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Hot and toxic: Accumulation dynamics and ecotoxicological responses of mussel *Mytilus galloprovincialis* exposed to marine biotoxins during a marine heatwave

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ABSTRACT

Climate change is increasing marine heatwaves (MHWs) frequency and severity worldwide. These extreme events often cause bivalves' mass mortality and facilitate the growth, proliferation and dispersion of toxin-producing microalgae blooms associated with threats to seafood safety. Yet, the interactive effects between MHW and uptake of marine biotoxins by biota are a novel topic still lacking thorough research, from both the ecotoxicological and seafood safety standpoints. This study assessed the effects of a MHW event on the accumulation/elimination dynamics of diarrhetic shellfish toxins in *Mytilus galloprovincialis* exposed to *Prorocentrum lima* and the ecotoxicological responses of mussels co-exposed to these two stressors. Results showed that acute exposure to +4 °C reduced toxins accumulation (−49 %) and elimination (−77 %) compared to control temperature. Moreover, exposure to MHW and toxins affected mussels' antioxidant activity, lipid and protein damage, and metabolism in a tissue-specific manner. These findings highlight that *M. galloprovincialis* can face higher vulnerability to toxins when MHW events strike.

1. Introduction

The oceans are presently undergoing significant transformations due to the impacts of climate change (IPCC, 2022). One of the most alarming consequences is the increasing frequency and intensity of marine heatwaves (Hobday et al., 2016; Frölicher et al., 2018).

Marine heatwaves (MHWs) are defined as extreme weather events during which seawater temperatures anomalously rise above the seasonally- varying threshold (90th percentile) for at least 5 consecutive days, or when successive temperature peaks occur with gaps of <2 days (Hobday et al., 2016). Global warming caused by increasing anthropogenic pressures, namely, greenhouse gas emissions, is presently considered the main driver of MHWs (Scannell et al., 2016). Since the beginning of the last century, the number of reported MHWs has been increasing in coastal areas worldwide, and so has increased their duration and intensity in the past decades (IPCC, 2021). Bivalves, such as mussels, constitute a significant source of animal protein consumed by humans. However, the stress induced by MHWs stands as the primary cause of mass die-offs in bivalve farming (Masanja et al., 2024). The enduring impact of MHWs across generations exerts a detrimental effect on bivalve populations, ultimately leading to widespread mortalities in bivalve aquaculture (Masanja et al., 2023). This can subsequently have severe implications from both the ecological (e.g., biodiversity loss) and ecosystems services (e.g., economical losses in fisheries and aquaculture sectors) standpoints. Hence, a deeper knowledge on the impacts and risks in marine ecosystems prompted by MHWs is essential to establish

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effective management and adaptation strategies that will allow building environmental resilience and assure seafood security (i.e. the production of safe and high-quality seafood in sufficient quantities to feed the populations; Frölicher et al., 2018). Yet, predicting marine species responses and ecosystems damages from MHWs is extremely challenging, as these events take place in fast, intense and interactive ways, often playing a synergistic or additive role on other existing environmental stressor, such as pollution, disease outbreaks and development of harmful algal blooms, HABs (Moore et al., 2008; Smith et al., 2023).

HABs are defined as the rapid and/or excessive growth of phytoplankton species under favorable environmental conditions. Some HAB species produce toxins that can potentially harm humans and biota (Moore et al., 2008). In addition, HABs can have negative effects on the environment, such as depleting oxygen levels in the water because of the accumulated biomass, causing the death of other marine species (Sanseverino et al., 2016). These phenomena are, therefore, an ecological and public health concern, that call for regular monitoring, effective risk assessment and modelling to forecast their occurrence and mitigate damages. Based on the public health problem that HABs can raise, the European Union has set limits for the presence of regulated toxins in shellfish. Such is the case of okadaic acid, a marine biotoxin frequently detected in seafood species (e.g., Mediterranean mussel, *Mytilus galloprovincialis*; Lee et al., 2016; Braga et al., 2023), being responsible for eliciting a wide range of health problems, from mild gastrointestinal symptoms to severe neurological effects, and for which the EU has set the limit of 160 μg toxin equivalent per kg shellfish (European Commission, 2004).

Considering the preponderant role that abiotic variables, namely temperature, have on growth, proliferation and survival of phytoplankton species, climate change related stressors, and MHW events in particular, have been pointed out as potential catalysts of HABs (Dale et al., 2006; Moore et al., 2008). Recent evidence suggests that these phenomena are already exhibiting disturbed seasonality worldwide (Wang et al., 2021), and projections for future outcomes consistently show that they will likely become more frequent and intense in the future, especially at high latitudes, as a result of ocean warming and tropicalization of phytoplankton species (Brandenburg et al., 2019; Ibarbalz et al., 2019), potentially having devastating consequences in marine ecosystems, fisheries, aquaculture, and human health. Still, the interactive effects that abrupt temperature rises can have on the accumulation, detoxification and ecotoxicological responses of marine organisms exposed to biotoxins produced by toxic microalgae species remain understudied. Studies in this direction are, thus, urgently needed as they enable setting early warning systems to detect, alert the population in due time, and establish effective response plans that mitigate impacts and prevent substantial animal, human and economic losses.

Prorocentrum lima is one of the most investigated benthic and toxic microalgae species. It is an important food item for a variety of benthic marine organisms, including zooplankton, fish larvae, and filter-feeding shellfish (Barkallah et al., 2020; Malika et al., 2021). The toxicity of *P. lima* is due to the production of diarrhetic shellfish toxins (DSTs) such as okadaic acid (OA) and its related dinophysistoxins compounds (DTXs; Nishimura et al., 2020). This cosmopolitan dinoflagellate typically found from tropical to temperate areas is thought to thrive under future climate change conditions, as it tends to be densely abundant in eutrophic and warm waters (Heil et al., 2005; Türkoğlu, 2010; Türkoğlu and Erdoğan, 2010).

