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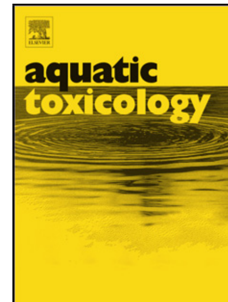

DNA damage and oxidative stress responses of mussels *Mytilus galloprovincialis* to paralytic shellfish toxins under warming and acidification conditions – Elucidation on the organ-specificity

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DNA damage and oxidative stress responses of mussels *Mytilus galloprovincialis* to paralytic shellfish toxins under warming and acidification conditions – elucidation on the organ-specificity

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Abstract

Commonly affected by changes in climate and environmental conditions, coastal areas are very dynamic environments where shellfish play an important ecological role. In this study, the oxidative stress and genotoxic responses of mussels (*Mytilus galloprovincialis*) exposed to paralytic shellfish toxin (PST) - producing dinoflagellates *Gymnodinium catenatum* were evaluated under *i*) current conditions (CC: 19 °C; pH 8.0), *ii*) warming (W: 24 °C; pH 8.0), *iii*) acidification (A:19 °C; pH 7.6) and *iv*) combined effect of warming and acidification (WA: 24 °C; pH 7.6). Mussels were fed with *G. catenatum* for 5 days, and to a non-toxic diet during the following 10 days. A battery of oxidative stress biomarkers and comet assay was performed at the peak of toxin accumulation and at the end of the post-exposure phase. Under CC, gills and hepatopancreas displayed different responses/vulnerabilities and mechanisms to cope with PST. While gills presented a tendency for lipid peroxidation (LPO) and genetic damage (expressed by the Genetic Damage Indicator - GDI), hepatopancreas seems to better cope with the toxins, as no LPO was observed. However, the mechanisms involved in hepatopancreas protection were not enough to maintain DNA integrity. The absence of LPO, and the antioxidant system low responsiveness, suggests DNA damage was not oxidative. When exposed to toxic algae under W, toxin-modulated antioxidant responses were observed in both gills and hepatopancreas. Simultaneous exposure to the stressors highlighted

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gills susceptibility with a synergistic interaction increasing DNA damage. Exposure to toxic algae under A led to genotoxicity potentiation in both organs. The combined effect of WA did not cause relevant interactions in gills antioxidant responses, but stressors interactions impacted LPO and GDI. Antioxidant responses and LPO pointed out to be modulated by the environmental conditions in hepatopancreas, while GDI results support the dominance of toxin-triggered process. Overall, these results reveal that simultaneous exposure to warming, acidification and PSTs impairs mussel DNA integrity, compromising the genetic information due to the synergetic effects. Finally, this study highlights the increasing ecological risk of harmful algal blooms to *Mytilus galloprovinciallis* populations.

Keywords: Harmful algal blooms; Climate change; Genotoxicity; Oxidative stress responses; Saxitoxins; *Gymnodinium catenatum*

1. **Introduction**

Coastal areas and estuaries are highly productive ecosystems where shellfish species play a fundamental role linking planktonic and nektonic communities and acting as ecosystem engineers capable of changing the abiotic environment around them (Sousa et al., 2009). However, coastal areas are recurrently under intense pressures that may affect shellfish well-being and survival. Therefore, shellfish are frequently exposed to pressures that range from anthropogenic contamination to pathogens and parasites, to variations on the abiotic environmental conditions, such as seawater temperature increase, pH decrease (a.k.a. acidification), salinity and nutrient fluctuations, changes in freshwater input, and exposure to toxic phytoplankton blooms (González and Puntarulo, 2016; Hégaret et al., 2007).

Harmful algal blooms (HABs) can have profound impacts on shellfish settlements and populations, leading in extreme cases to mass mortalities (Hégaret et al., 2011). HABs constitute a significant problem for shellfish farming, due to their adverse effects in metabolic fitness and organisms' health, and due to its impacts on human health as shellfish may act as vectors of HAB-toxins.

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There are a vast number of phytoplankton species capable of producing marine biotoxins, among these are the dinoflagellates from the genera *Alexandrium, Pyrodinium* and *Gymnodinium* producers of saxitoxin (STX) and their derivatives (Gedaria et al., 2007; Hallegraeff et al., 2012; Silva et al., 2015). STX and related compounds are among the most frequent biotoxins accumulating in filter-feeding organisms, such as shellfish, in the North Atlantic (Bricelj and Shumway, 1998; Brown et al., 2010). These toxins are responsible for the human illness paralytic shellfish poisoning (PSP) which is characterised by symptoms that range from nausea and diarrhoea to confused speech, paralysis and, in extreme concentrations, death (Landsberg et al., 2006; Manfrin et al., 2012).

While the impacts of these toxins in humans are well known, and their action mode almost completely described, the same cannot be said for biotoxins impacts in shellfish. Only in the last decades the effects of paralytic shellfish toxins (PST), and other biotoxins in shellfish gained relevance. Initially, this research started by focusing mostly on behavioural aspects and only some physiological parameters were investigated. A wide range of responses, ranging from reduction in the clearance rates, shell valves activity, byssus production, O_2 consumption and heart rate, as well as hatching and larval survival rates, have been reported for several shellfish species exposed to PST (Landsberg, 2002; Shumway and Gainey, 1992).

Shellfish defence mechanisms are composed by an innate immune system, that includes hemocytes and humoral elements, and is the first response to the presence of exogenous compounds based on neutrophil activation and inflammatory factors initiation, including peroxides and oxidative radicals production (Harikrishnan et al., 2011; Hégaret and Wikfors, 2005). Peroxides, oxidative radicals and other reactive oxygen species (ROS) have their origin in several biological processes, being essential to the physiological control of critical cellular functions (Fabioux et al., 2015; Guilherme et al., 2008a; Qiu et al., 2013). ROS also present a high potential to cause oxidative damage, since they can react with a wide range of biomolecules and induce protein degradation, enzymatic inhibition, lipid peroxidation (LPO), DNA damage and, in extreme conditions, cell death and tissue damage (Choi et al., 2006; Fabioux et al., 2015; Gerdol et al., 2014; Guilherme et al., 2008a). In order to maintain the redox balance and minimise oxidative damage, an antioxidant system evolved in the animals to prevent the overgeneration

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and neutralise ROS. This system includes antioxidant enzymes, as catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and glutathione reductase (GR), and non-enzymatic compounds as the reduced glutathione (GSH). Although the high efficiency of this system, not all ROS produced in the cells are deactivated, the imbalance between the formed and neutralised ROS is designated as oxidative stress (Fabioux et al., 2015; Guilherme et al., 2008b; Valavanidis et al., 2006). As a consequence of oxidative stress, LPO may occur from the attack of cellular polyunsaturated fatty acids by ROS, causing membrane destabilisation and disintegration. Also, LPO reaction originates malondialdehyde (MDA), known for causing protein synthesis inhibition, DNA adducts formation and for being genotoxic, mutagenic and carcinogenic (Melegari et al., 2012).

In shellfish exposed to PST, an increase of ROS was observed by several authors, depicting a species-specific response of the antioxidant system when considering Manila clam (*Ruditapes philippinarum*), mussels (*Mytilus galloprovincialis*) and scallops (*Patinopecten yessoensis*) feeding on *Alexandrium tamarense* (Abi-Khalil et al., 2017; Cao et al., 2018; Choi et al., 2006; Qiu et al., 2013). Mussels presented a faster response, taking advantage of CAT, SOD and GPx to reduce ROS, while scallops presented a slower response (Qiu et al., 2013). Also, PST exposure induced LPO and DNA damage in shellfish, namely in oysters (*Crassostrea gigas*) and scallops (*Chlamys farreri*) (Abi-Khalil et al., 2017; Cao et al., 2018; Choi et al., 2006; Qiu et al., 2013).

As mentioned above, shellfish have to deal with abiotic environmental changes, such as the increase of seawater temperature, acidification, and to recurrent exposure to HABs. Exposure to environmental drivers, either individually or combined, causes significant impacts in shellfish. While warming effects can be ambivalent depending on oxygen content and food availability, the balance between these conditions is difficult to occur, and adverse outcomes as behavioural and metabolic changes, growth rates reduction and increased xenobiotic accumulation are often reported (Anestis et al., 2007; Coppola et al., 2017; Filgueira et al., 2016). Acidification affects shellfish by compromising the structure and growth of the shell and altering several physiological and metabolic processes, from thermal stress tolerance to growth rates (Duarte et al., 2014; Griffith and Gobler, 2020; Nikinmaa and Anttila, 2015).

