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Can exposure to *Gymnodinium catenatum* toxic blooms influence the impacts induced by Neodymium in *Mytilus galloprovincialis* mussels? What doesn't kill can make them stronger?

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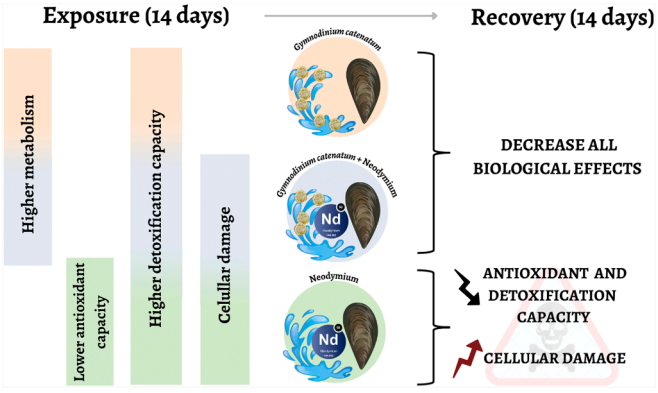
HIGHLIGHTS

- Exposure to G. *catenatum* cells increased mussels' metabolism.
- Restoration of lipid peroxidation after recovery to mixture treatment.
- After recovery, mussels were not able to eliminate the accumulated Nd.
- The mussels showed cell damage in both periods under the Nd treatment.
- Synergistic effects of *G. catenatum* and Nd treatments.

GRAPHICAL ABSTRACT



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ABSTRACT

The presence in marine shellfish of toxins and pollutants like rare earth elements (REEs) poses a major threat to human well-being, coastal ecosystems, and marine life. Among the REEs, neodymium (Nd) stands out as a widely utilized element and is projected to be among the top five critical elements by 2025. *Gymnodinium catenatum* is a phytoplankton species commonly associated with the contamination of bivalves with paralytic shellfish toxins. This study evaluated the biological effects of Nd on the mussel species *Mytilus galloprovincialis* when exposed to *G. catenatum* cells for fourteen days, followed by a recovery period in uncontaminated seawater for another

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fourteen days. After co-exposure, mussels showed similar toxin accumulation in the Nd and *G. catenatum* treatment in comparison with the *G. catenatum* treatment alone. Increased metabolism and enzymatic defenses were observed in organisms exposed to *G. catenatum cells*, while Nd inhibited enzyme activity and caused cellular damage. Overall, this study revealed that the combined presence of *G. catenatum* cells and Nd, produced positive synergistic effects on *M. galloprovincialis* biochemical responses compared to *G. catenatum* alone, indicating that organisms' performance may be significantly modulated by the presence of multiple co-occurring stressors, such those related to chemical pollution and harmful algal blooms.

Environmental implications: Neodymium (Nd) is widely used in green technologies like wind turbines, and this element's potential threats to aquatic environments are almost unknown, especially when co-occurring with other environmental factors such as blooms of toxic algae. This study revealed the cellular impacts induced by Nd in the bioindicator species *Mytilus galloprovincialis* but further demonstrated that the combination of both stressors can generate a positive defense response in mussels. The present findings also demonstrated that the impacts caused by Nd lasted even after a recovery period while a previous exposure to the toxins generated a faster biochemical improvement by the mussels.

1. Introduction

Rare earth elements (REEs) are a group of 17 elements with similar physical and chemical properties [23]. The high densities, melting points, electrical and thermal conductivity make these elements critical for a variety of electrical and electronic equipment (EEE) [23,35]. During the last decades, as industrial, economic and technological development has increased exponentially, the demand for REEs has been rising at the same time [2]. Combined with the growing demand for EEE, the volume of produced waste (e-waste) has increased exponentially, with 54 million metric tons in 2019 and an expected 30 % annual increase by 2030 [29]. The collection and recycling of e-waste can be challenging and thus, the presence of REEs in aquatic ecosystems is expected to increase over the years [37,47,54]. One of the REEs present in e-waste is neodymium (Nd) which has been documented in the literature in different aquatic systems. In mine waters, intermediate concentrations range from 0.01 to 52.67 μg L^{-1} in Guadiamar, Spain [52] and from 481 to 510 μg L^{-1} at Berkeley Pit Lake, a large acid mining lake in Butte, Montana (USA) [22]. Nd concentrations have been also documented in seawater on the Kona Coast of Hawaii (USA) and Labrador Beach (Australia) ranging from 24.4 μg L^{-1} [17] to 31.8 μg L^{-1} [28], respectively. Concentrations of this metal were also reported in marine organisms. Akagi and Edanami [3] evaluated the presence of Nd in soft tissues of *Ruditapes philippinarum* at different locations in Tokyo Bay: in Daiba, the Nd concentration was 411 ± 20 ng g^{-1} DW (dry weight) and in Kanazawa Hakkei it was 367.3 ± 5.7 ng g^{-1} DW. The concentrations of REEs, in particular Nd, were assessed in several common bivalves on the French coast [13]: in soft tissues of *Mytilus edulis*, Nd concentration ranged from 0.169 μg g^{-1} DW (Baie de la Fresnaye) to 0.671 μg g^{-1} DW (Pointe de Chémoulin estuary), in *Crassostrea gigas* concentrations ranged from 0.038 μg g^{-1} DW (Persuel Bay) to 2.088 μg g^{-1} DW (La Fosse estuary), while in *M. galloprovincialis* concentrations ranged from 0.062 μg g^{-1} DW (Etang du Prèvost lagoon) to 0.803 μg g^{-1} DW (Emb. De l'Hérault estuary). In the study of Figueiredo et al. [26], specimens of *M. galloprovincialis* were monitored on the Portuguese coast to determine the natural concentrations of REEs. The Porto Brandão site (south bank of the Tagus estuary) exhibited the highest Nd concentrations in mussels, with a mean value of 0.356 μg g^{-1} DW in fall and 0.502 μg g^{-1} DW in spring. In this site, concentrations showed to be influenced by anthropogenic pressures since it is located on the south bank of the Tagus estuary and crosses Lisbon, a dense Portuguese urban city (capital of Portugal). In addition to the literature available on field measurements, laboratory studies regarding the biochemical, physiological and behavioral impacts of Nd on marine organisms have been documented [30,45]. The study of Freitas et al. [30] evaluated the biochemical changes induced by Nd at different concentrations (2.5; 5.0; 10; 20; 40 μg L^{-1}) in *M. galloprovincialis* after 28 days of exposure. This study revealed that mussels exposed to this element showed an increase in metabolic capacity but an inefficient capacity of antioxidant and biotransformation enzymes in the elimination of reactive oxygen species, leading to cellular damage and loss of redox balance. Additionally, Leite et al. [45] found that exposure for 14 days to Nd (10 μg L^{-1}) caused histopathological injuries in the gills of mussels and cellular damage.