Mussels are filter-feeding and sessile benthic organisms inevitably exposed to environmental stressors. They play an important role in aquatic ecosystems, serving as a food source for a variety of organisms and contributing to nutrient cycling, as well as, in the human diet, being a much-appreciated seafood item around the world. A large fraction of mussels (*Mytilus* spp. in particular) produced for human consumption is assured by aquaculture, i.e., extensive farming in coastal areas (including estuaries and lagoons) using ropes, piles, trays or bags (Anderson et al., 2002; Buck et al., 2017). Given mussels' ecological and economical relevance, in face of the climate change effects already felt worldwide and expected to become increasingly more severe in the coming years, it is imperative to assure the conservation and sustainable production of these seafood species in the future.

Within this context, the present study aimed to assess the interactive effects of a MHW event typically registered in southwestern Europe (Sen Gupta et al., 2020) and exposure to *P. lima* on the accumulation of marine biotoxins (OA group toxins) and inherent ecotoxicological responses of Mediterranean mussel *M. galloprovincialis* to both stressors. The data herein gathered provides an important contribution towards a better understanding of climate change impacts for aquaculture and seafood safety.

2. Materials and methods

2.1. Animal collection

Live mussels *Mytilus galloprovincialis* (n = 260; 6.0 ± 1.6 cm total length; 3.2 ± 1.0 cm width; 21.8 ± 0.5 cm height; 22 ± 14 g total weight; mean ± standard deviation) were wild-caught, collected from the intertidal zone in the south of the Tagus River estuary, in Porto Brandão, Almada, Portugal (GPS coordinates: 38.675446, −9.219725). Upon collection, mussels were immediately transported to the Live Marine Organisms Bioterium (LABVIVOS) of the Portuguese Institute for the Sea and Atmosphere (IPMA I.P., Algés). Here, sediments, algae and barnacles attached to mussels' shells were carefully removed with a knife and then mussels were washed with seawater. To facilitate bivalves' handling processes, animals were randomly distributed into 1 L plastic boxes (n = 10 in each box, 6 replicate boxes per treatment), which were then placed in 12 different tanks within recirculation aquaculture systems (RAS). During cleaning and feeding (microalgae solutions were given all at once and the bivalves were allowed to filter microalgae solutions for 1 h, twice a day), the boxes were elevated from water to avoid contaminations between treatments during these critical steps.

Each rectangular experimental tank (64 L total capacity each) had independent functioning i.e., mechanical (nylon filtration socks), protein skimmer, and biological (sand filters and bioballs) filtration systems (all from TMC, Portugal), as well as, a Profilux system (± 0.1 pH units; GHL, Germany) to monitor and adjust the temperature in each tank whenever needed by means of thermostats (200 W, 226 V2Therm, TMC Iberia, Portugal) and refrigerators (± 0.1 °C; Frimar, Fernando Ribeiro Lda., Portugal). Mussels were subjected to an acclimation period of 20 days, during which they were fed with commercial freeze-dried microalgae *Tetraselmis* spp. (2 g L^{-1} stock solution; 2 mL day^{-1} $bivalve^{-1}$) obtained from Phytobloom prof, Necton, Olhão, Portugal.

Temperature, pH, dissolved oxygen and salinity were measured daily using a multiparameter equipment (Multi 3420, WTW, Germany) and kept at the following abiotic conditions: 20.0 ± 0.5 °C (average seawater temperature registered at the collection site; data from IPMA's monitoring program), 8.10 ± 0.10 pH units, 8.00 ± 0.20 mg L^{-1} dissolved oxygen level and 35.0 ± 1.0 ‰ salinity level. Ammonia, nitrites and nitrates levels were also measured every week using commercial kits (Tropic Marin, USA). Animal welfare and mortality was checked daily, and moribund or dead animals were discarded.

2.2. Experimental design

For the purposes of MHW event simulation, the average conditions (i.e., peak temperature, ramp temperature rise/decrease rate, duration of "plateau" phase at peak temperature) of a category II (strong; category I: moderate; category III: severe) MHW were used has a reference in the experimental design, as this corresponds to an intermediate and most frequently observed scenario, nowadays, in the southwestern Europe coastal zones (Sen Gupta et al., 2020), where *M. galloprovincialis* is extensively reared. Four experimental treatments, each carried out in

triplicate (2 boxes with $n = 10$ mussels × 3 replicate tanks = 60 animals/treatment; Fig. 1), were assigned:

Treatment 1 – Control: Mussels subjected to average seawater temperature in Porto Brandão (20 °C) and fed with non-toxic microalgae (*Tetraselmis* spp., ~2 g L^{-1} stock solution, 2 mL day^{-1} $bivalve^{-1}$);

Treatment 2 – MHW: Mussels subjected to a category II MHW (i.e., abrupt increase of +4 °C in relation to the average temperature, during 5 consecutive days, followed by another 5 days of temperature drop down, −0.8 °C per day) and fed with non-toxic microalgae (*Tetraselmis* spp., ~ 2 g L^{-1} stock solution, 2 mL day^{-1} $bivalve^{-1}$);

Treatment 3 – TOX: Mussels subjected to average seawater temperature in Porto Brandão (20 °C) and fed with dried toxic microalgae (*Prorocentrum lima*, ~3 g L^{-1} stock solution, 2 mL day^{-1} $bivalve^{-1}$; after T5 *Tetraselmis* spp., ~ 2 g L^{-1} stock solution, 2 mL day^{-1} $bivalve^{-1}$). *P. lima* (strain PL13v) was isolated in 1996 in the Ria of Vigo, NW Iberian and kindly provided by Dr. Catarina Braga (IPMA);

