 and 21 °C) in the absence (CTL) and presence of neodymium (Nd) for 14 days (Exp) followed by 14 days or recovery period without Nd (Rec). Results are means with standard deviations. Significant differences ($p < 0.05$) among treatments and experimental periods are identified by different letters (uppercase letters for 17 °C and lowercase letters for 21 °C). Significant differences ($p < 0.05$) between the two temperatures are represented with an asterisk ($n = 6$).

exposure, under a temperature of 17 °C, with increasing concentrations along the exposure gradient. The present results further demonstrated that higher accumulation was obtained at warming conditions (21 °C). Previous studies testing the influence of temperature on metal accumulation also demonstrated that clams *Ruditapes philippinarum* presented a higher concentration of mercury (Hg) under 22 °C compared to the ones at 17 °C (Coppola et al., 2020b). Although Coppola et al. (2020b) associated the increase of Hg at 21 °C with a higher metabolic capacity of clams, in the present study this was not observed as the electron transport system (ETS) activity was similar at both evaluated temperatures. However, the present results showed a slight decrease in the detoxification capacity (GSTs activity) of contaminated mussels at 21 °C compared to the ones at 17 °C also exposed to Nd, which may have contributed to increased Nd concentration at warming conditions. Such findings are in accordance with the study performed by Morosetti et al. (2020) that demonstrated the decrease of glutathione S-transferases (GSTs) activity in mussels exposed to cerium oxide nanoparticles (CeO_2 NPs) and Hg at increased temperature compared to the ones at CTL temperature.

The evaluation of histopathological alterations is an important tool to assess the impacts of stressful conditions in organisms (Cuevas et al., 2015), being the use of histopathological indexes (I_h) considered a common approach for that (among others, Coppola et al., 2020a; Costa et al., 2013; Leite et al., 2020). Regarding mussel's gills, this study showed a significantly higher I_h in mussels exposed to Nd at CTL temperature (17 °C), with enlargement of the central vessel and loss of cilia. According to Pagano et al. (2016), the loss of cilia indicates the beginning of gills exfoliation which leads, in short term, to difficulties in filtering food and in the mid-term to breathing problems. Previous studies conducted by Sunila (1988) also showed an increase in histopathological changes in mussel's gills when exposed to cadmium, copper, lead, cobalt, iron, and silver, while Pinto et al. (2019) demonstrated similar impacts due to lanthanum (La) exposure. At 21 °C, the gills of non-contaminated mussels showed significantly higher I_h values compared to the ones at 17 °C, where the main alteration was hemocytes infiltration, which is associated with the inflammatory response as a defensive reaction to cellular damage (Pagano et al., 2016). Similarly, Piscopo et al. (2021) revealed an increased histopathological alteration in non-contaminated clams *R. philippinarum* maintained at 21 °C compared to the ones at 17 °C. Because contaminated mussels at 21 °C did not show differences in gill's I_h compared to non-contaminated ones maintained at the same temperature and contaminated ones at 17 °C, we may hypothesize that the interaction of Nd at the highest temperature did not cause more damage in mussel's gills than each stressor acting separately.

Concerning mussel's digestive tubules, the results of I_h demonstrated no significant differences between the different treatments at 17 °C, however, contaminated mussels presented higher atrophy, and necrosis (characterized by cellular rupture (do Amaral et al., 2019)). At 21 °C, a slightly higher I_h was detected in mussels exposed to Nd compared to non-contaminated mussels, with higher atrophy and hemocyte infiltration. Although the present results revealed a low impact of Nd in mussel's digestive tubules, the combination of Nd and higher temperature exhibited higher impacts. Also, Leite et al. (2020) observed more effects in digestive tubules in *M. galloprovincialis* mussels exposed to titanium dioxide under 21 °C compared to non-contaminated mussels maintained at the same temperature.