Coastal ecosystems and marine organisms are threatened not only by pollutants such as REEs, but also by biotic factors such as harmful algal blooms (HABs), caused by the proliferation of toxic algal cells that produce marine toxins leading to impacts on fisheries, aquaculture, recreation and tourism in almost all aquatic environments [7,41]. The ingestion of phytoplankton and associated toxins by molluscs and planktivorous fish may trigger effects from the sub-cellular to the physiological level and, in extreme episodes, organisms' death [14,21]. Among the various marine organisms, bivalves are the main vector for the transfer of toxins, due to their accumulation by filtration, with studies revealing alterations in redox balance and oxidative status, particularly in antioxidant defense mechanisms [24,46,56]. Furthermore, the consumption by humans of toxins-contaminated bivalves may cause serious health problems [21,25,33]. There are several types of crustacean and mollusc poisoning syndromes, among which paralytic shellfish poisoning (PSP) is one of the most relevant due to its capability to block sodium channels [70]. Dinoflagellate species that produce paralytic shellfish toxins (PSTs) have the most significant contribution to the number of HABs events worldwide [44]. According to Hallegraeff et al. [39], 35 % of recorded seafood toxin events worldwide between 1985 and 2018 were from PSTs. PSTs-contaminated bivalves may cause neurological and gastrointestinal problems in humans when consumed [34]. Paralytic shellfish toxins are a broad group of neurotoxic alkaloids produced by various marine dinoflagellates such as *Gymnodinum catenatum*, *Pyrodinium bahamense* and *Alexandrium* spp. [50,69]. Among the different types of dinoflagellates, *G. catenatum* is distributed over a large geographical area from California, Mexico, Argentina, Venezuela, Japan, Philippines, Tasmania and the Iberian Peninsula [40]. This phytoplankton species is commonly linked to PSTs contamination in bivalves also in Portuguese coastal waters [10,66]. The occurrence of *G. catenatum* blooms in Portuguese coastal waters is associated with periodic relaxation of coastal upwelling in the western region and the presence of slow currents or coastal eddies that support this species near the coast [49,55,9]. Studies carried out by Freitas et al. [31] have already assessed biochemical responses in cockles (*Cerastoderme edule*), mussels (*M. galloprovincialis*) and razor shells (*Solen marginatus*) naturally exposed to a proliferation of *G. catenatum*, demonstrating that mussels with high PSTs levels exhibited antioxidant defenses activation, neurotoxicity, and cell damage.

Considering that the co-occurrence of REEs and PSTs can be more frequently expected in the coming years and no literature is available regarding the combined effects of these two stressors, the present study aimed to evaluate the biological effects of Nd (20 μg L^{-1}) on the mussel *M. galloprovincialis* when exposed to *G. catenatum* for fourteen days, followed by a recovery period in uncontaminated seawater for another fourteen days. The overall results were expected to provide novel

insights on the impact of these new emerging pollutants under a multiple- stressors scenario.

2. Materials and methods

2.1. Algal culture

The culture of the dinoflagellate *G. catenatum* (strain GYMN8 ESD1_18, IPMA collection) was maintained in natural seawater (salinity 28) supplemented with GSe medium [71]. The strain was mass cultured in 10-L culture flasks at a temperature of 18 °C under a 16 h light: 8 h dark photocycle with a light intensity of 15 μmol photons m^{-2} s^{-1} to supply to the mussel exposure experiment. Cells of *G. catenatum* were counted in Palmer-Maloney chambers under a Zeiss IM 35 inverted microscope and were harvested in the late exponential growth phase.

2.2. Sampling of mussels and experimental conditions

Specimens of *M. galloprovincialis* (width: 3.4 ± 0.3 cm; length: 6.0 ± 0.5 cm) were collected in June 2022 from the Ria de Aveiro coastal lagoon (Portugal) and kept in the laboratory where they spent ten days acclimating in artificial seawater (Coral Pro Salt Red Sea), constant aeration and conditions representative of those of the sampling site (temperature 17 ± 1 °C, pH 8.1 ± 0.1, salinity 28 ± 1 and natural photoperiod). During this period the water was renewed every three days after which the mussels were fed every day with AlgaMac Protein Plus (150,000 cells/mussel/day). The presence of PSTs in the mussels after depuration was assessed to ensure that the organisms were free of PSTs before the start of the experiment.

After the acclimation period, five mussels were placed in 3 L aquaria [15] so that the following treatments could be tested for fourteen days of exposure followed by a further fourteen days of recovery: i) **Control** (CTL, clean artificial seawater), ii) **Neodymium** (20 μg L^{-1} Nd), iii) ***G. catenatum*** (50,000 *G. catenatum* cells), iv) ***G. catenatum*** **+ Nd (**50,000 *G. catenatum* cells + 20 μg L^{-1} Nd) (Figure 1SM). An exposure period of fourteen days was selected based on Anderson et al. [4] and USEPA and USACE [65] guidelines while fourteen days of recovery period was chosen based on the study by Freitas et al. [32] showing that bivalves were able to depurate their metal content after seven days in clean seawater.

In the **CTL** and ***G. catenatum*** **+ Nd** treatments, twelve replicate aquaria were used per treatment. Six replicates were removed at the end of the exposure period and the remaining six replicates continued for the recovery period. Of the replicates removed at each time (exposure or recovery), three replicates (fifteen mussels, five mussels per aquarium) were used for PSTs quantification and the other three (nine mussels, three mussels per aquarium) for Nd quantification and biochemical analysis. In the **Nd** treatment, six replicates were used and at the end of the exposure period three replicates were removed and the remaining three continued for the recovery period. At the end of each period, nine mussels (three per aquarium) were used for Nd quantification and biochemical analysis. Paralytic shellfish toxins were not quantified in **Nd** treatment as it is assumed that the values will be similar to those found in the **CTL** treatment. To evaluate the accumulation of PSTs produced by *G. catenatum* in mussels over time, twenty-one replicates were used in the treatment with ***G. catenatum***. Three replicates were taken every four days (T2, T6, T10 and T14) for the quantification of PSTs in five organisms, and another three replicates were also taken after fourteen days of exposure for biochemical analysis and Nd quantification (nine mussels, three mussels per aquarium). The remaining six replicates were used for the recovery period: three replicates with five mussels were used for the quantification of PSTs and the other three replicates (three mussels per replicate) were used for biochemical analysis.

During the exposure period, the mussels subjected to *G. catenatum* treatments (*G. catenatum* and *G. catenatum* + Nd) were fed every two days with a 50,000 *G. catenatum* cells (1 *10^4 cell/individual), based on Botelho et al. [11]) and 1.90 mL of Algamac protein plus (0.38 mL per mussel); in the other treatments (CTL and Nd) only Algamac protein plus (0.75 mL per mussel, corresponding to 150,000 cells/animal/day) was given to the mussels. The profile of the *G. catenatum* culture supplied to the organisms was studied before the experiment. Algal mass was obtained through centrifugation (2300 *g* during 10 min) of 450 mL of harvested culture followed by the freezing of the cell pellet of *G. catenatum* in 0.1 M acetic acid at −80 ºC until analysis. The Nd concentration chosen (20 μg L^{-1}) was based on concentrations reported in marine environments [1,17,28] and previously tested to evaluate biochemical impacts on mussels [31].

In both the exposure and recovery periods, the seawater was weekly renewed, and the conditions of temperature, salinity, pH, photoperiod and Nd concentration (exposure period) were re-established. During the recovery period, mussels were maintained in clean seawater (without Nd and without the addition of the *G. catenatum* culture) and fed only with Algamac protein plus feed (previously described) to assess the recovery of biochemical performance and detoxification capacity of the organisms. When the mussels were removed from the aquaria, they were frozen in liquid nitrogen and stored at −80 ºC until further analysis.

2.3. Neodymium quantification in water samples and mussel's soft tissues

The quantification of Nd in water samples was carried out according to Viana et al. [68] while mussels were analysed following the protocol reported by Freitas et al. [30]. Neodymium quantification in water and mussels' tissues was performed by inductively coupled plasma mass spectrometry (ICP-MS). For the water samples, the quantification limit (LOQ) was 0.02 μg L^{-1} of Nd. Samples were acidified to pH < 2 with HNO_3 65 % and diluted by a factor of 20 before the analysis. For the quantification in mussels' tissues, the samples were freeze-dried, homogenized, and subjected to acid digestion with 1 mL HNO_3 65 % (v/v), 2 mL H_2O_2 (30 %), and 1 mL ultrapure H_2O. The digestion was performed in sealed Teflon vessels using a CEM MARS 5 microwave through a temperature ramp up to 180 ºC in 10 min, which was then maintained for 10 min. The digested samples were transferred to flasks and the volume was completed with ultrapure H_2O up to 25 mL. Quality control was assured through blanks (< 0.02 μg L^{-1} (LOQ)), the percentage of recovery (118 %) for certified reference material (CRM) BCR-668 (Mussel Tissue; 54 ± 4 μg/kg of Nd), and the coefficient of variation of duplicates (11 ± 5 %).