Treatment 4 – MHW + TOX: Mussels subjected to a category II MHW (i.e., abrupt increase of +4 °C in relation to the average temperature,

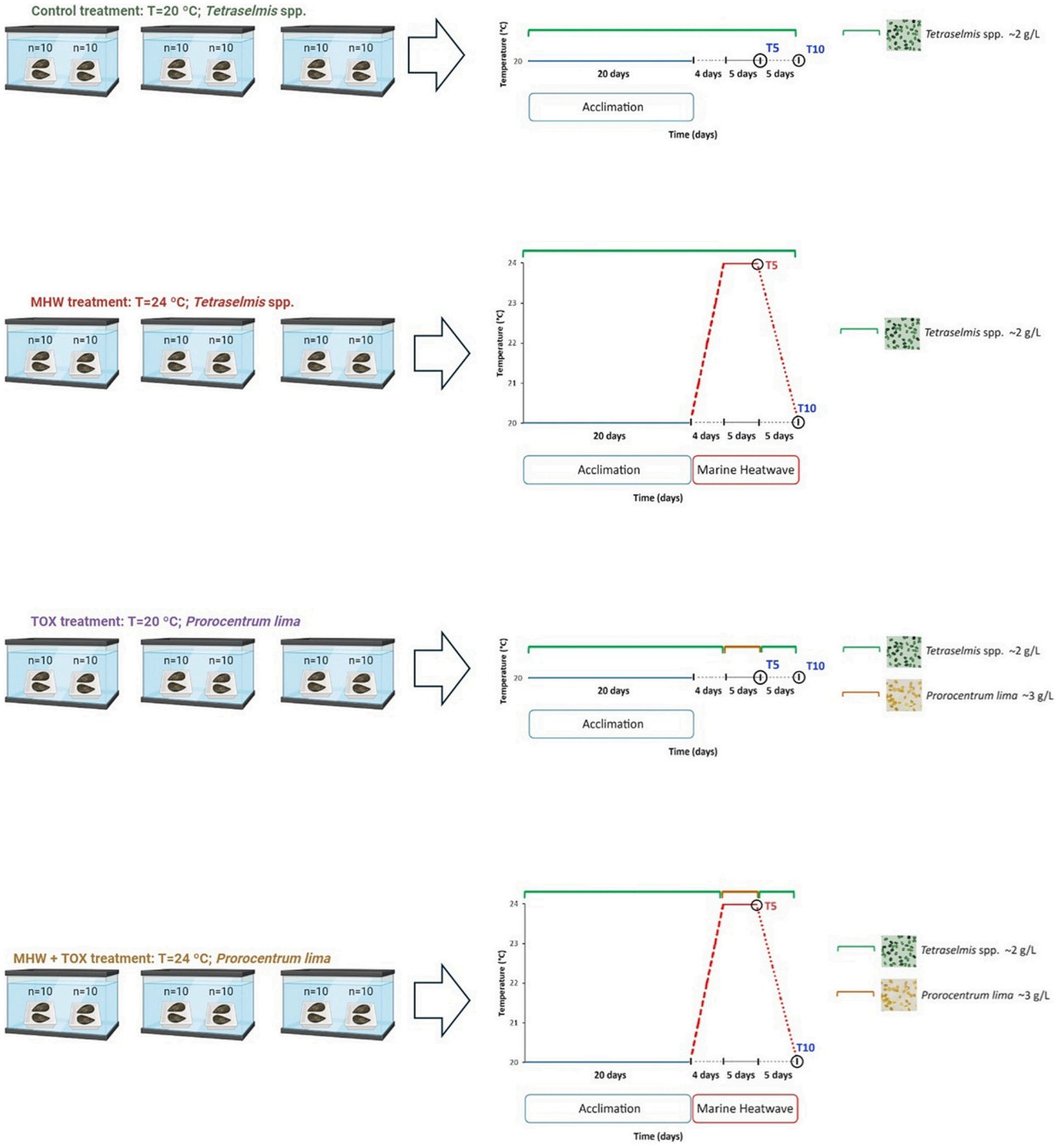

Fig. 1. Experimental setup. Abbreviations: Control - Mussels subjected to average seawater temperature in Porto Brandão (20 °C) and fed with *Tetraselmis* spp.; MHW - Mussels subjected to a category II marine heatwave (i.e., 24 °C) and fed with *Tetraselmis* spp.; TOX - Mussels subjected to average seawater temperature in Porto Brandão (20 °C) and fed with *P. lima*; MHW + TOX - Mussels subjected to a category II marine heatwave (i.e., 24 °C) and fed with *P. lima*; T5 – after 5 days of exposure to MHW and *P. lima*; T10 - after 5 days of recovery period from both stressors.

during 5 consecutive days, followed by another 5 days of temperature drop down, −0.8 °C per day) and fed with dried toxic microalgae (*Prorocentrum lima*, ~3 g L^{-1} stock solution, 2 mL day^{-1} $bivalve^{-1}$; after T5 *Tetraselmis* spp., ~ 2 g L^{-1} stock solution, 2 mL day^{-1} $bivalve^{-1}$). *P. lima* (strain PL13v) was isolated in 1996 in the Ria of Vigo, NW Iberian and kindly provided by Dr. Catarina Braga (IPMA).

After the acclimation period, seawater temperature was increased 1 °C every day, for 4 days, in treatments MHW and MHW + TOX to simulate the occurrence of a marine heatwave. Once the temperature reached 24 °C, the simulation of a 5 days' exposure to *P. lima* was initiated by replacing non-toxic feeding solution (*Tetraselmis* spp.) by toxic feeding solution (*P. lima*) in treatments TOX and MHW + TOX. After 5 days of exposure to toxic microalgae, the first sampling date took place, corresponding to the maximum exposure to toxic microalgae (T5). Finally, a 5-day period of recovery was carried out, by ceasing the feeding with toxic *P. lima* (replaced by *Tetraselmis* spp. solution, again) in TOX and MHW + TOX treatments, as well as, by slowly returning the temperature levels to 20 °C in MHW and MHW + TOX treatments. The second and last sampling was carried out at this stage (T10) (see Fig. 1).