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Despite the growing awareness for non-linear responses, and additive, synergistic or antagonistic interactions when exposure to multiple stressors occurs, limited data is available regarding the combine effects of warming and acidification in shellfish (Hu et al., 2015; Nardi et al., 2017; Wu et al., 2016). Even less is known when to warming and acidification a third stressor, such as HABs, is added.

Therefore, the present study aims to assess the organ-specific (gills and hepatopancreas) modulation of the antioxidant system and the eventual induction of LPO and DNA damage in mussels *Mytilus galloprovincialis* exposed to toxic dinoflagellate *Gymnodinium catenatum* under two relevant climate change drivers, ocean warming and acidification, also addressing the follow-up of the responses in the post-exposure period.

2. Material and methods

2.1. Mussels collection and acclimation

One hundred and forty immature mussels *Mytilus galloprovincialis* (53.8 ± 6.2 mm shell length) were harvested from the Aveiro Lagoon (Portugal) in July 2016, during a period of no PSP toxicity in shellfish (Braga et al., 2018). Mussels were cleaned from macro-algae and epibionts, and placed in four 150-L tanks, under the conditions described in Braga et al. (2018).

Each tank simulated an environmental scenario of current conditions of temperature and pH (CC: 19 °C; pH 8.0), warming (W: 24 °C; pH 8.0), acidification (A: 19 °C; pH 7.6) and warming and acidification combined (WA: 24 °C; pH 7.6) (Fig. 1). These treatments correspond to the current environmental conditions and scenarios predicted by the Intergovernmental Panel on Climate Change (IPCC, 2013). Each tank was subdivided into 6 sections to allow an experimental design encompassing toxin-exposed and control groups in triplicate ($2 \times 3 = 6$).

Mussels were allowed to gradually adjust to the new conditions, increasing 1 °C and decreasing 0.1 pH unit per day, and then were let to acclimate for 21 days. Temperature and pH levels were automatically adjusted whenever needed, through automatic

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seawater refrigeration systems, submerged digital heaters and a computerised pH control system, as described in Braga et al. (2018).

The following abiotic conditions were maintained in the tanks: i) dissolved oxygen (DO) > 5 mg L^{-1}; ii) salinity = 35.7 ± 0.4 ‰; iii) photoperiod 12 h light : 12 h dark. Temperature, pH, salinity and DO were daily checked using a multi-parameter measuring instrument (Multi 3420 SET G, WTW, Germany). Ammonia, nitrite and nitrate levels were daily checked using colourimetric tests (Tropic Marin, USA), and kept below detectable levels with daily water changes, except nitrates, which were kept below 2.0 mg L^{-1}. Seawater total alkalinity was also weekly measured in every tank, spectrophotometrically at 595 nm, following a protocol described by Sarazin et al. (1999) and the combination of total alkalinity and pH was used to calculate carbonate system parameters. A summary of seawater parameters is reported in Table S1 and Fig. S1 (Supplementary material). During acclimation, the mussels were fed with 200,000 cells per day of the freeze-dried *Tetraselmis* sp., a non-toxic diet (Necton, Olhão, Portugal).

2.2. G*ymnodiniun catenatum* cultivation

The strain IO-13-04 of *G. catenatum*, isolated in 2005 in the NW Portuguese coast, was obtained from the Lisbon University (ALISU) algae culture collection. Cells were cultivated as in Braga et al. (2018). Briefly, cells were grown in filtered and autoclaved seawater (30 ‰ salinity) and enriched with GSe medium (Doblin et al., 1999), at 18 °C with a 12 h light : 12 h dark cycle under fluorescent lights. Cells were concentrated (10 µm mesh sieve) and harvested at a density of approximately 2.5 × 10^6 cells per litre.

The toxin concentrations were determined in algae cell culture as described in Braga et al. (2018), whose toxin profile included the sulfocarbamoyl toxins C1+2 (with a concentration of 40.80 fmol.cell^{-1}, corresponding to 95.7 % of the toxin molar fraction) and GTX5 (1.01 fmol.cell^{-1}, 2.4 %), and the decarbamoyl toxins dcNeo (0.42 fmol.cell^{-1}, 1.0 %), dcGTX2+3 (0.31 fmol.cell^{-1}, 0.7 %) and dcSTX (0.1 fmol.cell^{-1}, 0.2 %).

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2.3. Exposure of mussels to toxic dinoflagellates

In the exposed groups, mussels were fed with approximately 91 000 *G. catenatum* cells per day per mussel, for 5 days. At the end of the exposure period, the feeding was changed to a non-toxic diet (*Tetraselmis* sp., as described for acclimation), which lasted for 10 days to allow toxin elimination and the recovery of the eventual effects. Mussels from the control groups were fed as described above for the acclimation period.

On the fifth day of exposure to toxic dinoflagellates *G. catenatum*, mussels from the exposed and control groups were harvested *per* condition to assess oxidative stress and genetic damage. The same sampling procedure was repeated on day 15, at the end of the post-exposure period (10 days). The exposure period and the algal concentration was chosen in order to simulate a natural bloom and to ensure that the toxin accumulation in mussels reached the regulatory limits of 800 µg of STX equivalents per kg of shellfish.

The toxin concentrations in the mussels (whole soft tissues) at days 5 and 15 are presented in table 1, as described in Braga et al. (2018).

2.4. Evaluation of oxidative stress and genetic damage

Mussel tissues sampling and preparation

Seven mussels were harvested in each sampling moment for antioxidants, LPO and genetic damage assessment. The gills and hepatopancreas were excised from each specimen and divided into two subsamples: one for oxidative stress analyses, which was immediately frozen in liquid nitrogen and stored at -80 °C until analyses. The other subsample used for genetic damage evaluation was slowly frozen in an antifreeze solution of PBS and DMSO (9:1, v:v) and kept in thermal insulation material, initially at -20°C for 1 week and then at -80 °C until analyses.

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Oxidative stress analyses

The oxidative stress parameters were analysed following the procedures previously described by Marques et al. (2017, 2016). The tissues were homogenised and divided into two aliquots: for post-mitochondrial supernatant (PMS) and the LPO sample preparation.

The PMS fraction was obtained by centrifugation, while LPO aliquot was obtained by adding butylated hydroxytoluene (BHT) and phosphate buffer to the PMS fraction to prevent oxidation (Bird and Draper, 1984; Ohkawa et al., 1979; Wilhelm Filho et al., 2001a, 2001b). Both PMS and LPO aliquots were stored at -80 °C until further analyses.

The total protein content was determined according to the Biuret method (Gornall et al., 1949), using bovine serum albumin (E. Merck-Darmstadt, Germany) as a standard the results were expressed in mg mL^{-1}.

Catalase (CAT) activity was determined following Claiborne (1985) method, with Marques et al. (2016) modifications. The assay mixture was adjusted for 5 µL of PMS samples. The absorbance variation was measured in a microplate reader (SpectraMax 190), at 240 nm. The CAT activity was calculated in µmol H_2O_2 consumed min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 43.5 M^{-1} cm^{-1}.

The superoxide dismutase (SOD) activity was assessed using a Ransod kit (Randox Laboratories Ltd., UK). SOD activity was detected in the microplate reader at 505 nm, and the results were expressed as SOD units mg $protein^{-1}$.

Glutathione reductase (GR) activity was determined following Cribb et al. (1989) with Marques et al. (2016) modifications. The enzyme activity was determined by measuring the oxidation of NADPH at 340 nm in the microplate reader and calculated as nmol NADPH oxidised min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 6.22 $\times 10^3$ M^{-1} cm^{-1}.

Glutathione peroxidase (GPx) activity was determined according to Claiborne (1985) and modified as in Athar and Iqbal (1998) as described in (Marques et al., 2017). NADPH oxidation was recorded in the microplate reader at 340 nm. GPx activity was calculated in nmol NADPH oxidised min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 6.22 $\times 10^3$ M^{-1} cm^{-1}.

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Glutathione-S-transferase (GST) activity was determined following the Habig et al. (1974) method as described in (Marques et al., 2017). The absorbance variation was recorded in the microplate reader at 340 nm. GST activity was calculated as nmol CDNB conjugate formed min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 9.6×10^3 M^{-1} cm^{-1} (Bird and Draper, 1984).

The total glutathione content (GSHt) was determined following Baker et al. (1990) and Tietze (1969). Proteins were precipitated, centrifuged, and GSHt was analysed in the supernatant as in (Marques et al., 2017). The GSHt content was assessed through the formation of 5-thio-2-nitrobenzoic acid (TNB), measured in the microplate reader at 415 nm. The results were express as nmol of TNB formed min^{-1} mg $protein^{-1}$ using a molar extinction coefficient of 14.1×10^3 M^{-1} cm^{-1}.