2.4. Paralytic Shellfish Toxins quantification in toxic algae cells and mussel's soft tissues

Toxins of harvested *G. catenatum* cells were extracted according to the method described in Botelho et al. [11]. At the end of the exposure and recovery periods, the mussels were dissected to obtain whole soft tissue samples for toxin determination. Afterwards, the homogenized tissue was divided into three aliquots (5 g) and stored at −20 °C until toxin extraction and analysis. According to the AOAC method described by Lawrence et al. [43] with modifications made by Botelho et al. [12] the extraction, clean-up and oxidation procedures for PSTs (GTX5, GTX6, C1 +2, C3 +4, dcSTX, dcNEO, dcGTX2 +3, STX, NEO and GTX2 +3) determination by LC-FLD were performed as detailed in [18]. The same procedure of sample oxidation (peroxide and periodate acid) was followed substituting the oxidant reagent with ultrapure water to detect natural fluorescent compounds.

The analyses were carried out using an Agilent system comprising: a quaternary pump and a column oven (1290 Infinity series); a refrigerated autosampler (1100 series) and a fluorescence detector (1200 series). OpenLAB CDS (Rev. C) software performed the data acquisition and peak integration. The details of the chromatographic conditions for the determination of PSTs are reported by Botelho et al. [10].

For quantification of PSTs in mussel tissues, matrix-matched

calibration curves with cleaned-up bivalve tissue extract were used. Calibration curves prepared in ultrapure water were used for toxin quantification in *G. catenatum* samples. Certified reference materials in solution (C1 +2-b, GTX2 +3-d, GTX1 +4-d, GTX5-c, GTX6, STX-f, NEO-d, dcSTX-b, dcNEO-d and dcGTX2 +3-c) were provided through the National Research Council. The concentration of C3 + 4 was determined by the conversion into GTX1 + 4, through hydrolysis. The limits of detection in C18-cleaned extracts varied for each of the individual toxins: 4 nmol L^{-1} for GTX2 + 3, GTX5, STX and dcSTX, 8 nmol L^{-1} for GTX6, 20 nmol L^{-1} for dcGTX2 + 3 and C1 + 2, 30 nmol L^{-1} for NEO, and 40 nmol L^{-1} for GTX1 + 4 and dcNEO. Spiking of mussel tissues with dcGTX2 + 3, C1 + 2, dcSTX, GTX2 + 3, GTX5 and STX solutions were prepared at final concentrations ranged from 0.24 μmol L^{-1} (dcGTX2 +3) to 0.75 μmol L^{-1} (C1 +2). Obtained recovery values varied from 93 % (dcGTX2 +3) to 102 % (GTX2 +3).

2.5. Biochemical parameters

The evaluated biochemical parameters were related to mussels' metabolic capacity (electron transport system activity, ETS) [20,42]; energy reserves (protein content, PROT) [61]); antioxidant defenses (activity of the enzymes superoxide dismutase, SOD [6,16] and glutathione peroxidase, GPx [53]) (Fig. 1A); redox balance (total oxyradical scavenging capacity, TOSC toward peroxyl radicals ROO•, hydroxyl radicals HO• and peroxynitrite ONOO- [36]; oxidized glutathione content, GSSG [57]); detoxification capacity (activity of the enzymes glutathione S-transferases activity, GSTs [16,38] and carboxylesterases, CbEs [64]) (Fig. 1B); and cellular damage (lipid peroxidation levels, LPO [51]). For the biochemical parameters analysis, different extraction buffers were used in the ratio of 1:2 (w v^{-1}) or 1:5 (w v^{-1}) (TOSC). After the extraction with the corresponding buffers, the samples were analyzed in duplicate in a microplate reader (Synergy™HT, Biotek Instruments, Inc.) or a Gas Chromatography (GC) system (Agilent Technologies 7820 A) for TOSC. Detailed experimental procedures can be found in the supplementary material.

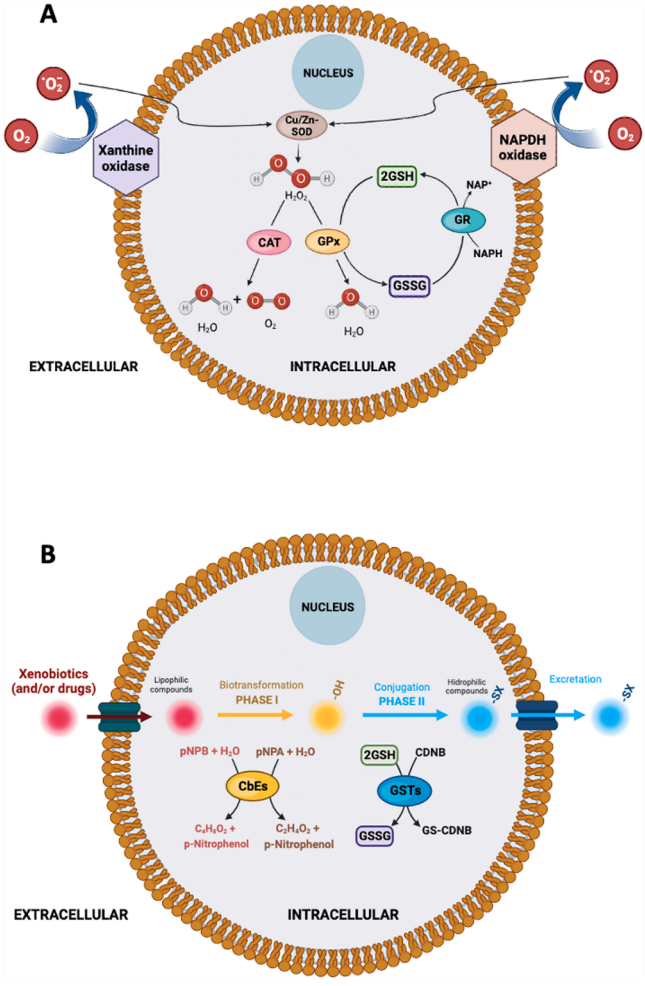

Fig. 1. A: Enzymatic and Non enzymatic antioxidant mechanisms: antioxidant enzymes (Cu/Zn-SOD- Superoxide dismute; CAT – Catalase; GPx – Glutathione peroxidase; GR – Glutathione reductase) and non-antioxidant scavengers (GSSG – oxidized glutathione and GSH – reduced glutathione) and B: Detoxification mechanisms (CbEs – Carboxilesterases and GSTs - Glutathione S-transferases).

2.6. Data analysis

2.6.1. Statistical analyses

The biological responses obtained were subjected to hypothesis testing through multivariate permutational analysis of variance. To assess whether the combination of *G. catenatum* and Nd modulates the effects caused by individual stressors on the mussels, the biochemical results were submitted to the PERMANOVA routine of the PRIMER v6 software [5], testing the null hypotheses: i) no significant differences exist among treatments (CTL, *G. catenatum,* Nd, *G. catenatum* and Nd) after exposure. Significant differences are identified in the figures with lower-case letters; ii) no significant differences exist among treatments (CTL, *G. catenatum*; Nd; *G. catenatum* + Nd) after the recovery period. Significant differences are identified in the figures with upper-case letters; iii) for each treatment (CTL; *G. catenatum*; Nd; *G. catenatum* + Nd) no significant differences exist between both experimental periods (exposure vs recovery). Significant differences are identified in the figures with an asterisk. The significance level was set at $p < 0.05$ and if the main test was significant, pairwise comparisons were performed.