Regarding biochemical responses, energy balance plays an important role in an organism's survival, adaptation, and tolerance to stress (Sokolova et al., 2012). The organism's energy balance can be assessed

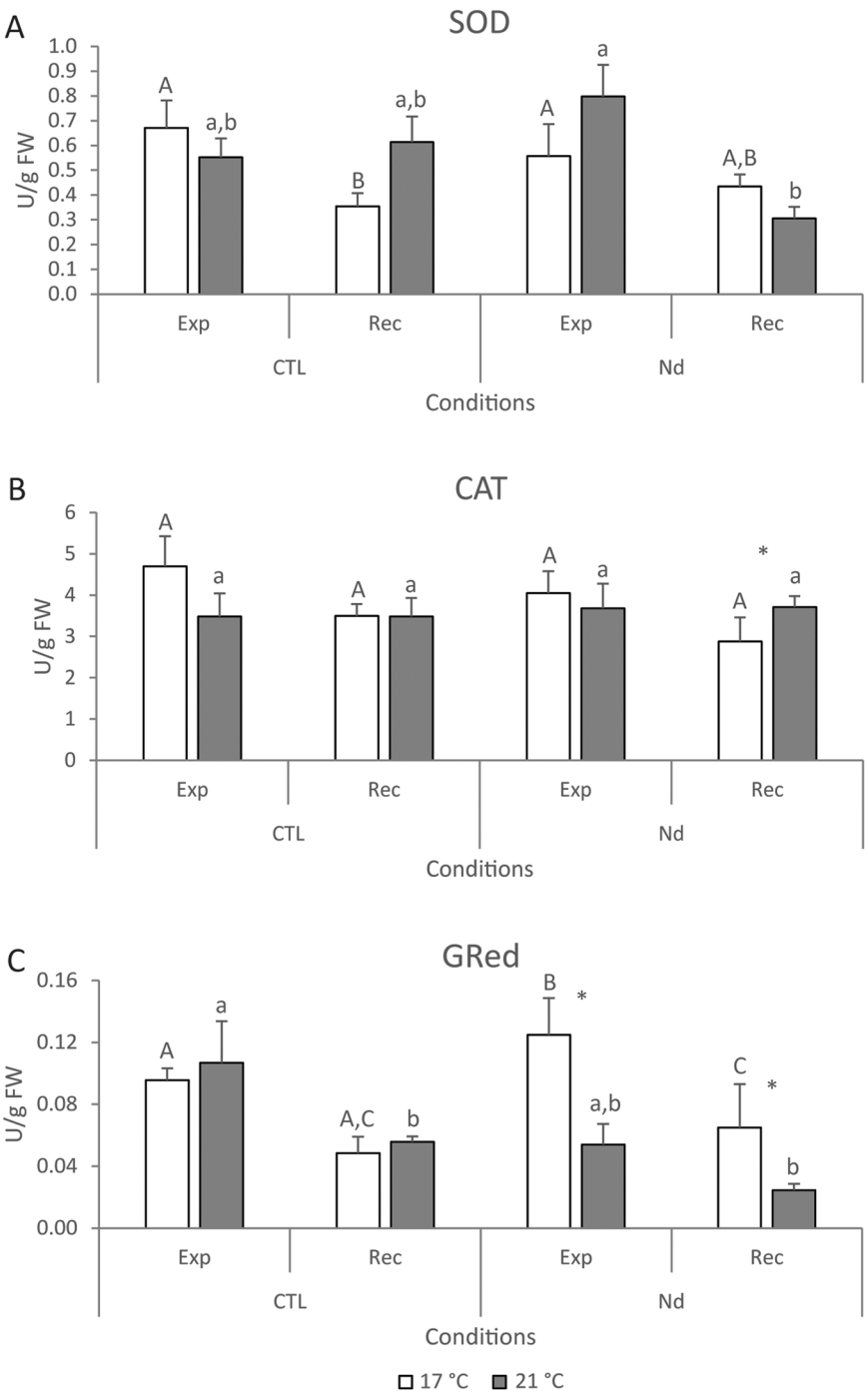


Fig. 5. A: Superoxide dismutase (SOD) activity; B: Catalase (CAT) activity; C: Glutathione reductase (GRed) activity in *Mytilus galloprovincialis* exposed to different temperatures (17 and 21 °C) in the absence (CTL) and presence of neodymium (Nd) for 14 days (Exp) followed by 14 days or recovery period without Nd (Rec). Results are means with standard deviations. Significant differences ($p < 0.05$) among treatments and experimental periods are identified by different letters (uppercase letters for 17 °C and lowercase letters for 21 °C). Significant differences ($p < 0.05$) between the two temperatures are represented with an asterisk (n = 6).

by the evaluation of energy consumption and energy reserves. Electron transport system (ETS) activity can be used to estimate the energy consumption at a mitochondrial level, indicating the organism's metabolic *status* (De Coen and Janssen, 1997) and the results obtained in this study showed that the metabolism was not altered when mussels were exposed to Nd, regardless of the temperature, indicating the possibility that the concentration tested or the exposure