Lipid peroxidation (LPO) was determined through thiobarbituric acid reactive substances (TBARS) quantification as in Ohkawa et al. (1979) and Bird and Draper (1984) adapted by Wilhelm Filho et al. (2001a, 2001b). Briefly, protein determination was performed on the aliquot prepared for LPO determination after homogenisation. LPO was then determined as described in (Marques et al., 2017). The absorbance was measured at 535 nm in the microplate reader. LPO was expressed in nmol of TBARS formed mg $protein^{-1}$ using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Genetic damage evaluation

Genotoxicity caused by PST and the altered environmental conditions was assessed by the comet assay. The alkaline version of this methodology was performed following Collins (2004) procedures with Guilherme et al. (2010) modifications. The tissues were cut (gills two times and hepatopancreas one time) in a PBS bath (pH 7.4; 0,01 M). 1 mL of cell suspension in PBS was collected in each sample and centrifuged at 1,500 rpm, at 4 °C, for 5 minutes. The supernatant was then discarded (990 µL) and the pellet resuspended in a new PBS (1 mL). Again, the cell suspension was centrifuged and 990 µL of the supernatant discarded. To the remaining 20 µL of cell suspension, was added 70 µL of 1% agarose LMP (in PBS). From this solution, two mini-gels, with 6 µL of cell suspension, were placed onto an agarose NMP pre-coated slide and refrigerated for 5

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minutes at 4 °C. In order to improve the assay output, each slide contained four samples, in a system of eight gels per slide with two replicates per sample

The slides were immersed in a lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris and 1% Triton X-100, and 10% of DMSO, pH 10) at 4 °C, for at least 1 h, in the dark. The slides were then moved to the electrophoresis tank (Sub-Cell® GT, Bio-Rad), immersed in the electrophoresis solution, for 20 min, to alkaline treatment. The DNA migration was performed for 15 minutes, at a fixed voltage of 25 V, a current of 300 mA (power supply PowerPac™, Bio-Rad), which results in 0.7 V cm^{-1} (achieved by adjusting the total volume of buffer). The slides were then neutralised in PBS for 10 minutes, followed by 10 minutes in distilled water and 10 minutes in ethanol (100%) at 4 °C, and left to dry at room temperature overnight. For nucleoids counting and DNA damage evaluation, the slides were stained with ethidium bromide (20 µg mL^{-1}) and observed using a Leica DMLS fluorescence microscope (× 400 magnification).

Visual classification of nucleoids into five comet classes, according to the tail intensity and length (0 - no tail; 4 - almost all DNA in tail), was performed to quantify DNA damage. The Genetic Damage Indicator (GDI) was calculated according to the formula:

$$\mathrm{GDI} = \sum \%\ \text{nucleoids class}\ i\ \times i$$

Where *i* is the number of each defined class (ranging within 0-4).

GDI values were expressed as “arbitrary unit” in a range of 0–400 per 100 scored nucleoids (average value for the two mini-gels observed per sample).

2.5. Statistical analysis

Statistica 7.0 software was used for statistical analysis. The three sets of data, antioxidants, lipid peroxidation and genetic damage, were tested for outliers through the Grubbs Statistic Test, normality through the Shapiro-Wilks test, and homogeneity of variance through Levene’s Test, to assess statistical demands. When the statistical demands were not satisfied, the data were transformed. The different sets of data were analysed only within each sampling moment (Exposure or Post-exposure). A two-way ANOVA followed by Tukey HSD test as post-hoc comparison tests were applied to assess

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the effects of each factor and the interaction of the factors in mussels' responses. The factors considered were "toxins exposure" (mussels exposed to toxic algae~~,~~ and negative control), and "environmental condition" (mussels under four different conditions, CC: 19 °C and 8.0 pH; W: 24 °C and 8.0 pH; A: 19 °C and 7.6 pH; WA: 24 °C and 7.6 pH). Statistical differences were considered significant at $p < 0.05$ (Zar, 1996).

3. Results

3.1. Oxidative stress responses of mussels exposed to *Gymnodinium catenatum* under different conditions of temperature and pH

Antioxidant system modulation: Gills responses

Mussels with a diet restricted to non-toxic algae (*Tetraselmis* sp.) showed no significant responses of the gills antioxidant system on day 5 when comparing the different environmental conditions tested (CC, W, A and WA) (Fig. 2 and Table S2). However, on day 15, a significant increase of GPx activity was observed in mussels kept under W, A, and WA when compared to CC, as well as an increase of GSHt content in mussels under the combined effect of warming and acidification (WA) when compared to W.

Analysing the effects of exposure to the toxic algae *G. catenatum* (through statistical comparisons with the unexposed group) within each environmental scenario, significant changes are noticeable (Fig. 2 and Table S2). At the peak of toxin accumulation (day 5), a decrease of SOD activity and GSHt content, as well as induction of GPx activity, were observed in mussels maintained under the current conditions (CC). Similarly, a decrease of SOD and induction of GPx activity was observed in mussels under warming conditions (W). However, an inversion of GSHt content variation was registered, being significantly augmented in mussels maintained in W. Mussel gills were particularly impacted under the acidification conditions (A), showing induction of CAT, SOD and GPx activity, as well as GSHt content increase. Responses of mussels to the toxic algae under the combined

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effect of warming and acidification (WA) were similar to CC, with a reduction of SOD activity and GSHt content, coupled with induction of GPx activity.

Following toxin exposure, the antioxidant responses of mussels at the end of 10 days feeding on *Tetraselmis* sp. (post-exposure period) were limited to induction of GPx activity under CC and its reduction under WA.

Antioxidant system modulation: Hepatopancreas responses

Mussels maintained under different environmental conditions (CC, W, A and WA) did not show significant responses of the antioxidant system on day 5, when the diet was constituted by non-toxic algae (*Tetraselmis* sp.). An exception was the increase of GST activity observed under WA when compared with the mussels kept under A (Fig. 3 and Table S3). However, on day 15, CAT activity was significantly reduced in mussels under warming (W) and the combined effect of warming and acidification (WA). In contrast, an increase in CAT activity was observed for mussels under acidification (A) conditions, always in comparison with CC. A reduction of GPx activity was also observed in mussels under acidification (A) conditions comparing to warming (W).

When *G. catenatum* was introduced into mussels' diet, significant changes were observed for two parameters (Fig. 3 and Table S3). Increasing GR activity and GSHt content were observed in mussels (toxin-exposed *vs* unexposed) under CC, while under the remaining conditions (W, A and WA), the only parameter affected was GR activity, which decreased in W and increased in A and WA. After switching the diet to non-toxic algae (*Tetraselmis* sp.), which corresponded to the post-exposure period, the antioxidant responses of mussels were limited to induction of GPx activity in CC and its reduction under the remaining environmental conditions.

Peroxidative damage induction: LPO in gills

Mussels fed on non-toxic algae revealed no significant alterations on LPO levels when comparing the different environmental conditions on day 5, with the exception of

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an LPO decrease observed in group W comparing to CC (Fig. 4 and Table S4). However, on day 15 LPO increased in all altered conditions tested (W, A, and WA) *versus* CC, with particularly high levels being observed in mussels under W and A (also significantly higher than WA).

In the case of mussels exposed to *G. catenatum*, a significant increase of LPO values was observed in animals maintained under the combined effect of warming and acidification (WA) (Fig. 4 and Table S4). After replacing toxic diet by non-toxic algae, a significant increase in the LPO level was measured in mussels under CC conditions, while mussels maintained under the remaining environmental conditions (W, A, and WA) presented significantly lower values.

Peroxidative damage induction: LPO in hepatopancreas

When non-toxic algae constituted the diet, no significant changes in LPO levels were observed at any environmental condition (CC, W, A or WA) tested (Fig. 5 and Table S5). Also, no significant changes in LPO were observed in mussels exposed to *G. catenatum* in comparison with those unexposed (Fig. 5 and Table S5), in any condition.

3.2. Genetic damage in mussels exposed to *Gymnodinium catenatum* under different conditions of temperature and pH

DNA damage induction in gills

Mussels fed on non-toxic algae did not display significant changes in the genetic damage indicator (GDI) at any of the environmental condition that were tested (CC, W, A or WA) (Fig. 6 and Table S6). However, when the toxic dinoflagellate *G. catenatum* was introduced into mussels' diet, significantly higher values of GDI were observed in gills in all the environmental conditions tested, except for CC (Fig. 6 and Table S6). After replacing the toxic diet by non-toxic algae, simulating an elimination stage after an algae bloom, the GDI measured in the gills did not present significant differences in this experiment.