2.6.2. Principal coordinate ordination (PCO)

To create the Euclidean distance similarity matrix, the comprehensive matrix encompassing all biomarker responses was used. To simplify it further, the distance between centroids pertaining to different treatments (CTL, *G. catenatum*, Nd, *G. catenatum* + Nd) during both the exposure and recovery periods were calculated. Next, this matrix was subjected to a principal coordinates ordination (PCO) analysis. Additional variables were incorporated into the PCO graph, including Pearson correlation vectors of biochemical descriptors with a correlation value greater than 0.75.

3. Results

3.1. Mortality

Overall, at the end of the 28 days of the experience, the mortality recorded was 23 % in the co-exposure treatment (*G. catenatum* + Nd) and 7 % in the recovery period treatment with *G. catenatum*. No mortality was recorded in the remaining treatments.

3.2. Neodymium quantification in water samples and mussel's soft tissues

In the artificial seawater samples collected from the uncontaminated treatments (CTL), the measured Nd concentration was below the quantification limit of < 0.02 μg L^{-1}, as shown in Table 1. On the other hand, the mean Nd concentration in the artificial seawater samples collected immediately after spiking ranged between 26.0 ± 2.8 and 20.8 ± 4.8 μg L^{-1} in the Nd and *G. catenatum* + Nd treatments, respectively, corresponding to 26 % and 3 % (respectively) higher than the nominal concentration.

The concentration of Nd in CTL mussels' tissues at the end of the exposure period was 0.3 ± 0.2 μg g^{-1} DW, while in those exposed to Nd, without or with *G. catenatum*, the concentrations were 1.0 ± 0.2 μg g^{-1} DW and 1.0 ± 0.3 μg g^{-1} DW, respectively (Table 2). After the recovery

Table 1
Neodymium concentration (μg L^{-1}) in the artificial seawater collected immediately after spiking from Control (CTL) andcontaminated aquaria with mussels (Nd and *G. catenatum* + Nd treatments). Results are the mean of three water samples ± standard deviation. The Nd quantification limit (LOQ) was 0.02 μg L^{-1}.

Treatments	After spiking 20 μg L^{-1} of Nd
CTL	< 0.02
Nd	26.0 ± 2.8
Nd + *G. catenatum*	20.8 ± 4.8

Table 2
Neodymium (Nd) concentrations (μg g^{-1}, dry weight (DW)) in mussels' soft tissues after 14 days of exposure and 14 days of recovery. Values are the mean of nine mussels analized per treatment (three mussels per replicate) ± standard deviation. The Nd quantification limit (LOQ) was 0.02 μg L^{-1}.

Treatments	Exposure (μg g^{-1})	Recovery (μg g^{-1})
CTL	0.3 ± 0.2	0.3 ± 0.1
Nd	1.0 ± 0.2	1.0 ± 0.4
Nd + *G. catenatum*	1.0 ± 0.3	0.9 ± 0.4

period, the concentration of Nd in the tissues of CTL mussels was 0.3 ± 0.1 μg g^{-1} DW, while in those exposed to Nd, without or with *G. catenatum*, the concentrations were 1.0 ± 0.4 μg g^{-1} DW and 0.9 ± 0.4 μg g^{-1} DW, respectively (Table 2).

3.3. Paralytic Shellfish Toxins quantification in toxic algae cells and mussel's soft tissues

The toxins GTX6, C1 + 2, C3 + 4, dcNEO, dcGTX2 + 3, STX, NEO and GTX2 + 3 were not detected either in the culture of *G. catenatum* or in the mussels. The toxins dcSTX (23 %) and GTX5 (77 %) were the ones that contributed to the toxin profile in the *G. catenatum* culture at the initial time (T0, Fig. 2). No toxins were detected two days after the mussels were fed with *G. catenatum* culture, but after six (T6) and ten days (T10) only the dcSTX was observed, and after fourteen days (T14) dcSTX (58 %) and GTX5 (42 %) were accumulated (Fig. 2). After the recovery period (T28, Fig. 2), both toxins were still present, with GTX5 contributing the most to the toxin profile (62 %, Fig. 2). In the treatment in which the mussels were fed with *G. catenatum* and exposed to Nd, the

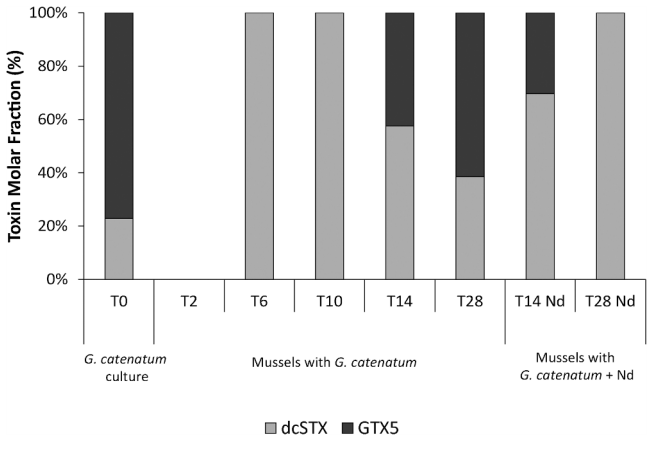

Fig. 2. Toxin profiles of the quantified toxins (dcSTX and GTX5) expressed as molar ratios (%) in *Gymnodinium catenatum* culture at initial time (T0) and in *Mytilus galloprovincialis* exposed to *G. catenatum* treatment after 2, 6, 10, 14 days (T2, T6, T10 and T14) and *G. catenatum* + Nd treatment after 14 days (T14 Nd) followed the recovery period (T28, T28 Nd) in both treatments (*G. catenatum* and *G. catenatum* + Nd treatments). Results are the mean of three values (n = 3, wherein one test unit was regarded as an aquarium).

presence of the dcSTX (70 %) and GTX5 (30 %) were found after fourteen days (T14), while, after the recovery period, only the dcSTX was detected (Fig. 2). Regarding the profile of PSTs present in *G. catenatum* cells and accumulated into the mussels, it was found that after two days (T2) the organisms did not accumulate detectable toxins, after six days (T6) there was an increase of dcSTX (59.6 ± 2.7 ng g^{-1}) and it remained at a similar concentration (61.7 ± 2.2 ng g^{-1}) after ten days (T10) and at the end of fourteen days (T14) the concentration of dcSTX decreased to 16.5 ± 7.2 ng g^{-1} (Fig. 2). At the end of the exposure period, the concentration of dcSTX was similar in both treatments, being 16.5 ± 7.2 ng g^{-1} in the treatment with *G. catenatum* and 27.4 ± 11.8 ng g^{-1} in the treatment with *G. catenatum* + Nd (Fig. 3A). After twenty-eight days (T28, recovery period), in the *G. catenatum* treatment, the concentration of dcSTX was 9.7 ± 2.6 ng g^{-1}, and in the *G. catenatum* + Nd treatment a similar concentration was detected corresponding to 11.9 ± 1.4 ng g^{-1} (Fig. 3A). After fourteen days (T14, exposure period), concentrations of GTX5 in the *G. catenatum* treatments were similar, with 60.3 ± 8.2 ng g^{-1} in the *G. catenatum* treatment and 60.6 ± 0.9 ng g^{-1} in the *G. catenatum* + Nd treatment. In the recovery period, GTX5 was 51.3 ± 27.8 ng g^{-1} in the *G. catenatum* treatment, while in the *G. catenatum* + Nd treatment this toxin was no longer detected (Fig. 3B).

3.4. Biochemical parameters

Throughout the experimental periods, the values of the biochemical parameters evaluated between the CTL treatments (exposure and recovery) did not show significant differences, showing that the organisms maintained their biochemical performance after 28 days of experimental period.