period used were not enough to impact the mussel's metabolic capacity. However, a previous study conducted by Freitas et al. (2020) demonstrated that at 17 °C mussels exposed to an increasing gradient of Nd increased their metabolic capacity, with significantly higher ETS activity at 10 μg/L of Nd compared to the control mussels (with no Nd). The difference between the findings of these two studies may result from the fact that in the present study mussels were exposed to Nd for 14 days, while Freitas et al. (2020) conducted a 28-days experiment. Thus, impacts on metabolism at low Nd concentrations may depend on the exposure duration. In terms of warming, this study did not present differences in ETS activity, probably because mussels are adapted to daily changes of temperature in the environment and the exposure period was not long enough to generate differences. The daily average water temperature in the sampling area ranges between 14 °C in the Winter and 18 °C in the Summer (Weather Spark, 2022).

The obtained results further revealed that mussels exposed to Nd at 17 °C were able to avoid the expenditure of proteins (PROT) and the production of proteins exceed their use, while at the highest temperature contaminated mussels showed similar PROT content to non-

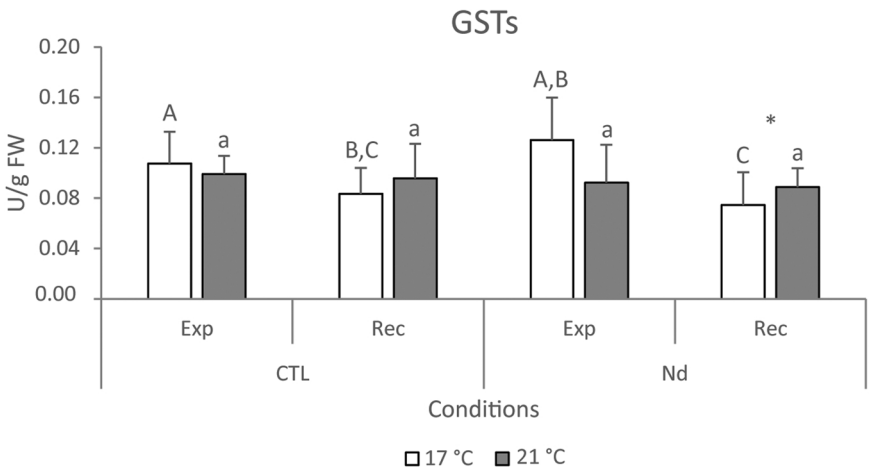
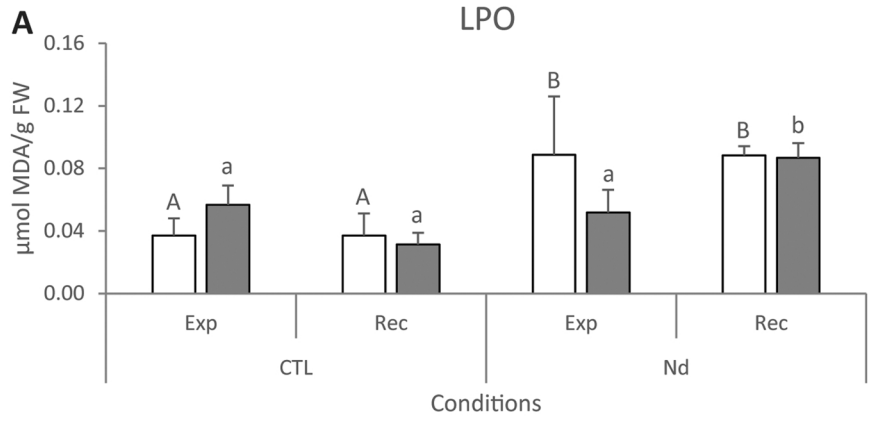