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DNA damage induction in hepatopancreas

Analysing the GDI levels in mussels fed on non-toxic algae according to the environmental scenario (CC, W, A or WA), significant differences were observed between all the conditions tested, on day 5, with mussels under A presenting the highest GDI (Fig. 7 and Table S7). At the end of the experimental period, the highest GDI value was observed in mussels under WA, though all the altered conditions displayed higher DNA damage than CC.

Exposure to *G. catenatum* also produced significant changes in the hepatopancreas GDI, disclosing increased values in all the environmental conditions tested on day 5 (Fig. 7 and Table S7). On the recovery period, only mussels under WA presented significant differences, with an increase in the GDI value.

4. Discussion

4.1. DNA damage and oxidative stress responses of mussels exposed to toxic algae *G. catenatum* under the current conditions of seawater temperature and pH

The present work evaluated the biochemical and cytogenetic responses in two key organs, *viz*. gills and hepatopancreas, of mussels exposed to the toxic dinoflagellate *Gymnodinium catenatum*. Both gills and hepatopancreas antioxidant systems were modulated at the end of 5 days simulating the exposure to a toxic algal bloom.

Gills showed ambivalent responses of the antioxidant system, which, together with the absence of LPO and GDI increments, suggests a low risk of paralytic shellfish toxins (PST). Gills are the first organ in contact with the toxic dinoflagellates, but toxins may only reach gills after systemic circulation or due to eventual cell lysis of *G. catenatum*. By this reason, the amount of toxins reaching the gills on day 5 were not high enough to substantially induce ROS production, promote changes on antioxidant activities (CAT, GR, and GST), and induce damage in key molecules like lipids and DNA.

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On the other hand, the observed decrease in SOD activity and GSHt content may be an early sign of toxicity, as the induction of ROS and antioxidant responses is a relatively rapid phenomenon (Qiu et al., 2013). The antioxidant depletion reported could be related to SOD protein degradation, particularly when the presence of PST may compromise enzyme and co-factors renewal (Mat et al., 2013; Mello et al., 2013). As for GSHt, the overall responses of the glutathione redox cycle (GPx and GR) suggest the occurrence of active removal of GSSG from the cells (Fabioux et al., 2015; Kaplowitz, 1981; Soldatov et al., 2007). Also, GSH sequestration through the formation of PST-GSH conjugates may be occurring, as GSH is closely related to the PST biotransformation (Sakamoto et al., 2000; Sato et al., 2000). The increase of LPO detected 10 days after the diet change, disclosing a late effect, cannot be dissociated from the exposure route (toxin reaches the gills mainly systemically). It can also be a consequence of the above-hypothesised impairments occurring at the peak of exposure, affecting SOD action and glutathione redox cycle. Therefore, the statement that, under the current environmental conditions (CC), *G. catenatum* toxins do not possess a pro-oxidant potential on gill cells must be assumed cautiously. The hepatopancreas, which is the main toxins storage organ, showed an effective capacity to deal with PSTs. The observed increase of GSHt content can be related with the need for ROS elimination and with toxin metabolism and biotransformation. In addition, no LPO was detected throughout the experiment suggesting the hepatopancreas ability to cope with pro-oxidant challenges associated with PST.

Nevertheless, an increase in DNA damage was observed in the hepatopancreas at day-5 of the exposure period. The possible adaptation of this organ to cope with PST by increasing toxins biotransformation and elimination via glutathione-dependent pathways (as denunciated by the GSHt increase) does not seem enough to protect DNA integrity under high PST concentrations. In fact, the increasing damage detected in the DNA could be related to the glutathione-dependent elimination pathways. Several *in vitro* studies indicated that GSH might be involved in shellfish transformation of PST analogues (Sakamoto et al., 2000; Sato et al., 2000). These studies reported a spontaneous reaction of several STX analogues after incubation with GSH, under heavy heating conditions (70 °C), resulting in chemically simpler compounds.

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The role of GSH is usually related to the increase of hydrosolubility of xenobiotics, enhancing their elimination and decreasing their toxicity (Kaplowitz, 1981). In the case of PSTs, the structurally less complex compounds, products of toxins transformation, such as dcSTX and STX, present higher toxic potential. It is thus possible that the observed increase of DNA damage is related to the increase in glutathione content and lower elimination of the most potent PST. However, it is important to note that at the end of the experiment, when most of the toxins were eliminated, GDI decreased to control levels, pointing out the activation of DNA repair processes, through base excision repair (BER) and nucleotide excision repair (NER) mechanisms, and/or damaged cells turnover.

Exposure to the toxic algae seems to cause more damage in the DNA than in the lipids, as no significant variation was observed in LPO. This lack of LPO increase in the hepatopancreas, combined with low responsiveness of the antioxidant system, suggests that the DNA integrity loss, was not of oxidative origin and other mechanisms independent of ROS and oxidative stress may be causing the damage in the genetic material. Not much is known on how the toxins react with shellfish DNA, but, recently, oysters exposed to PST showed that DNA fragmentation associated apoptosis is a caspase-dependent process rather than an oxidative phenomenon (Abi-Khalil et al., 2017).

Gills and hepatopancreas seem to have different responses/vulnerabilities and mechanisms to cope with PST. This idea is in line with the differential PST metabolism theory proposed by Jaime et al. (2007) to justify distinct PST biotransformation in several tissues/organs of shellfish species.

4.2. Interference of warming and acidification on DNA damage and oxidative stress responses of mussels to toxic algae *G. catenatum*

Several studies have been carried out to assess the impact of environmental changes in shellfish. Most of these studies were focused on a single stressor, such as the temperature, pH or salinity (Anestis et al., 2007; Múgica et al., 2015; Nikinmaa and Anttila, 2015; Parker et al., 2013), and few approaches covered multiple factors or tried

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to understand their combined effects in bivalves (Freitas et al., 2017; Hu et al., 2015; Matozzo et al., 2013). In the present work, the effects of exposure to the toxic dinoflagellate *G. catenatum* were studied under scenarios of warming (W) and acidification (A), and with the two factors combined (warming and acidification - WA).

Modulation of mussels' DNA integrity and oxidative stress endpoints by warming and/or acidification

Mussels kept under warming (W) conditions and feeding on non-toxic algae only reflected alterations in the antioxidant system responses at the end of the experimental period. Despite some previous studies indicating that increased temperature affects mussels metabolic of mechanisms (Anestis et al., 2010, 2007), the results of this study are in accordance with data obtained by Kamel et al. (2012) showing no significant different antioxidant enzymes responses. It should be highlighted that a significant GDI reduction in hepatopancreas was observed at the peak of toxin accumulation (day 5) and the opposite variation at the end of the post-exposure period (day 15), suggesting an early activation of DNA repair mechanisms and their exhaustion with the continuation of the stimulus.

The effect of an acidified environment on the antioxidant system of mussels has been under debate, and while, some authors reported the induction of the activity of antioxidant enzymes (Hu et al., 2015; Matozzo et al., 2013), others suggested unaltered activities (Freitas et al., 2017; Nardi et al., 2017). In this study, a similar response was found in mussels under acidification (A) and W conditions, as no effects were detected in the antioxidant system. Regarding LPO, acidification seems to cause a delayed effect in the gills, but not affecting hepatopancreas, revealing a tissue-dependent response. However, acidification caused a GDI increase in hepatopancreas, suggesting that the DNA repair machinery is unable to respond effectively to the stressor, which may lead to degradation of the population genetic information.

Under the combined effect of warming and acidification (WA), significant differences were only detected at the end of the experiment. Results obtained in the present work are in line with those reported by Hu et al. (2015) and Matozzo et al. (2013) where the

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antioxidant system response seems to increase with shellfish exposure to a higher temperature and lower pH levels, and where the significant interactions between the effects of the stressors were detected for some of the parameters tested, namely GPx. On the other hand, some studies are presenting contrasting information, with an opposite response in GPx modulation in the gills (Nardi et al., 2017), highlighting the need for more studies considering the organ-specific impacts of exposure to multiple stressors.