3.4.1. Metabolic capacity and energy reserves content

After the exposure period, significantly higher ETS activity was found in mussels exposed to *G. catenatum* and *G. catenatum* + Nd in comparison with CTL organisms, while after the recovery period

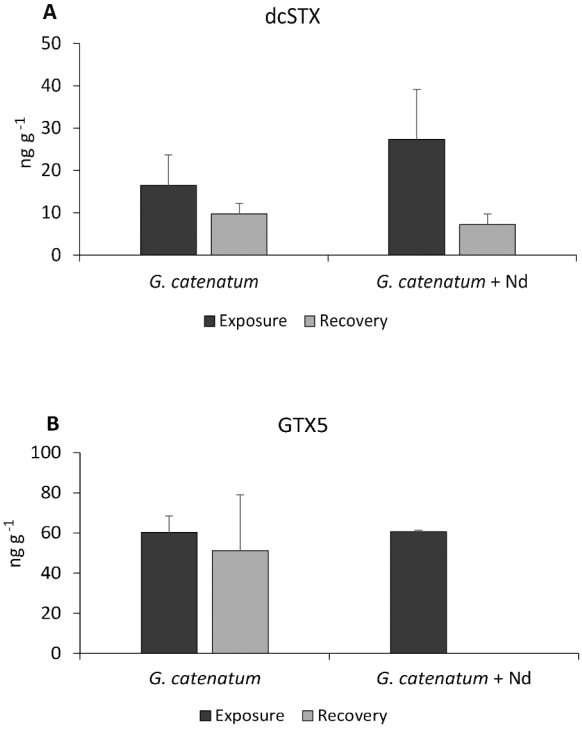

Fig. 3. A: Concentrations of dcSTX and B: Concentrations of GTX5 (ng g^{-1}, mean + standard deviation; n = 3, wherein one test unit was regarded as an aquarium) in *Mytilus galloprovincialis* during exposure (fourteen days, T14) and recovery (fourteen days followed exposure time, T28) periods.

significantly higher and lower ETS activity was detected in Nd and *G. catenatum* +Nd treatments respectively, in comparison with *G. catenatum* treatment. Comparing both experimental periods, significantly lower values were found in the recovery period in *G. catenatum* treatment while under the Nd treatment, an opposite response was observed (Fig. 4A).

After both experimental periods, significantly higher PROT content was found in the Nd treatment compared with the remaining treatments. No significant differences were observed between experimental periods for each treatment (Fig. 4B).

3.4.2. Antioxidant enzymes

After the exposure period, significantly lower SOD activity was detected in Nd treatment in comparison to CTL. After the recovery period, SOD activity was significantly lower in Nd and *G. catenatum* + Nd treatments, in comparison to CTL and *G. catenatum* treatments. Comparing both experimental periods, significantly lower values were detected in the recovery period in Nd and *G. catenatum* + Nd treatments (Fig. 5A).

In terms of GPx activity, after the exposure period, no significant differences were found among treatments. After the recovery period, GPx activity was significantly lower in Nd and *G. catenatum* + Nd treatments in comparison to CTL and *G. catenatum* treatments. Comparing both experimental periods, significantly lower values were detected in the recovery period in Nd and *G. catenatum* + Nd treatments (Fig. 5B).

3.4.3. Redox balance

After the exposure period, no significant differences in terms of TOSC ROO•, HO• and ONOO- were detected among treatments and no significant differences were found among treatments in TOSC ROO• and ONOO- after the recovery period. On the other hand, a reduction of TOSC HO• was observed in *G. catenatum* + Nd treatment compared to

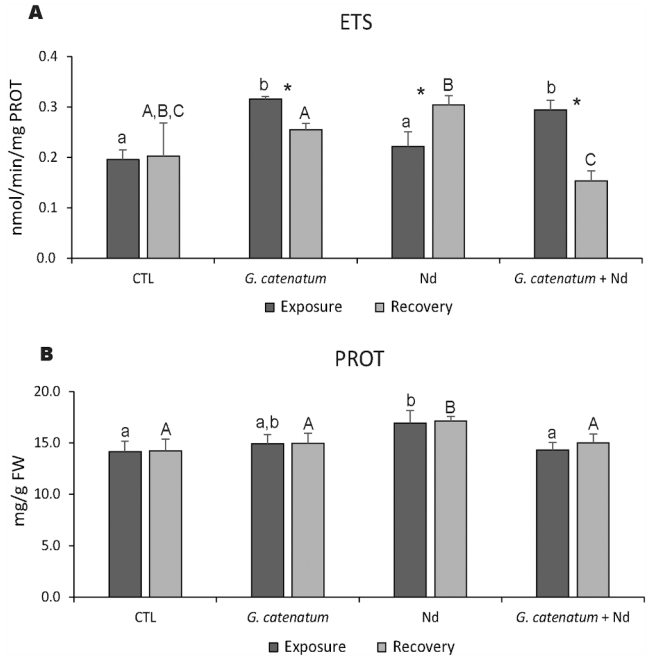

Fig. 4. A: Electron transport system activity (ETS) and B: Protein content (PROT), in *Mytilus galloprovincialis* exposed to different treatments (CTL, *G. catenatum*, Nd, *G. catenatum* + Nd) for fourteen days (Exposure) followed by fourteen days (Recovery). Results are mean + standard deviation (n = 3, wherein one test unit was regarded as an aquarium). Significant differences ($p<0.05$) among treatments are shown with different lowercase (Exposure period) and uppercase (Recovery period) letters. Asterisks represent significant differences between exposure and recovery periods.

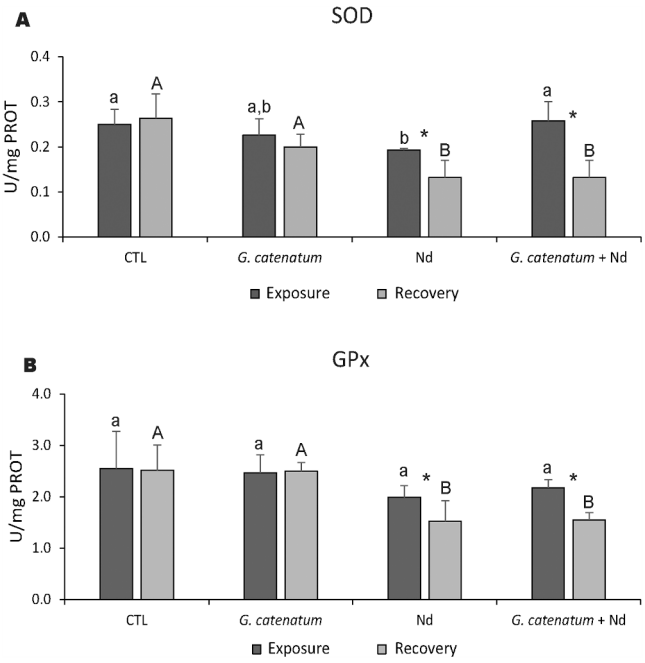

Fig. 5. A: Superoxide dismutase activity (SOD) and B: Glutathione Peroxidase activity (GPx), in *Mytilus galloprovincialis* exposed to different treatments (CTL, *G. catenatum*, Nd, *G. catenatum* + Nd) for fourteen days (Exposure) followed by fourteen days (Recovery). Results are mean + standard deviation (n = 3, wherein one test unit was regarded as an aquarium). Significant differences ($p < 0.05$) among treatments are shown with different lowercase (Exposure period) and uppercase (Recovery period) letters. Asterisks represent significant differences between exposure and recovery periods.