In each sampling event, *M. galloprovincialis* were collected from each treatment to determine: 1) toxins concentration and profile; and 2) tissue-specific ecotoxicological responses. Sampled animals were weighted, measured and euthanized by incision of a scalpel in the adductor muscle. The soft tissue weight was recorded. To determine toxins concentration, whole bivalves' edible fraction was thoroughly washed with running tap water to eliminate any shell and sand residues, drained and pooled (6 animals per pool) to perform 3 composite samples per treatment, and promptly homogenized using a blender. Composite samples were performed due to mass requirements of the methodologies used; see Section 2.4.1. For biomarker responses, animals were dissected, and muscle, gills and digestive gland were individually collected to be analysed ($n = 6$ animals/treatment). Samples were frozen in liquid nitrogen and kept at −80 °C until further analysis.

2.3. Determination of biotoxins by liquid-chromatography

2.3.1. Toxins extraction

Toxins extraction from freeze-dried cells of *P. lima* was carried out with methanol, as described in Braga et al. (2021), to confirm the presence and determine the concentration of okadaic acid (OA) and dinophysistoxin-1 (DTX1). The toxin content was 326.4 ± 23.2 μg g^{-1} (dw) OA and 165.1 ± 5.7 μg g^{-1} (dw) DTX1 (Braga et al., 2021). Specifically, 52.2 mg of dried algae was vortexed with 5 mL of methanol (MeOH pa, Honeywell, Germany) during 15 min. Subsequently, the mixture underwent centrifugation at 2000*g* for 10 min. Following centrifugation, the extract was filtered and then stored at −20 °C until LC-MS/MS analysis.

Toxins extraction from mussel samples was performed according to the Standard Operating Procedure (SOP) of the European Reference Laboratory for Marine Biotoxins (EURLMB) for the determination of marine lipophilic biotoxins in bivalve molluscs (EURLMB, 2015). Briefly, five individuals from each treatment and sampling point were selected and prepared in triplicate. The process began by opening the mussels and removing the entire soft tissue from the shell.

Homogenized mussel samples (2 g) were placed in 30 mL centrifuge tubes mixed with 9 mL MeOH and vortexed for 5 min. Following this step, centrifugation at 2000 *g* for 10 min was performed (Fisher Scientific, accuSpin Micro 17 R, US), and the supernatant was collected and carefully transferred to new 30 mL centrifuge tubes. The remaining tissue underwent homogenization by scatter and were subsequently re-extracted repeating the same steps previously described. The overall volume was adjusted to 20 mL with MeOH. An aliquot of this extract was filtered using a 0.2 μm syringe filter (Labfill, China) into a chromatography vial and stored at −20 °C until LC-MSMS analysis.

2.3.2. Hydrolysis of DTX3

Due to the lack of reference standards for the multiple 7-O-acyl ester derivatives (DTX3) of both OA and DTX1, an alkaline hydrolysis step was carried out to convert these compounds into their respective parental toxin (Braga et al., 2021). The hydrolysis started by adding 125 μL of 2.5 M NaOH to a 1 mL aliquot of the sample extract in a test tube, which was homogenized for 30 s in the vortex and heated at 76 °C for 40 min in a heating block. The sample was let to cool down until reaching room temperature and neutralised with 125 μL of 2.5 M HCl (37 % pa, Honeywell, Germany). The sample was vortexed for 30 s, and an aliquot was filtered through a 0.2 μm syringe filter and stored at −20 °C until LC-MS/MS analysis.

2.3.3. LC-MS/MS analysis

The LC-MS/MS equipment consisted of an Agilent 1290 Infinity liquid chromatograph coupled to a triple quadrupole mass spectrometer Agilent 6470 (Agilent Technologies, Germany). The chromatographic separation was conducted with a Zorbax SB- C8 RRHT column (2.1 × 50 mm, 1.8 μm), protected with a guard column (2.1 × 5 mm, 1.8 μm). The mobile phase A was water with 2 mM ammonium formate (Sigma-Alrich, USA) and 50 mM formic acid (LC-MS, Carlo Erba, Germany) and the mobile phase B was 95 % acetonitrile (LC-MS grade, Carlo Erba, Germany) with 2 mM ammonium formate and 50 mM formic acid. An elution gradient at a flow rate of 0.4 mL/min was used as follows: 0–3 min, gradient from 88 to 50 % eluent A; 3–6.5 min gradient 50 to 10 % eluent A; 6.5–8.9 min 10 % eluent A; 8.9–10 min, gradient 10 to 88 % eluent A. The detection was carried out in Multiple Reaction Monitoring (MRM) acquisition mode. Two MRM transitions were monitored in negative polarity: m/z 803 > 255 and m/z 803 > 563 for OA and m/z 817 > 255 and m/z 817 > 563 for DTX1, quantification and confirmation, respectively. Five calibration standard solutions ranging from 2.0 to 22.0 ng mL^{-1} with a correlation >0.990 was set up for quantification using a matrix match calibration and certified OA and DTX1 reference standards purchased from CIFGA (Lugo, Spain).