Fig. 6. Glutathione S-transferases (GSTs) activity in *Mytilus galloprovincialis* exposed to different temperatures (17 and 21 °C) in the absence (CTL) and presence of neodymium (Nd) for 14 days (Exp) followed by 14 days or recovery period without Nd (Rec). Results are means with standard deviations. Significant differences ($p < 0.05$) among treatments and experimental periods are identified by different letters (uppercase letters for 17 °C and lowercase letters for 21 °C). Significant differences ($p < 0.05$) between the two temperatures are represented with an asterisk ($n = 6$).



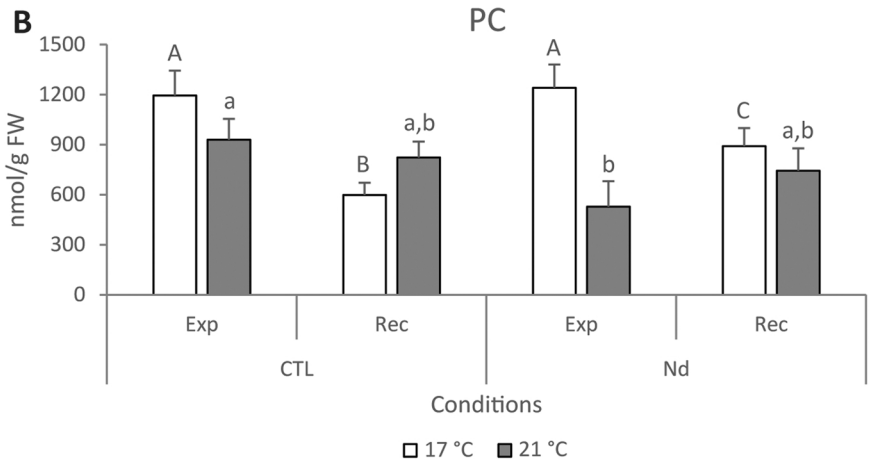


Fig. 7. A: Lipid peroxidation (LPO) levels; B: Protein carbonylation (PC) levels in *Mytilus galloprovincialis* exposed to different temperatures (17 and 21 °C) in the absence (CTL) and presence of neodymium (Nd) for 14 days (Exp) followed by 14 days or recovery period without Nd (Rec). Results are means with standard deviations. Significant differences ($p < 0.05$) among treatments and experimental periods are identified by different letters (uppercase letters for 17 °C and lowercase letters for 21 °C). Significant differences ($p < 0.05$) between the two temperatures are represented with an asterisk ($n = 6$).

contaminated ones at the same temperature. Monteiro et al. (2019) demonstrated that at 17 °C *M. galloprovincialis* exposed to 5 and 50 µg/L of Ti maintained their metabolism while increasing energy reserves (GLY and PROT content) after 96 h and 14 days. The present study further revealed that Nd-exposed mussels showed lower PROT content at 21 °C than at 17 °C, which may indicate the limited capacity of bivalves to increase the production of proteins under higher stressful conditions (Nd exposure and higher temperature). Morosetti et al. (2020) also demonstrated that mussels under warmer conditions, exposed to CeO_2 NPs, Hg, and the combination of the two were not able to increase the production of proteins.