G. catenatum and Nd treatments (Fig. 6A, B, C). Comparing experimental periods, TOSC ROO•, TOSC HO• and TOSC ONOO- significantly increased in the recovery period in Nd treatment (Fig. 6A, B, C), while a significant decrease of TOSC ONOO- at the end of the recovery was observed in *G. catenatum* + Nd (Fig. 6C).

After the exposure period, no significant differences in GSSG content were found among treatments. After the recovery period, significantly higher values were found in the Nd treatment in comparison to *G. catenatum* and *G. catenatum* + Nd treatments, while GSSG content was significantly lower in *G. catenatum* + Nd treatment in comparison with the remaining treatments. The GSSG content observed in *G. catenatum* treatment was similar to CTL. Comparing both experimental periods, significantly lower values were found after the recovery period in *G. catenatum* and *G. catenatum* + Nd treatments (Fig. 6D).

3.4.4. Biotransformation enzymes activity

After the exposure and recovery period, no significant differences were found among treatments for GSTs activity. Comparing both experimental periods, significantly lower values were observed in Nd treatment after the recovery period (Fig. 7A).

After the exposure period, significantly higher CbEs activity was detected in the presence of *G. catenatum* treatment, while significantly lower values were measured in the Nd treatment compared to the CTL treatment. After the recovery period, significantly lower CbEs activity was observed in Nd treatment in comparison to CTL mussels. Comparing both experimental periods, significantly lower values were observed after the recovery period in *G. catenatum* + Nd treatment, while the opposite was observed for Nd treatment (Fig. 7B).

3.4.5. Cellular damage

After the exposure period, significantly higher LPO levels were detected in Nd and *G. catenatum* + Nd treatments in comparison to CTL. After the recovery period, significantly higher LPO levels were found in

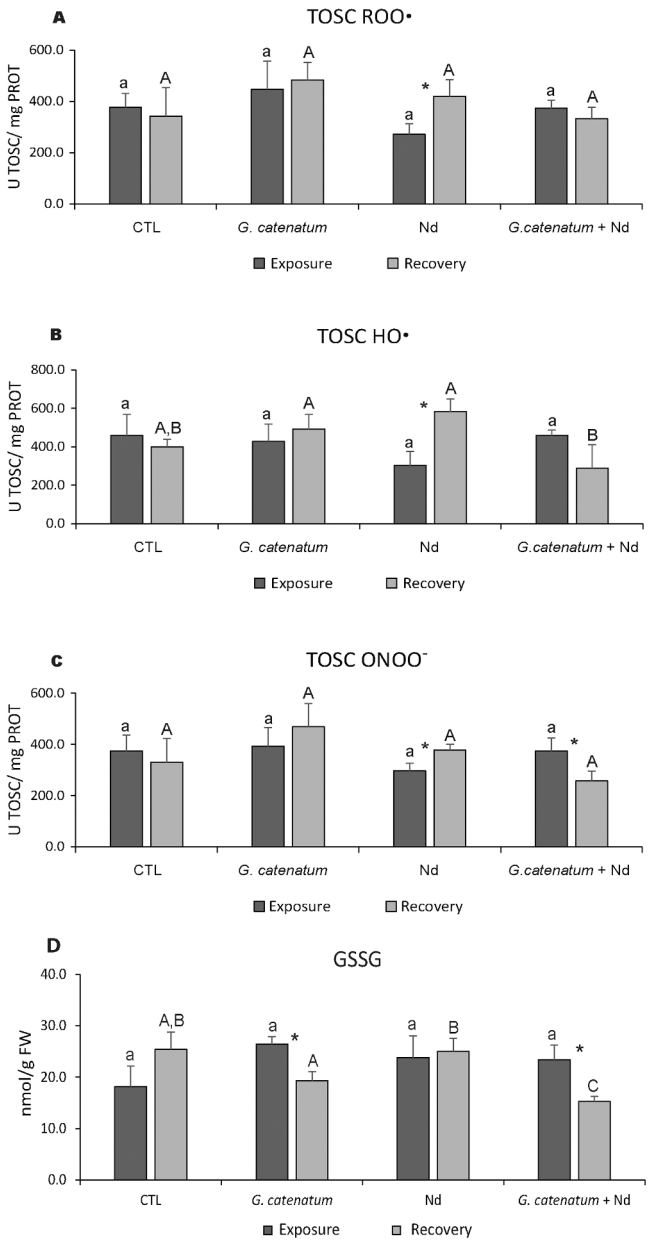

Fig. 6. A: Total oxyradical scavenging capacity toward peroxyl radical (TOSC ROO•); B: Total oxyradical scavenging capacity toward hydroxyl radical (TOSC HO•); C: Total oxyradical scavenging capacity toward peroxynitrite (TOSC ONOO-) and D: Oxidized glutathione (GSSG), in *Mytilus galloprovincialis* exposed to different treatments (CTL, *G. catenatum*, Nd, *G. catenatum* + Nd) for fourteen days (Exposure) followed by fourteen days (Recovery). Results are mean + standard deviation (n = 3, wherein one test unit was regarded as an aquarium). Significant differences ($p < 0.05$) among treatments are shown with different lowercase (Exposure period) and uppercase (Recovery period) letters. Asterisks represent significant differences between exposure and recovery periods.

Nd treatment in comparison to CTL while LPO values observed in *G. catenatum* + Nd treatment were similar to the ones at the CTL. Comparing both experimental periods, significantly lower values were found after the recovery period in *G. catenatum* + Nd treatment with no significant differences between experimental periods in the remaining treatments (Fig. 8).

3.5. Principal coordinate ordination (PCO)

The principal coordinate ordination (PCO, Fig. 9) revealed a generally clear distinction between the four exposure treatments, regardless of the experimental period: CTL, *G. catenatum*, Nd and *G. catenatum* + Nd. The PCO1 axis explained 38.5 % of the total variation, while the PCO2 axis explained 31.6 % of the total variation. According to PCO1, Group II (Nd, exposure and recovery) and Group IV (*G. catenatum* + Nd, recovery), on the positive side of the axis, were separated from Group I (CTL, exposure and recovery) and Group III (*G. catenatum,* exposure and recovery, and *G. catenatum* + Nd, exposure) on the negative side of the axis. This separation is associated with the SOD, GPx and CbEs activities, which showed the highest correlation value with this axis negative side (−0.773, −0.855, −0.899, respectively). Regarding the PCO2 axis, Groups I and IV on the negative side were separated from Groups II and III on the positive side of the axis. The separation was mainly related to higher metabolic capacity, lipid damage and total oxyradical scavenging capacity, with higher correlation values with PCO2 positive side (0.857, 0.828, 0.680, respectively).

4. Discussion

The present study highlights the biochemical changes in the mussel *M. galloprovincialis* when exposed for fourteen days to Nd in the presence of high densities of *G. catenatum* cells followed by a recovery period in uncontaminated seawater for a further fourteen days. Previous studies demonstrated that during the filtration process, bivalves can accumulate both PSTs from algal cells and Nd found from seawater [30,31]. Understanding the effects of the combined exposure of bivalves to a toxic algal bloom and Nd contamination represents a scientific advance, since the only available studies report the biochemical effects on *M. galloprovincialis* exposed to each stressor individually [30,31]. Furthermore, this study highlights the importance of assessing the ability of mussels to recover their biochemical performance when stressors are removed during the recovery period, to better evaluate the long-term consequences of a contamination event on wildlife.

Overall, based on mussels' biochemical responses, the principal coordinate ordination (PCO) analysis distinguished 4 main groups, clearly separating CTL, Nd and *C. catenatum* exposed groups: **Group I**, constituted by CTL (exposure and recovery); **Group II**, comprising Nd (exposure and recovery) treatments; **Group III,** including *G. catenatum* (exposure and recovery), *G. catenatum* + Nd (exposure) and **Group IV**, containing *G. catenatum* (recovery) treatment.