2.4. Ecotoxicological responses (biomarkers)

2.4.1. Measurements of mussels and sample preparation

Muscle, gills and digestive gland collected from each specimen were homogenized in an Ultra-Turrax (Ika, T 10 basic, German) scatter, under ice-cold conditions, with 2 mL of homogenization buffer: i) phosphate buffered saline (PBS; 3 mM of potassium chloride, KCl, 10 mM of potassium phosphate monobasic, KH_2PO_4 and 140 mM of sodium chloride, NaCl, pH adjusted to 7.4 ± 0.02 units; all reagents from Sigma Aldrich, Germany), for analysis of oxidative stress biomarkers; ii) Imidazole 150 mM, EDTA (triplex, Merck, USA) 1 mM, Triton 1 % (Sigma- Aldrich, USA), pH 7.4, for analysis of lactate dehydrogenase activity; or HEPES 20 mM (Gibco, USA), EDTA Titriplex III (ethylenediaminetetraacetic acid disodium salt, Merck, Germany) 1 mM, Tripton 1 %, pH 7.4 for the analysis of citrate synthase activity. Tissue homogenates underwent centrifugation at 10.000 *g* for 10 min. The resulting supernatants were then promptly frozen and stored at −80 °C for subsequent analysis.

2.4.2. Biomarker analyses

All samples were analysed in duplicate, and standards in triplicate. Analyses were carried out in 96-well microplates and using the microplate reader (ThermoScientific, Multiskan GO, US). All reagents were of analytic or superior grade.

2.4.2.1. Total protein content. To determine the total protein levels in each sample, the Bradford assay (Bradford, 1976) was conducted. This was done to normalize the subsequent biomarker results, which were reported in mg of protein. The absorbance of each sample was measured at 595 nm. To generate a calibration curve, a standard solution of bovine serum albumin (BSA; Sigma Aldrich, Germany) at various dilutions (at

least 7 different concentrations, ranging from 0 to 2 mg mL^{-1}) was used. Results were expressed as mg protein mL^{-1}.

2.4.2.2. Catalase (CAT). To measure catalase (CAT) activity, a modified version (Maulvault et al., 2017) of the protocol described by Johansson and Håkan Borg (1988) was employed, using 96-well microplates. A calibration curve was established using formaldehyde standards at seven concentrations (ranging from 0 to 75 μM). Enzyme activity was calculated based on the formation of 1.0 nmol of formaldehyde (Sigma- Aldrich, Germany) per minute, and one unit of catalase was defined as the amount required to produce this content of formaldehyde. Absorbance was measured at 540 nm and results were expressed as nmol min^{-1} mg^{-1} protein.

2.4.2.3. Glutathione S-transferase (GST). The activity of glutathione S-transferase (GST) was assessed by adapting the method of Habig et al. (1974) to 96-well microplates as described by Maulvault et al. (2017), with 1-Chloro-2,4-dinitrobenzene (CDNB; Sigma- Aldrich, Germany) at a concentration of 100 mM serving as the substrate. Absorbance was measured at 340 nm at one-minute intervals for a total of 6 min, with the increase in absorbance reflecting the activity of GST. The reaction rate was calculated based on the molar extinction coefficient of 1-Chloro-2,4-dinitrobenzene (CDNB; Sigma- Aldrich, Germany), and the results were reported as nmol min^{-1} mg^{-1} protein.

2.4.2.4. Superoxide dismutase (SOD). Superoxide dismutase (SOD) activity was determined through the method described by Sun et al. (1988) adapted to 96-well microplates as described by Cereja et al. (2023), which utilizes nitro blue tetrazolium (NBT; Merck, USA) and xanthine oxidase (XOD; Sigma- Aldrich, Germany). Bovine erythrocyte superoxide dismutase was utilized as the standard and positive control. Samples were analysed by measuring the absorbance at 560 nm at one-minute intervals for 5 min, followed by additional readings at 10 and 15 min. The percentage (%) of enzyme inhibition was determined by comparison against the absorbance per minute of the negative control (blank).

2.4.2.5. Lipid peroxidation (LPO). Lipid peroxidation was determined through the thiobarbituric acid (TBA 1 %, Sigma- Aldrich, USA) reactive substances (TBARS) protocol (Uchiyama and Mihara, 1978). This method uses an eleven-point calibration curve (0–0.3 mM TBARS) that was obtained using malondialdehyde bis-(dimethyl acetal) (MDA; Merck, USA) as standard. The absorbance of each sample was measured at 532 nm, and the results were expressed as MDA concentration in nmol mg^{-1} protein.

2.4.2.6. Citrate Synthase (CS) and Lactate dehydrogenase (LDH). The activities of citrate synthase (CS; an indicator of aerobic potential) and lactate dehydrogenase (LDH; an indicator of anaerobic potential) were assessed as described in Rosa et al. (2016). For CS, 10 μL of sample, 200 μL of buffer [0.25 mM DTNB (Sigma- Aldrich, Germany), 75 mM TRIS-HCL (Merck, USA; pH 8.0)], 10 μL of acetyl CoA (Roche, Germany) at a concentration of 0.4 mM and 10 μL of oxaloacetic acid (Sigma- Aldrich, USA) at 0.5 mM were added to each microplate well. After a 2 min incubation at room temperature, absorbance was measured at 412 nm at one-minute intervals for a total of 5 min.

For LDH, 5 μL of sample, 200 μL of NADH (Sigma- Aldrich, USA) solution (0.15 mM) and 10 μL of pyruvic acid (1 M; Sigma-Aldrich, USA) were added to the microplate well. Absorbance was measured at 340 nm at one-minute intervals for a total of 6 min. CS and LDH activities were expressed in U mg^{-1} protein.

2.4.2.7. Heat shock proteins (HSP). To quantify Heat Shock Protein 70 (HSP70/HSC70), an indirect Enzyme Linked Immunosorbent Assay (ELISA) was employed based on a protocol established by Njemini et al. (2005). In this assay, anti-HSP70/HSC70 primary and secondary antibodies were utilized, diluted to 1.0 μg mL^{-1} in a 1 % BSA solution. The substrate employed was a PnPP (Sigma- Aldrich, USA) solution containing an alkaline/acid phosphatase conjugate (Sigma- Aldrich, Germany). A calibration curve was created using at least seven concentrations of purified HSP70 active protein (Acris, USA), ranging from 0 to 2 μg mL^{-1}. The absorbance was measured at 405 nm, and the results were reported in μg mg^{-1} protein.