Effects of exposure to toxic algae under warming conditions

Environmental changes and their effects on marine organisms are a complex issue that results from the interaction of multiple factors, that may lead to either decreased (antagonism) or increased (synergism or potentiation) toxicity (Duarte et al., 2014; Lischka et al., 2011). The most purposive approach to shed light on the combined impact of toxins and warming, elucidating causality relationships and hypothetical factor interactions, goes through comparing the effects of both stressors acting simultaneously with those resulting from isolated exposures (see Table S8 - supplementary material, for a summary of all interactions). Hence, in what concerns gills and addressing first the antioxidant responses, it is clear that no relevant differences emerged (no interactions) as a result of the combination of factors (toxic algae exposure and warming). The exception was GPx at the PE, when an antagonistic interaction of both factors was evident, neutralising the activity elevation.

Regarding LPO in gills, the interpretation is more complex. No effect was detected in mussels fed with toxic algae under W, as was observed for CC, suggesting a dominance of the toxin-triggered processes. However, an antagonistic interaction (with mutual interference) was detected at the end of the experiment, when an LPO reduction was measured, which contrast with induction of LPO measured when factors were evaluated individually.

Concerning genetic damage, the occurrence of a synergistic interaction on day 5, when simultaneous exposure induced DNA damage, must be highlighted. Indeed, it is important to recognise that while no interactions were detected for the antioxidant

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responses, the increase of GDI values was parallel to the increase in GSHt content in mussels gills exposed to the toxic algae. This increase in GDI levels is an environmentally relevant finding, highlighting the increased risk of mussels exposed to the interaction of harmful algal blooms and warming.

On the other hand, no signs of increased risk resulting from the concomitant exposure to toxins and warming were observed from the LPO analysis in hepatopancreas. Overall, deeming the damage indicators LPO and GDI, gills showed to be more sensitive to the interaction of environmental factors than the hepatopancreas, reinforcing the previous findings and stating the existence of organ-specific response patterns.

Effects of exposure to toxic algae under acidification conditions

Mussels under the combined impact of toxic algae and acidification showed relevant differences on the gills' antioxidant responses when compared to the effects observed in mussels exposed to non-toxic algae under the acidification treatment (Table S8). The interactions detected for these parameters while measurable do not fit the typified patterns (*i.e.*, antagonism, synergism and potentiation). In terms of LPO, which is indicative of damage mainly on cellular membranes, an antagonistic effect was found at the end of the experiment in gills. This antagonistic response is related to the activation of repair mechanisms to minimise the membrane damage (Fabioux et al., 2015; Guilherme et al., 2008b). However, these repair mechanisms may be specific for LPO damage as no clear sign of apoptosis, as a reduction of DGI, was observed. At the peak of toxin exposure (day 5), a significant increase in DGI was observed under acidification, indicating a genotoxicity potentiation under these conditions.

It is noteworthy that the simultaneous increase of GSHt and GDI previously found in the hepatopancreas of mussels exposed to *G. catenatum* under CC was also observed in the gills of mussels exposed to the toxic algae under warming and under acidification conditions. This result emphasises the existence of a possible relation between the role of GSHt in toxins elimination and the increased DNA damage, as suggested above, reinforcing the idea that glutathione-dependent elimination pathways may increase the

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risk to shellfish when PSP analogues are concerned (Sakamoto et al., 2000; Sato et al., 2000).

The only significant response in hepatopancreas after 5 days of exposure to the toxic algae under acidification was GR activity, which seems to be modulated by the toxin presence since it was similar to the response obtained in mussels exposed to the toxins under CC. Also, the genetic damage was clearly modulated by the toxin presence with the increase of GDI. In this case, an underlying addictive effect may be occurring since mussels exposed to acidification and toxins independently already revealed a significant increase in genetic damage.

Effects of exposure to toxic algae under combined conditions of warming and acidification

The combined effect of exposure to the three stressors (W + A + Toxin) did not originate any relevant interaction in the antioxidant response system of gills at the peak of toxic algae exposure. The main differences in the gills' response profile were observed for LPO and GDI. A significant increase in both damages was found after exposure to toxic algae, warming and acidification due to the stressors' interaction. This interaction profile, probably, fits better on the additivity then on synergism and reveals that under combined exposure to warming and acidification mussels' gills are particularly vulnerable to PST. These responses changed at the end of the post-exposure period when only LPO exhibited interaction effects. Interestingly, a pattern was perceptible, revealing that after 10 days of toxin elimination, under any of the altered environmental conditions (W, A or WA), there was an antagonistic effect in the gills, presented as a decrease of LPO levels. This pattern may be associated with LPO damage repair, though the lack of DNA damage and the reduced response exhibited by the antioxidant system pointed out the cell renewal as a possible cause to this decrease. The late effects detected in LPO may be related with the need of repairing the DNA damage observed at the peak of toxin accumulation, or it may be related with toxin-triggering apoptosis mechanisms (Abi-Khalil et al., 2017, 2016). Again, the reduced antioxidant response coupled with the LPO and DNA damage modulation suggests that the PST affected the gills cells through several pathways.

In the hepatopancreas, the responses showed a clear organ-specific pattern. An antagonistic response was observed for GSHt on day 5. Despite the increase in the co-factor content of mussels fed with toxic algae under CC, no response was observed under the combined effect of exposure to toxic algae and WA. These results emphasise the complexity of the interaction effects and highlight the need for a better understanding of how GSH and PST react with each other. The involvement of GSH in the toxin metabolism and biotransformation may also be related to the toxin elimination (Braga et al. 2018). Therefore, studies analysing the individual GSH and GSSG contents with the assessment of a potential formation of PST-GSH conjugates under different environmental conditions may be essential to understand the toxins effects and the elimination process in mussels.

While the antioxidant system responses were modulated by the environmental conditions during exposure, the results observed for GDI levels, support a toxin-triggered process dominance, as increased damage was observed in mussels under CC and in mussels under WA when the toxic algae were introduced.

Notwithstanding, at the end of the post-exposure period, an antagonistic response was observed in the antioxidant system. The GPx activity, which decreased in mussels under WA, was induced in mussels under CC. GPx activity decrease may have been related either with enzyme exhaustion, due to a continuing stimulus; or with recovery to basal levels, due to reduction of the stressor, since during post-exposure toxin elimination occurred in the mussels (Braga et al., 2018). The latter is, however, the most likely hypothesis since GPx exhaustion would lead to a significant increase in LPO damage, not detected in the data (Fabioux et al., 2015; Guilherme et al., 2008b).

An increase in ROS content could have explained the increase of GDI levels occurring at this stage. However, as referred above, the data analysis does not point to an increase in ROS, on the contrary, as no LOP was not observed, and in the antioxidant system, only GPx was altered, ROS seems to be decreasing in the cells. Therefore, this study reveals that under combined warming and acidification, mussels' capacity for recovery and repair of DNA damage is compromised. Nevertheless, further studies are needed to understand whether the impairment of DNA recovery capacity is due to the reduction of DNA repair mechanisms, or due to a decrease in cell turnover and apoptosis. The inhibition of repair enzymes has been described in mussels for other xenobiotics, such

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as Cd, which interferes in the final ligation step of BER, leading to the accumulation of DNA breaks in mussels (Emmanouil et al., 2007; Lynn et al., 1997). So, PST interference on BER and NER mechanisms should not be discarded, especially when these toxins are known to cause enzyme inhibition or transcription modulation (Mat et al., 2013; Mello et al., 2013).

Overall, the integrity of the genetic information was seriously compromised with the synergetic effects caused by the exposure of mussels to *G. catenatum* under projected conditions of warming and acidification.

5. Conclusions

To the best of our knowledge, this is the first study addressing the biochemical and cytogenetic responses of mussels *Mytilus galloprovincialis,* exposed to the toxin-producing dinoflagellate *Gymnodinium catenatum*, under four environmental conditions simulating current and predicted environmental conditions of seawater warming and acidification.

Overall, exposure to *Gymnodinium catenatum* under CC caused an active modulation of the antioxidant systems in both organs, which have different responses/vulnerabilities and mechanisms to cope with the paralytic shellfish toxins (PST). Under CC, the toxins systemic distribution in gills seems to contribute to a low risk of incurring genetic damage from PST exposure. In contrast, hepatopancreas, which seems to be better adapted to cope with the pro-oxidant challenges, was more prone to genetic damage, suggesting that PST originate non-oxidative damage in this organ cells.

The different responses in mussels exposed to PSTs under one single environmental condition (W or A) and those observed under combined environmental conditions (WA) highlights the importance and need for more studies focusing on the interaction of exposure to multiple stressors. Under altered environmental conditions (W, A or WA), toxin accumulation caused an increase in the genetic damage both in gills and hepatopancreas, with the non-correspondent response of LPO and the antioxidant system s, reinforcing that non-oxidative DNA damage might be occurring. Living under

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WA conditions increased mussels vulnerability to PST, with a dominance of toxin-triggered processes and the DNA damage increase, compromising the integrity of the genetic information and the mussels' DNA repair capacity.