In the present study, the metabolic capacity of the organisms was assessed by measuring the activity of a complex of enzymes identified as the electron transport system (ETS) [20,62]. This is a biochemical measure of the potential metabolic activity of aerobic organisms and represents the amount of oxygen consumption that would occur if all enzymes were functioning at their maximum capacity [63]. The results showed that, during the exposure period, the intake of toxic algal cells and/or the accumulation of PSTs (dcSTX and GTX5) in the tissues of the mussels caused a significant increase in ETS activity in the organisms exposed to the *G. catenatum* and *G. catenatum* + Nd treatments (Group III); these effects may represent a protective behavior associated with an increase in the activity of the CbEs to cope with the harmful conditions caused by the accumulated toxins. On the other hand, at the end of the recovery period (T28), mussels previously exposed only to the *G. catenatum* cells did not eliminate most of the PSTs (dcSTX and GTX5) accumulated during the exposure period (Group III) which could resulted from the co-occurrence of toxins elimination and biotransformation processes in bivalve tissues [14]. In contrast, after the recovery (T28 Nd), mussels previously exposed to the mixture (*G. catenatum* + Nd) eliminated GTX5 and exhibited lower concentration of dcSTX in their tissues (Group IV), which might indicate greater mussels' conversion rate of GTX5 into dcSTx when previously exposed to both stressors. In fact, after 14 days of exposure the toxins profile was similar in

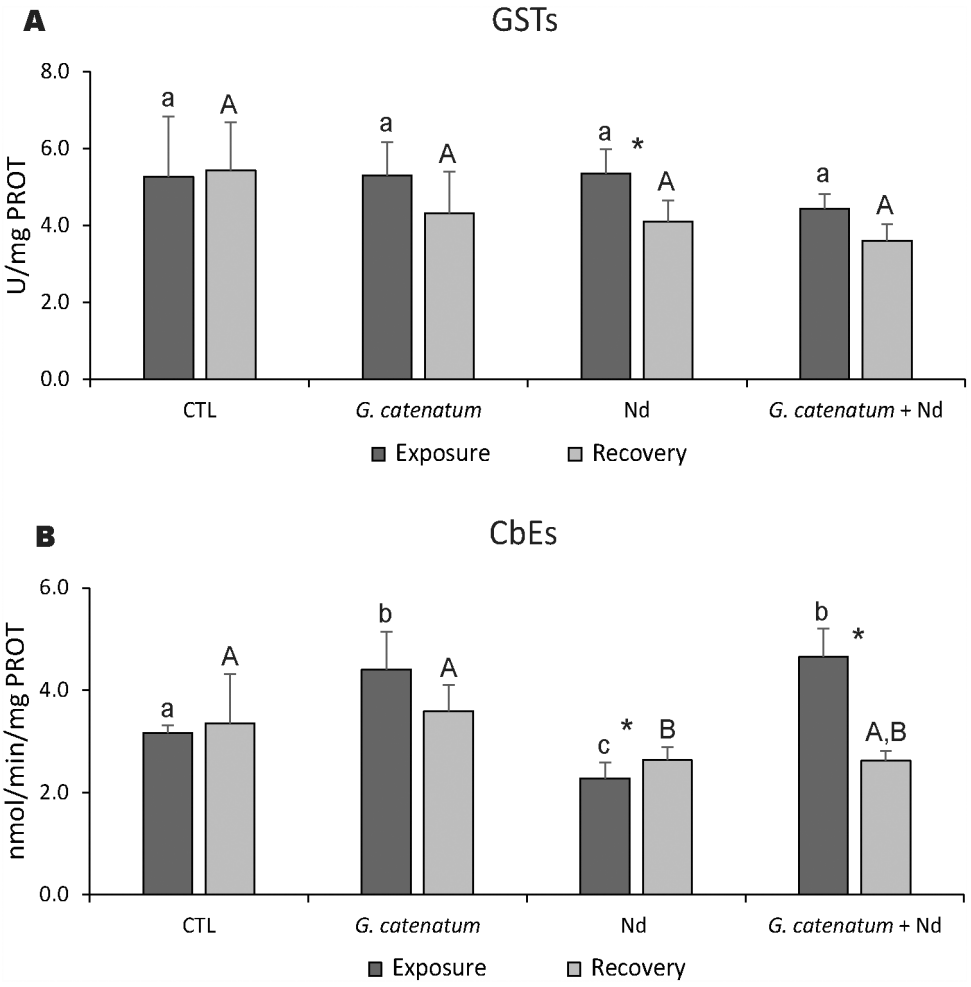

Fig. 7. A: Glutathione S-transferases activity (GSTs) and B: Carboxylesterases activity (CbEs), in *Mytilus galloprovincialis* exposed to different treatments (CTL, *G. catenatum*, Nd, *G. catenatum* + Nd) for fourteen days (Exposure) followed by fourteen days (Recovery). Results are mean + standard deviation (n = 3, wherein one test unit was regarded as an aquarium). Significant differences ($p < 0.05$) among treatments are shown with different lowercase (Exposure period) and uppercase (Recovery period) letters. Asterisks represent significant differences between exposure and recovery periods.

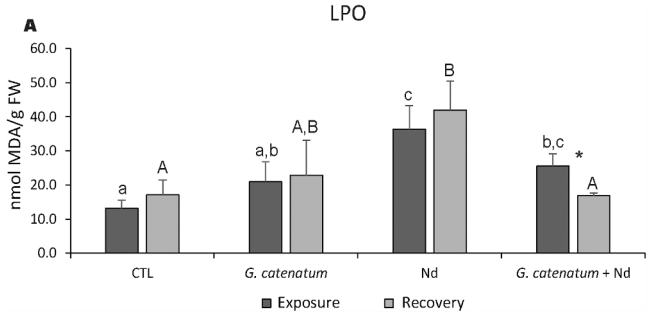

Fig. 8. A: Lipid peroxidation levels (LPO), in *Mytilus galloprovincialis* exposed to different treatments (CTL, *G. catenatum*, Nd, *G. catenatum* + Nd) for fourteen days (Exposure) followed by fourteen days (Recovery). Results are mean + standard deviation (n = 3, wherein one test unit was regarded as an aquarium). Significant differences ($p < 0.05$) among treatments are shown with different lowercase (Exposure period) and uppercase (Recovery period) letters. Asterisks represent significant differences between exposure and recovery periods.

G. catenatum and *G. catenatum* + Nd treatments, while after the recovery this patten changed with mussels previously exposed to both stressors presenting only dcSTX. The present study did not explore the conversion capacity, particularly through PST-transforming enzymes [58], but our

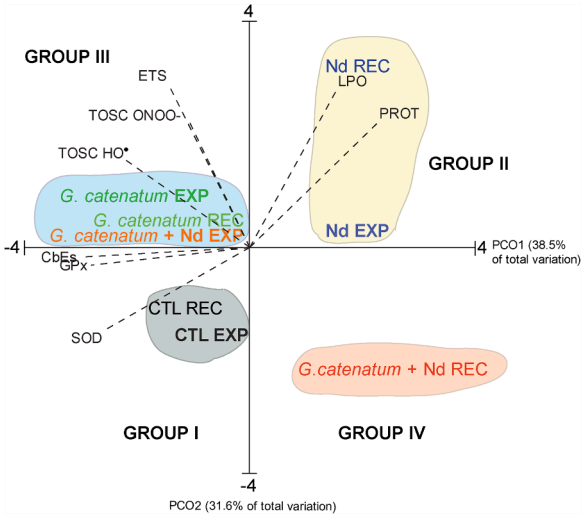

Fig. 9. Biochemical affinity groups (Group I (CTL (exposure and recovery), Group II (Nd (exposure and recovery), Group III (*G. catenatum* (exposure and recovery) and *G. catenatum* + Nd exposure and Group IV (*G. catenatum* + Nd recovery)) represented in the principal coordinate ordination.

results suggest that under more severe stress conditions, this pathway may have been the preferred method for mussels to restore their health status, potentially due to its lower metabolic cost. In fact, these results were accompanied with a significant decrease in ETS activity in the *G. catenatum* treatments after the recovery period compared to the exposure values, with a greater difference between exposure and the recovery period in the mixture (*G. catenatum* + Nd). This decrease may suggest that in the absence of the *G. catenatum* as food, there was no need to activate detoxification mechanisms and the mussels were able to return to CTL metabolic condition. In contrast, the increased ETS activity observed after the recovery period in mussels exposed to Nd, may indicate that organisms were still coping with the effects of Nd that was not eliminated from their tissues after recovery. Freitas et al. [30] also demonstrated that the exposure of *M. galloprovincialis* to Nd for 28 days enhanced mussels' metabolic capacity.