2.5. Statistical analysis

Univariate permutational analyses of variance (PERMANOVA) were performed for the tested variables [i.e., biochemical biomarkers and toxins bioaccumulation after the maximum exposure and recovery periods (T5 and T10, respectively)]. Euclidean distances were used to calculate the similarity matrix that was used to test whether the tested variables were affected by treatments. Values of the pseudo-F statistic were run with partial sums of squares (Type III), with unrestricted permutation of raw data, and computed using 9999 permutations. As PERMANOVA is based on permutations it is more robust to the assumptions of ANOVA (Anderson, 2001). Post-hoc pair-wise comparisons were then performed using PERMANOVA to compare between treatments and variables. Differences were considered significant at $p < 0.05$. These analyses were performed in PERMANOVA+ for PRIMER v6 (PRIMER-E Ltd., Plymouth).

3. Results

3.1. Toxins accumulation, transformation and elimination dynamics

Mussels exposed to the MHW exhibited reduced performance compared to those maintained at 20 °C. Under the MHW conditions, mussels accumulated lower toxin levels, showed a slightly diminished ability to biotransform both OA and DTX1 into their acyl derivatives, and were less effective in eliminating the toxins accumulated during 5 days of feeding on toxic algae (Fig. 2). At the end of the uptake period, mussels maintained at 20 °C (TOX) reached a toxicity of 131.1 ± 22.4 μg OA eq. kg^{-1} whereas those exposed to the MHW (24 °C, MHW + TOX) reached 67.0 ± 9.5 μg OA eq. kg^{-1}. Mussels converted 76.4 % of the toxins into their acyl derivatives at T5 under TOX treatment, while mussels exposed to the MHW + TOX converted 68.4 %. Nevertheless, at the end of the experiment (T10) only esterified compounds were observed in both treatments.

While higher toxin levels were detected at sampling point T5 for mussels maintained at 20 °C, similar levels were observed between TOX and MHW + TOX at the elimination phase (T10). This is the result of a lower ability to eliminate the toxins when facing a drastic change in temperature as simulated in this study with a MHW II. Toxin levels decreased 30.2 % in the TOX treatment between the two sampling points T5 and T10, while mussels under the MHW effect only eliminated 6.9 % of the toxins.

3.2. Mussels ecotoxicological responses

3.2.1. Interactive ecotoxicological effects of marine heatwaves and P. lima (T5)

Antioxidant enzymes activities upon 5 days of exposure to *P. lima* and heatwaves (T5) are shown in Fig. 3A-I and Table SM1.

In general, CAT, SOD and GST activities in bivalves' muscle and digestive gland were not significantly affected by thermal stress and/or the presence of the toxin ($p > 0.05$; Fig. 3A-C, 3G-I). The gills were somewhat more responsive, with CAT activity showing a significant increase in mussels exposed to *P. lima* regardless of temperature (i.e., in TOX and TOX + MHW treatments in relation to Control treatment; $p = 0.026$ and $p = 0.028$, respectively; Fig. 3E).

Despite antioxidant scavengers' activity not being significantly affected in most cases, significantly higher LPO levels occurred in the

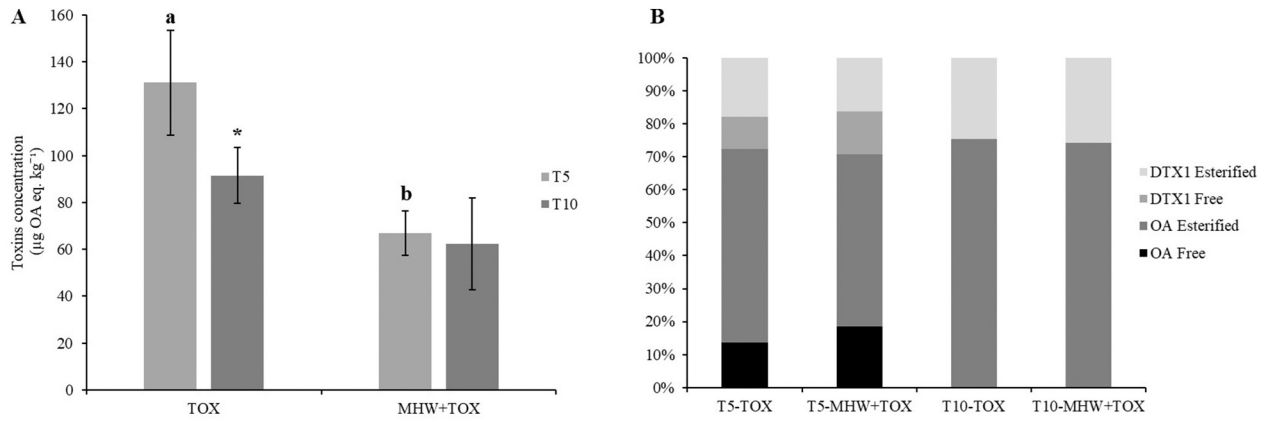

Fig. 2. Shellfish toxicity (µg OA eq. kg^{-1}) determined in mussels at current temperature of 20 °C (TOX) and mussels exposed to the effect of a category II marine heatwave (24 °C, MHW + TOX), both fed with toxic dinoflagellate *Prorocentrum lima* for 5 days (T5) and subsequent period of 5 days with non-toxic algae, *Tetraselmis* spp. (T10). Different letters denote significant differences between treatments, at the same sampling point (T5: lower-case; T10: upper-case; $p < 0.05$). Asterisks indicate significant differences between T5 and T10 for a given treatment ($p < 0.05$) (A); the toxin profile composed by okadaic acid (OA), dinophysistoxin-1 (DTX1) in their free and esterified form (B).