Finally, this study highlights the role of defence mechanisms protecting shellfish from marine biotoxins, such as saxitoxin, remarking that exposure to multiple stressors predicted in a climate change scenario coupled with the increasing harmful algal blooms potentiates the risk to shellfish *Mytilus galloprovincialis* populations.

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Sofia Guilherme: Conceptualization, Methodology, Formal analysis - Statistical approach, Writing - Review & Editing;

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Declaration of interests

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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.

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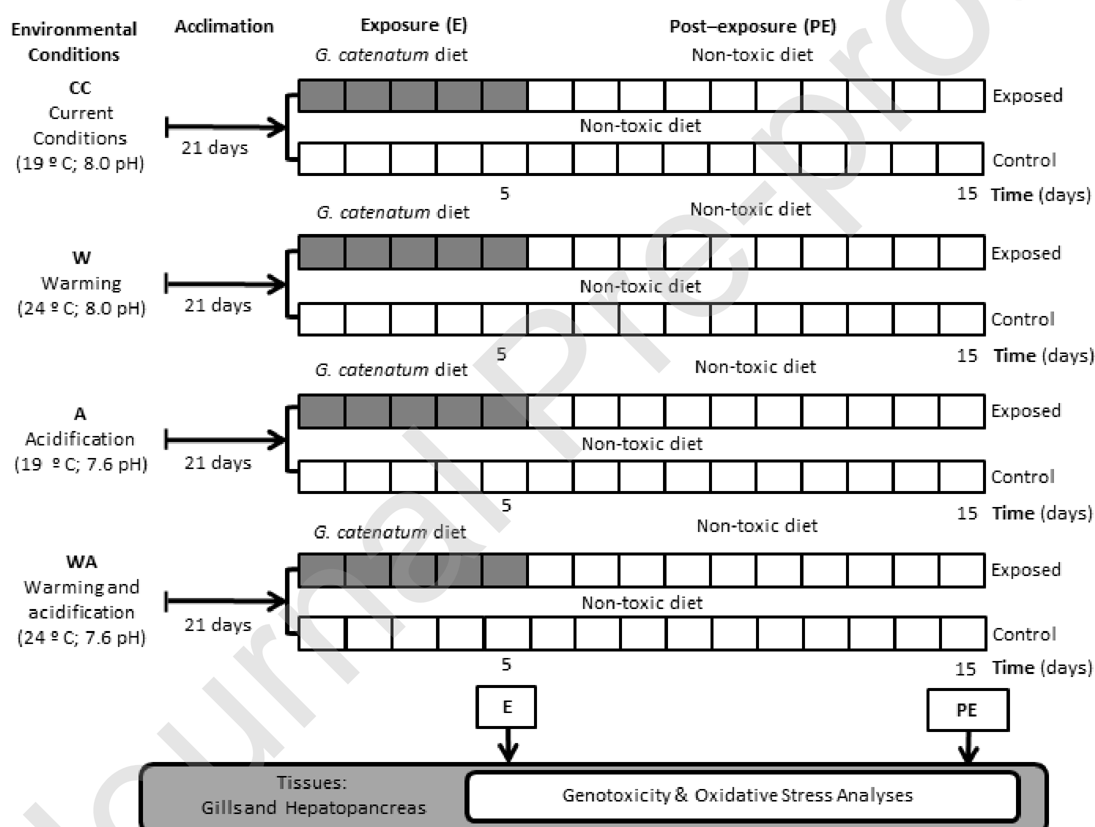

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Figure 1. Design of mussel feeding experiment, with toxic *Gymnodinium catenatum* diet (in grey) and non-toxic diet - *Tetraselmis* sp. (in white), under four environmental conditions (CC: 19 °C and 8.0 pH; W: 24 °C and 8.0 pH; A: 19 °C and 7.6 pH; WA: 24 °C and 7.6 pH). In the timescale, the numbers 5 and 15 identify the sampling days, on the fifth day of exposure to toxic algae (E) and on the tenth day of post-exposure (PE), respectively.

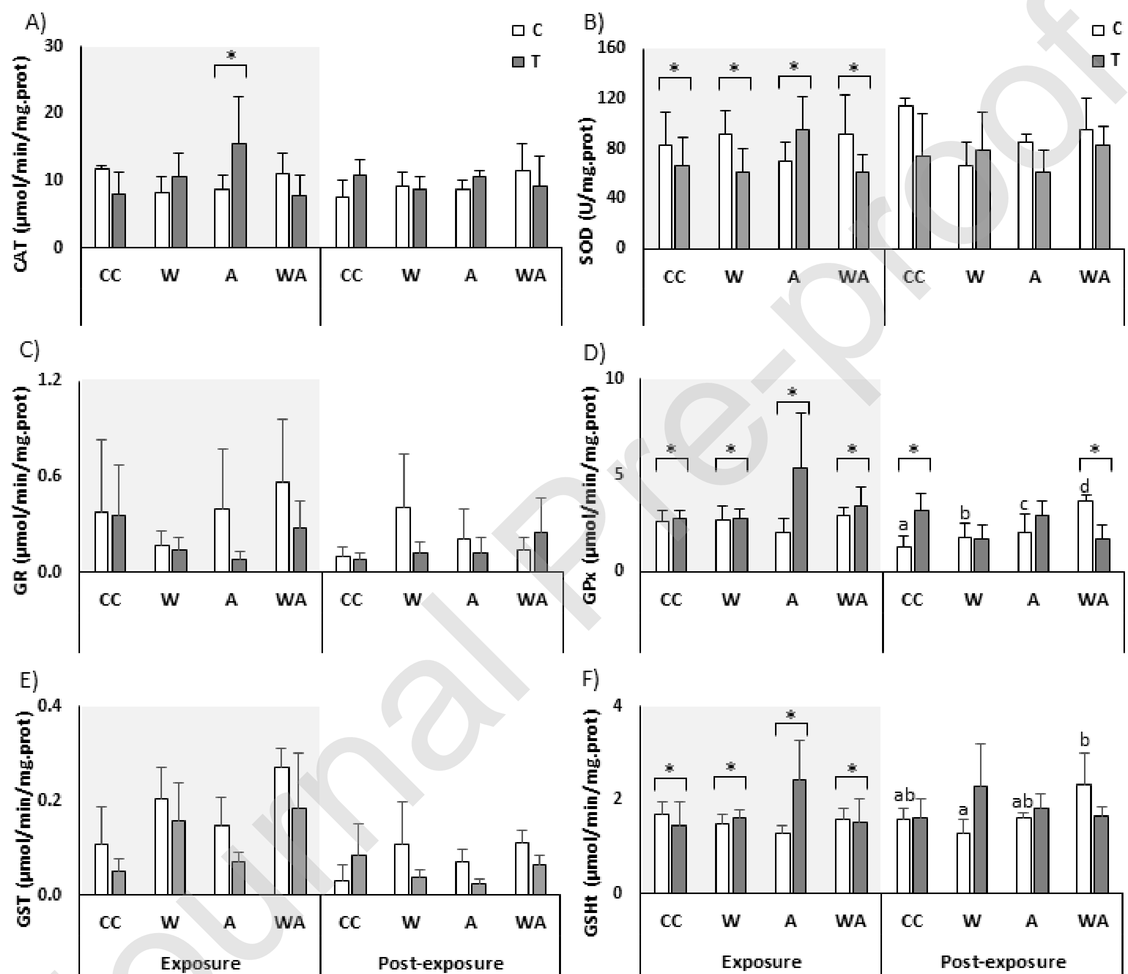

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Figure 2. Antioxidant responses (mean ± SD) in gills of mussels exposed to toxin producing-dinoflagellates *G. catenatum* during 5 days (E - Exposure period) and to non-toxic algae in the 10 days afterwards (PE – Post-exposure period), under four environmental conditions: current conditions (CC) 19 °C and 8.0 pH; warming (W) 24 °C and 8.0 pH; acidification (A) 19 °C and 7.6 pH; warming and acidification (WA) 24 °C and 7.6 pH. Parameters include: A) catalase (CAT), B) superoxide dismutase (SOD), C) glutathione reductase (GR), D) glutathione peroxidase (GPx), E) glutathione-S-transferase (GST) activities, and F) total glutathione content (GSHt). Asterisks (*) represent significant differences ($p < 0.05$) between mussels exposed to *G. catenatum* (T; Toxic) and control group (C; exposed to *Tetraselmis* sp.) within the same environmental condition (CC, W, A and WA) tested in E and PE period. Different lowercase letters indicate significant differences ($p < 0.05$) between the different environmental conditions tested (CC, W, A and WA), in E and PE period, within the groups exposed to non-toxic algae (C). No letter in the columns indicates the absence of significant differences.