Under non-stressful conditions, reactive oxygen species (ROS) are naturally produced and eliminated by the defense mechanisms (such as activation of antioxidant defenses, including the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GRed) as well as biotransformation defenses, like GSTs) (Regoli and Giuliani, 2014). However, under stressful conditions, the production of ROS can be enhanced and if not eliminated can cause damage to lipids, proteins, and DNA (Regoli and Winston, 1999). Under 17 °C (control temperature), our findings showed that mussels exposed to Nd maintained SOD,

CAT, and GSTs activity in comparison to values observed in non-contaminated exposed mussels, but increased GRed activity, which was not enough to avoid peroxidation of cellular membrane lipids, although oxidation of proteins was not observed. A similar pattern was found by Trapasso et al. (2021) that observed increased GRed activity in *M. galloprovincialis* exposed to the rare earth element gadolinium (Gd), leading to higher LPO levels although PC levels were maintained. Also, Freitas et al. (2020) showed that Nd exposed mussels to the same concentration of Nd increased CAT and GSTs activity, which was not enough to prevent LPO. Mussels exposed to Nd under warming conditions slightly increased their SOD activity and although mussels in this condition showed the highest accumulation of Nd, it seemed that the increase in SOD activity might be sufficient to avoid lipid peroxidation and protein carbonylation (PC). Nevertheless, the present findings may indicate that the Nd concentration tested (10 μg/L) and the exposure period (14 days) were sufficient to induce oxidation of the membrane lipids in mussels but the combination with higher temperature led to the activation of SOD which was enough to avoid cellular damage.

4.2. Effects after the recovery period

Under 17 °C (control temperature), after the recovery period mussels were not able to decrease the Nd that was accumulated, presenting no significant differences to mussel's Nd concentration observed after the exposure period. However, under 21 °C the concentration of Nd was lower than the values observed after the exposure period. Figueiredo et al. (2022) and Maulvault et al. (2018) reported that higher temperature facilitates the elimination of certain contaminants. In particular, Figueiredo et al. (2022) showed that under a warming scenario the elimination of La by the species *Spisula solida* was more efficient than at the control temperature; and the study by Maulvault et al. (2018), demonstrated that the elimination rate of inorganic arsenic was higher in a warming scenario than under CTL temperature in the species *M. galloprovincialis*. Nevertheless, in terms of Nd accumulation, differences between temperatures were not associated with different detoxification strategies or metabolic capacity at 17 and 21 °C, thus further investigation must be carried out to understand why under warming conditions mussels were able to decrease their Nd concentration.

Histopathological alterations in mussel's gills, showed that despite the changes observed after the exposure period can be considered moderate (Bernet et al., 1999), no significant improvements in the gill's *status* were observed after the recovery period. Thus, this may be related to the short recovery time tested (14 days) or due to the fact that no chelating agent was added into the media, aiming to prevent the mussels from reabsorbing the excreted Nd. The results of the I_h of mussel's digestive tubules revealed that mussels maintained at both studied temperatures were not able to recover from the exposure to Nd. Moreover, no improvements were observed in mussels maintained at 21 °C at the end of the recovery period compared to those from the exposure period. Taking this into account, it is possible to hypothesize that it is not only important what the organisms are exposed to but also the duration of exposure.