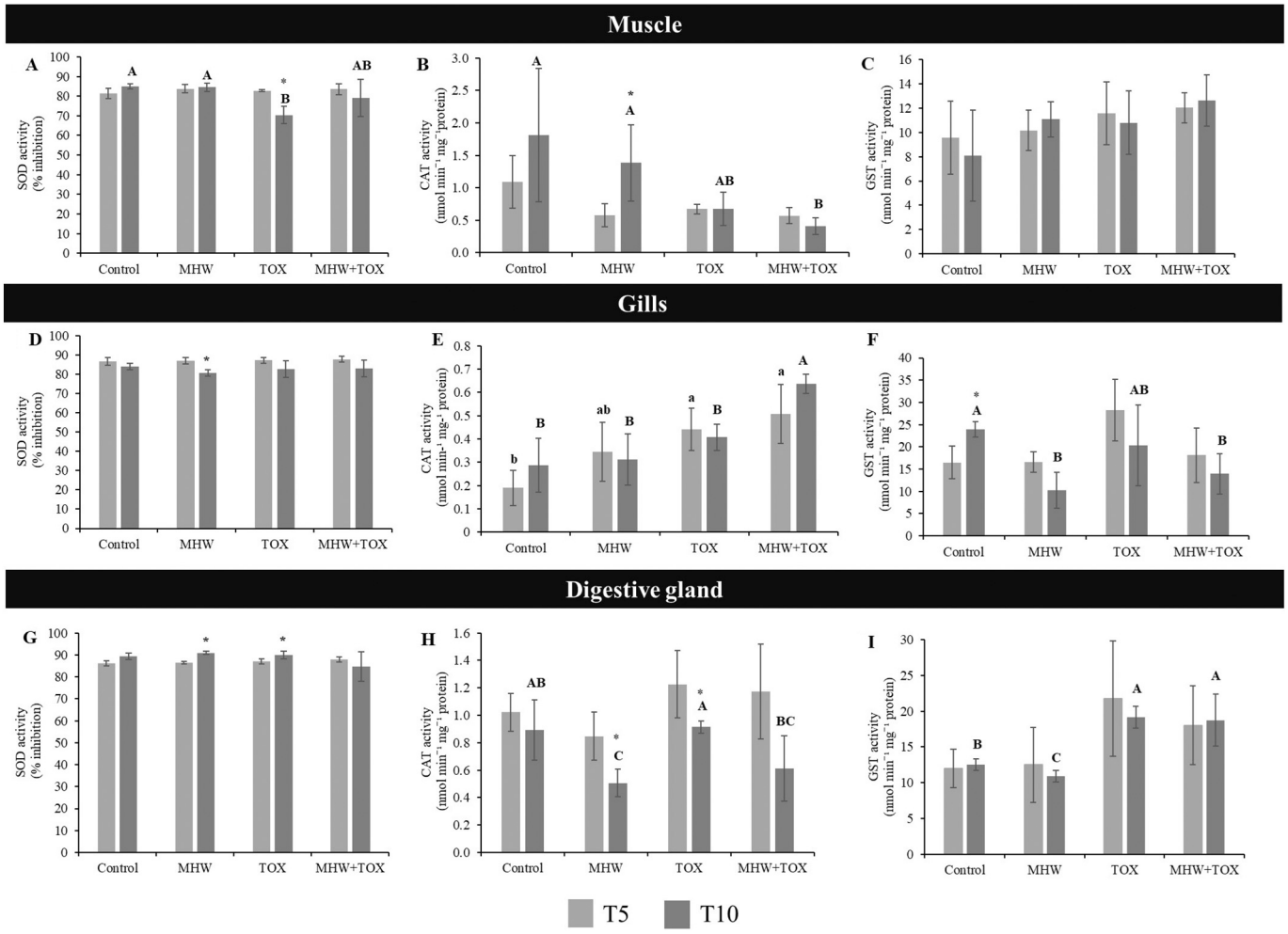

Fig. 3. Antioxidant enzymes activity (A, D, G – SOD, % inhibition; B, E, H – CAT, nmol min^{-1} mg^{-1} protein; C, F, I – GST, nmol min^{-1} mg^{-1} protein; mean ± standard deviation; $n = 6$) in *M. galloprovincialis* muscle (A-C), gills (D—F) and digestive gland (G-I), after 5 days of exposure to MHW and *P. lima* (T5) and after 5 days of recovery period from both stressors (T10). Different letters denote significant differences between treatments, at the same sampling point (T5: lower-case; T10: upper-case; $p < 0.05$). Asterisks indicate significant differences between T5 and T10 for the same tissue ($p < 0.05$). Abbreviations: Control - Mussels subjected to average seawater temperature in Porto Brandão (20 °C); MHW- Mussels subjected to a category II marine heatwave (24 °C); TOX- Mussels fed *P. lima* and subjected to average seawater temperature in Porto Brandão (20 °C); MHW + TOX- Mussels fed *P. lima* and subjected to a category II marine (24 °C); SOD – superoxide dismutase; CAT – catalase; GST – glutathione S-transferase.

muscle of bivalves exposed to MHW + TOX ($p = 0.029$, 77 % increase; Fig. 4A; Table SM1). As for heat shock proteins (HSP70/HSC70) content upon 5 days of exposure to *P. lima* and heatwaves (T5) are shown in Fig. 4B, D, F and Table SM1. Results were not consistent, with an inhibition being observed in mussels exposed to MHW (muscle: $p = 0.027$; 29 % decrease; digestive gland: $p = 0.028$, 60 % decrease in relation to Control; Fig. 4B and F) or toxin (muscle: $p = 0.031$, 52 % decrease in relation to Control; Fig. 4B) acting alone, while an induction was triggered by the combination of MHW and toxins in the gills ($p = 0.025$; >100 % increase in relation to Control; Fig. 4D).