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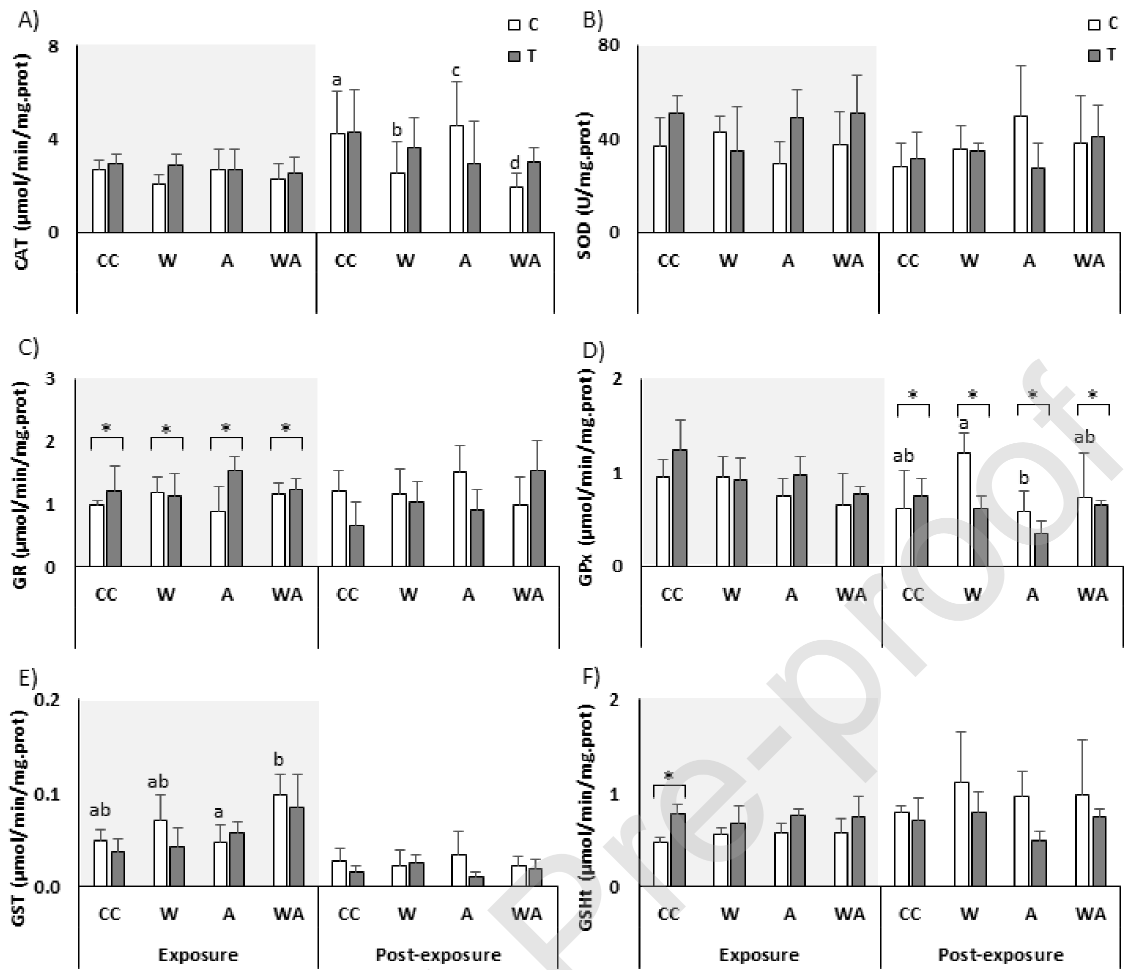

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Figure 3. Antioxidant responses (mean ± SD) in hepatopancreas of mussels exposed to toxin producing-dinoflagellates *G. catenatum* during 5 days (E - Exposure period) and to non-toxic algae in the 10 days afterwards (PE – Post-exposure period), under four environmental conditions: current conditions (CC) 19 °C and 8.0 pH; warming (W) 24 °C and 8.0 pH; acidification (A) 19 °C and 7.6 pH; warming and acidification (WA) 24 °C and 7.6 pH. Parameters include: A) catalase (CAT), B) superoxide dismutase (SOD), C) glutathione reductase (GR), D) glutathione peroxidase (GPx), E) glutathione-S-transferase (GST) activities, and F) total glutathione content (GSHt). Asterisks (*) represent significant differences ($p < 0.05$) between mussels exposed to *G. catenatum* (T; Toxic) and control group (C; exposed to *Tetraselmis* sp.) within the same environmental condition (CC, W, A and WA) tested in E and PE period. Different lowercase letters indicate significant differences ($p < 0.05$) between the different environmental conditions tested (CC, W, A and WA), in E and PE period, within the groups exposed to non-toxic algae (C). No letter in the columns indicates the absence of significant differences.

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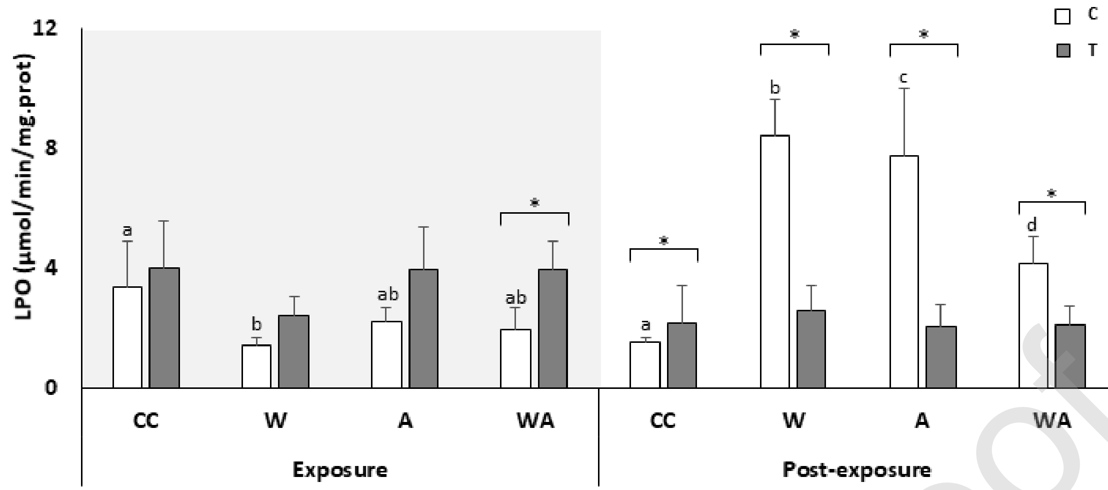

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Figure 4. Lipid peroxidation damage (LPO) (mean ± SD) in gills of mussels exposed to toxin producing-dinoflagellates *G. catenatum* during 5 days (E - Exposure period) and to non-toxic algae in the 10 days afterwards (PE – Post-exposure period), under four environmental conditions: current conditions (CC) 19 °C and 8.0 pH; warming (W) 24 °C and 8.0 pH; acidification (A) 19 °C and 7.6 pH; warming and acidification (WA) 24 °C and 7.6 pH. Asterisks (*) represent significant differences ($p < 0.05$) between mussels exposed to *G. catenatum* (T; Toxic) and control group (C; exposed to *Tetraselmis* sp.) within the same environmental condition (CC, W, A and WA) tested in E and PE period. Different lowercase letters indicate significant differences ($p < 0.05$) between the different environmental conditions tested (CC, W, A and WA), in E and PE period, within the groups exposed to non-toxic algae (C).–No letter in the columns indicates the absence of significant differences.