Regardless of the temperature, Nd-exposed mussels submited to recovery period presented ETS values significantly lower than those during the exposure period. These results were similar to the ones observed in control mussels after the recovery period, highlighting the capacity of mussels to restore their metabolic activity. Valves closure, a behavior usually observed in bivalves under stressful conditions (Gosling, 2003) may be used as an attempt by mussels to reestablish their normal oxidative *status*. Lower ETS activity after the recovery period may also indicate no need for the activation of defense mechanisms with the decrease of stressful conditions. Nunes et al. (2017) also observed a decrease in metabolism and no activation of defense mechanisms after 10 days of recovery in *R. philippinarum* previously exposed to 0.25 μg/L of paracetamol compared to the ones collected after 96 h of exposure to the same concentration of paracetamol. At 17 °C, accompanying the decrease of ETS after the recovery period, mussels also decreased their PROT content which could result from lower production of enzymes as a consequence of a decreased metabolic capacity. This pattern was also observed by Nunes et al. (2017), where previously contaminated clams demonstrated lower PROT content compared to exposed ones. Additionally, Velez et al. (2016) observed a decrease in GLY content after 28 days of the recovery period in *R. philippinarum* previously exposed to arsenic. Similarly, Anacleto et al. (2015) observed a decrease in GLY content in *M. galloprovincialis, R. philippinarum, and Scrobicularia plana* during a depuration period. Nevertheless, at 21 °C no changes in PROT content were observed between Nd exposure and recovery periods, which could be due to the use of another energy reserve. Since no literature exists in this regard, further investigation should be conducted on this topic.

Concerning defense mechanisms, similar or even lower antioxidant and biotransformation levels in Nd contaminated mussels after the recovery period in comparison to the ones after the exposure may evidence the re-establishment of mussel's oxidative *status*. Furthermore, after the recovery, values of enzymatic activities were similar between the control and previously exposed mussels, indicating that the stress imposed was no longer sufficient to activate defense mechanisms. Nevertheless, cellular damage occurred in mussels previously contaminated, highlighting the impacts caused by previous exposure to Nd. Nunes et al. (2017) observed that clams previously exposed to paracetamol managed to recover their oxidative *status* up to values similar to mussels in control conditions with no LPO levels registered.

5. Conclusions

The present study highlights that Nd induced biochemical and histopathological alterations in *M. galloprovincialis*, revealing oxidative stress and injuries in gills. Furthermore, temperature rise influenced the uptake of Nd by mussels, the energy balance, and the activation of antioxidant enzymes, however, no cellular damage was observed. Additionally, temperature rise, acting alone and in combination with Nd, induced histopathological changes in mussel's gills and digestive tubules. Therefore, this study pointed out that both Nd exposure and higher temperature cause negative impacts on mussels, which may compromise population maintenance and eventually affect ecosystem biodiversity. Nevertheless, mussels were mostly able to recover their biochemical performance, but histopathological and cellular damage was still present. Additionally, mussels were not able to eliminate Nd during the recovery period, except under increased temperature. The present findings may highlight that even a short exposure period to Nd, can change mussel's health *status* with a limited capacity to totally recover their physiological and biochemical capacities, which can eventually affect reproduction and population growth. Overall, this study emphasizes the need to assess the presence of this and other REEs in aquatic systems and the impacts of environmentally relevant concentrations on *M. galloprovincialis* or other bivalve species, considering future climate change-related factors including the increase of temperature, salinity shifts, and the decrease in pH, thus providing a realistic environmental risk assessment.

CRediT authorship contribution statement

Carla Leite: Methodology; Data analyses; Writing – original draft. **Francesca Coppola:** Methodology; Data analyses. **Vanessa Queirós:** Methodology; Data analyses**. Tania Russo:** Methodology; Data analyses. **Gianluca Polese:** Conceptualization; Funding; Supervision; Writing – review & editing. **Carlo Pretti:** Conceptualization; Funding; Supervision; Writing – review & editing. **Eduarda Pereira:** Conceptualization; Funding; Supervision; Writing – review & editing. **Rosa Freitas:** Conceptualization; Funding; Supervision; Writing – review & editing.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2022.104029.

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