Aerobic metabolism, through various cellular pathways such as oxidative phosphorylation, and electron transport chains in mitochondria and microsomes, naturally produces reactive oxygen species (ROS) which can increase when organisms are under stressful conditions [59]. Among the various ROS produced, the prominent ones are the singlet oxygen ($^1O_2$), the superoxide anion ($O_2^{\bullet-}$), the hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO•) which rapidly react to form other molecules such as peroxynitrite (HOONO), hypochloric acid (HOCl), peroxyl radicals (ROO•) and alkoxyl radicals (RO•) [59]. The adverse effects caused by produced oxyradicals are counteracted by antioxidant defense mechanisms, such as SOD, GPx and other enzymes, but also low molecular weight scavengers such as glutathione. However, when organisms are exposed to stressful conditions, an overproduction of ROS may occur and, if not promptly neutralized by the antioxidant system, can cause damage to lipids, proteins and DNA [8,59] and under extremely stressful conditions may inhibited the enzymes [67]. In this study, the results showed that after the exposure period, the increase of ETS activity in the treatments with *G. catenatum* (Group III) was not paralleled by an increase in antioxidant capacity. After the recovery period, antioxidant enzymes decreased in the *G. catenatum* + Nd treatment compared to the exposure period and to the CTL, accompanying the significant decrease in ETS activity and lower accumulation of toxins in this treatment. In the Nd treatments an inhibition of antioxidant enzymes (Group II) was observed after a period of recovery, indicating that even after the removal of this metal, the effects remained. The increase in PROT observed in this treatment proves that the enzymes are in fact being inhibited, since greater production of PROT is not reflected in greater enzymatic activity.

In order to have a global perspective on the role of antioxidant defenses and cellular redox status, the overall capability of neutralizing different types of ROS was assessed through the total oxyradical scavenging capacity assay (TOSCA) [59,60]. Our findings revealed that, despite the Nd-mediated inhibition of the investigated antioxidant enzymes (SOD and GPx), there was an increase in total oxyradical scavenging capacity (TOSC) in these treatments after the recovery period indicating a greater effort of other antioxidant enzymes and scavengers that were not measured in this study. These findings may be related to the accumulation of Nd, which was not eliminated by mussels in the recovery period and remained in mussels' tissues. Despite this, higher levels of oxidized glutathione (GSSG) in the exposure to *G. catenatum* diet compared to the other treatments reveals that *G. catenatum* causes redox homeostasis disturbance in organisms. However, when *G. catenatum* was removed from the mussels' diet, organisms were able to restore their redox balance. Mussels exposed to arsenic (As) [67], lanthanum (La) and gadolinium (Gd) [19] also showed the ability to restore their redox balance and therefore improve their oxidative performance after a short recovery period.

The biotransformation enzymes like glutathione S-transferases (GSTs), which participate in conjugation, and carboxylesterases (CbEs), involved in hydrolysis, play a crucial role in the detoxification and elimination processes. These enzymes do not solely target foreign substances (xenobiotics), but also affect certain endogenous compounds [59,64]. This dual role significantly contributes to the overall safeguarding mechanism against chemical stress [59]. Since the concentration of Nd after the exposure was similar to the values found in mussels after the recovery, the increase in mussel's metabolism after recovery may be related to defense mechanisms such as GSTs. Also, the study by Leite et al. [45] revealed that *M. galloprovincialis* exposed to 10 μg L^{-1} of Nd at 17 ºC during the recovery period was unable to reduce the accumulated Nd, and the authors hypothesized that no chelating agent was added to the media to prevent these organisms from reabsorbing the excreted Nd. The present study showed that GSTs activity was higher in Nd treatments (Group II), which indicates that these enzymes are more effective in detoxifying this metal, while CbEs activity seemed to have an important detoxification role in mussels exposed to a bloom of *G. catenatum*. The study of Freitas et al. [30] also evidenced an increase in GSTs activity when mussels were exposed to 5 and 10 μg L^{-1} of Nd for 28 days, while Freitas et al. [31] showed that mussels contaminated with paralytic shellfish toxins had very low GSTs activity values, corroborating our findings.

As a consequence of the disturbance of redox homeostasis, cellular damage was observed in Nd treatments (Nd and *G. catenatum* + Nd), here assessed by lipid peroxidation levels (LPO, oxidative degradation of lipids) [59]. Also, studies by Freitas et al. [30], found that cell damage occurred in *M. galloprovincialis* when exposed to Nd for 28 days. However, in the recovery period, mussels previously exposed to the combination of *G. catenatum* with Nd (Group IV) were able to recover up to CTL values: these effects could be possibly due to a lower accumulation of dcSTX and the complete elimination of GTX5, which caused the organisms to diminish their metabolism and therefore produce lower ROS, resulting in reduced levels of LPO. This response was not observed in mussels subjected to Nd alone, where LPO levels remained constant, highlighting the persistence of effects caused by Nd. Similarly, [27] observed a more efficient recovery of lipid values in *Spisula solida* when exposed to La at high temperatures, and Maulvault et al. [48] reported a higher rate of elimination of inorganic As in *M. galloprovincialis* in a warming scenario.

5. Conclusion

This study investigated the recovery of mussel biochemical performance following exposure to *G. catenatum* cells and Nd. Results indicated that while mussels could recover from *G. catenatum* exposure alone, co-exposure to Nd hindered this recovery, potentially jeopardizing population maintenance and survival. Moreover, during the recovery phase, mussels failed to eliminate accumulated Nd, leading to persistent cellular damage even after exposure to clean seawater. This study also revealed synergistic effects when *G. catenatum* and Nd were present concurrently, suggesting that the presence of one stressor might influence the impact of the other. Nevertheless, further research is warranted to address emerging pollutant contamination in aquatic ecosystems, emphasizing the need for holistic approaches to ecosystem management and conservation. Comprehensive analysis of stressors on marine life, such as the one conducted in the present study, will contribute to achieve the Sustainable Development Goals (SDGs) 13, 14, and 15 ("Climate Action," "Life Below Water," and "Life on Land," respectively), promoting climate action and ecosystem protection. This study also contributes to SDGs 1 and 2 ("No Poverty" and "Zero Hunger"), benefiting coastal communities reliant on marine habitats. Understanding pollutant impacts on marine life is vital for human well-being (SDG 3).

CRediT authorship contribution statement

Amadeu Soares: Funding acquisition, Resources. **Eduarda Pereira:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing. **Rosa Freitas:** Conceptualization, Formal analysis, Funding

acquisition, Supervision, Writing – review & editing. **Francesco Regoli:** Funding acquisition, Resources. **Alessandro Nardi:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Marta Cunha:** Data curation, Formal analysis, Methodology, Writing – original draft. **Sabrina Sales:** Formal analysis, Methodology. **Maria Botelho:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, 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.jhazmat.2024.134220.

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