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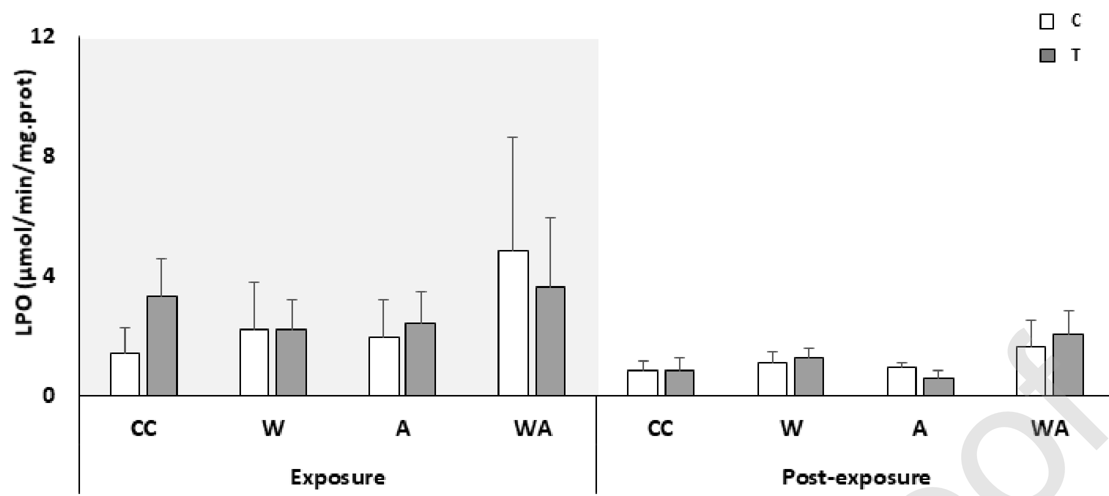

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Figure 5. Lipid peroxidation damage (LPO) (mean ± SD) in hepatopancreas of mussels exposed to toxin producing-dinoflagellates *G. catenatum* during 5 days (E - Exposure period) and to non-toxic algae in the 10 days afterwards (PE – Post-exposure period), under four environmental conditions: current conditions (CC) 19 °C and 8.0 pH; warming (W) 24 °C and 8.0 pH; acidification (A) 19 °C and 7.6 pH; warming and acidification (WA) 24 °C and 7.6 pH. Asterisks (*) represent significant differences ($p < 0.05$) between mussels exposed to *G. catenatum* (T; Toxic) and control group (C; exposed to *Tetraselmis* sp.) within the same environmental condition (CC, W, A and WA) tested in E and PE period. Different lowercase letters indicate significant differences ($p < 0.05$) between the different environmental conditions tested (CC, W, A and WA), in E and PE period, within the groups exposed to non-toxic algae (C). No letter in the columns indicates the absence of significant differences.

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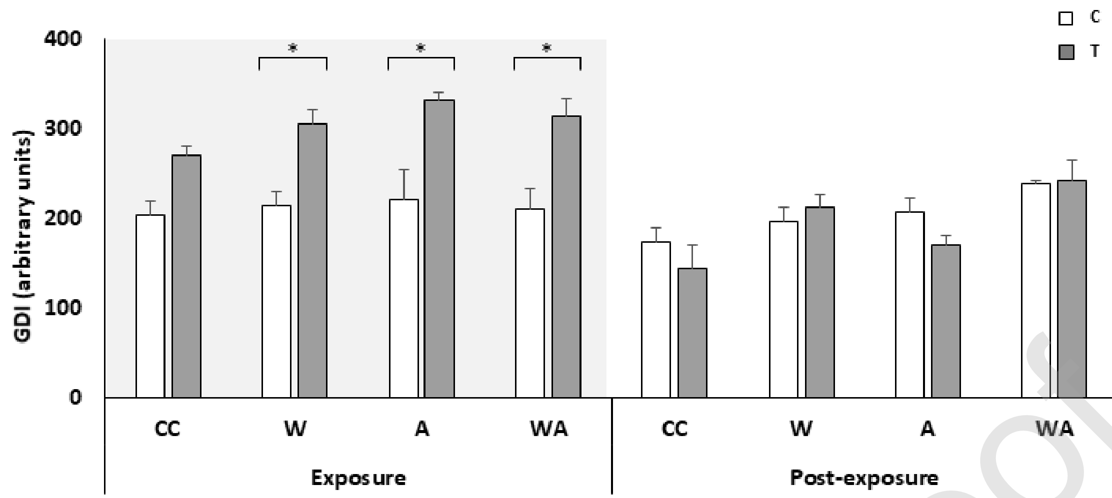

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Figure 6. Genetic Damage Indicator (GDI) (mean ± SD, expressed as arbitrary units) in gills of mussels exposed to toxin producing-dinoflagellates *G. catenatum* during 5 days (E - Exposure period) and to non-toxic algae in the 10 days afterwards (PE – Post-exposure period), under four environmental conditions: current conditions (CC) 19 °C and 8.0 pH; warming (W) 24 °C and 8.0 pH; acidification (A) 19 °C and 7.6 pH; warming and acidification (WA) 24 °C and 7.6 pH. Asterisks (*) represent significant differences ($p < 0.05$) between mussels exposed to *G. catenatum* (T; Toxic) and control group (C; exposed to *Tetraselmis* sp.) within the same environmental condition (CC, W, A and WA) tested in E and PE period. Different lowercase letters indicate significant differences ($p < 0.05$) between the different environmental conditions tested (CC, W, A and WA), in E and PE period, within the groups exposed to non-toxic algae (C). No letter in the columns indicates the absence of significant differences.

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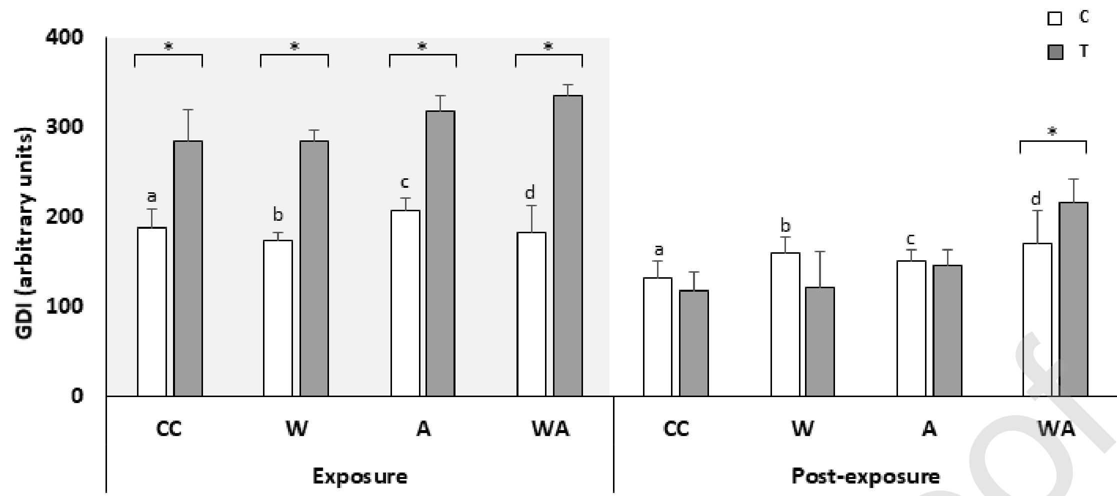

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Figure 7. Genetic Damage Indicator (GDI) (mean ± SD, expressed as arbitrary units) in hepatopancreas of mussels exposed to toxin producing-dinoflagellates *G. catenatum* during 5 days (E - Exposure period) and to non-toxic algae in the 10 days afterwards (PE – Post-exposure period), under four environmental conditions: current conditions (CC) 19 °C and 8.0 pH; warming (W) 24 °C and 8.0 pH; acidification (A) 19 °C and 7.6 pH; warming and acidification (WA) 24 °C and 7.6 pH. Asterisks (*) represent significant differences ($p < 0.05$) between mussels exposed to *G. catenatum* (T; Toxic) and control group (C; exposed to *Tetraselmis* sp.) within the same environmental condition (CC, W, A and WA) tested in E and PE period. Different lowercase letters indicate significant differences ($p < 0.05$) between the different environmental conditions tested (CC, W, A and WA), in E and PE period, within the groups exposed to non-toxic algae (C). No letter in the columns indicates the absence of significant differences.

Table 1. PSP toxicity (µg STX eq. kg^{-1}, mean ± SD) determined in mussels (whole soft tissues) exposed to toxic *Gymnodinium catenatum* for 5 days (E) followed by a non-toxic diet during the 10 days (PE), under four environmental conditions (CC: 19 °C and 8.0 pH; W: 24 °C and 8.0 pH; A: 19 °C and 7.6 pH; WA: 24 °C and 7.6 pH), adapted from Braga et al. (2018).

Environmental Conditions	**STX concentration** (µg STX eq.kg^{-1})	
	Exposure (E)	**Post-exposure (PE)**
CC (19 °C; 8.0 pH)	1494 ± 202	414 ± 140
W (24 °C; 8.0 pH)	662 ± 23	261 ± 7
A (19 °C; 7.6 pH)	761 ± 63	268 ± 71
WA (24 °C; 7.6 pH)	856 ± 61	272 ± 99

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