Regarding metabolic enzymes activities upon 5 days of exposure to *P. lima* and heatwaves (T5), they are shown in Fig. 5A-F and Table SM1.

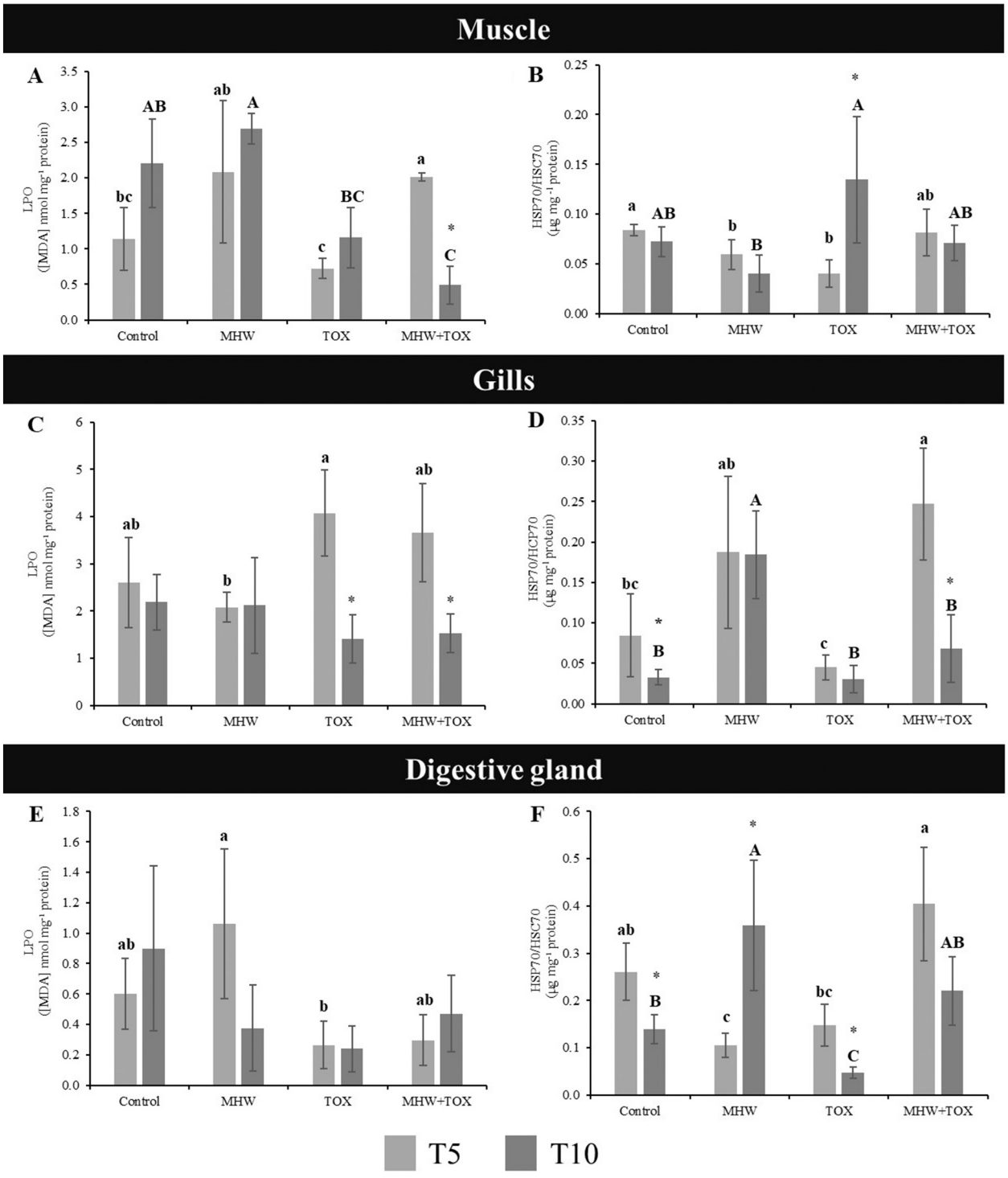

Fig. 4. Lipid peroxidation (A, C, E – LPO, [MDA] nmol mg^{-1} protein) and heat shock proteins (B, D, F - HSP70/HSC70, µg mg^{-1} protein) in *M. galloprovincialis* muscle (A-B), gills (C—D) and digestive gland (*E*-F), after 5 days of exposure to MHW and *P. lima* (T5) and after 5 days of recovery period from both stressors (T10) (mean ± standard deviation; $n = 6$). Different letters denote significant differences between treatments, at the same sampling point (T5: lower-case; T10: upper-case; $p < 0.05$). Asterisks indicate significant differences between T5 and T10 for the same tissue ($p < 0.05$). Abbreviations: Control - Mussels subjected to average seawater temperature in Porto Brandão (20 °C); MHW- Mussels subjected to a category II marine heatwave (24 °C); TOX- Mussels fed *P. lima*; and subjected to average seawater temperature in Porto Brandão (20 °C); MHW + TOX- Mussels fed *P. lima*. and subjected to a category II marine heatwave (24 °C); MDA – malondialdehyde.

Overall, CS activity was enhanced by MHW or toxins exposure alone in the muscle ($p = 0.031$, 63 % increase in MHW and $p = 0.029$, 38 % increase in TOX against Control; Fig. 5A) and by toxins exposure alone in the digestive gland ($p < 0.05$, in TOX against all treatments; Fig. 5E). A different trend was observed in the gills, with MHW (regardless of toxin exposure) significantly inhibiting CS activity ($p = 0.027$ and $p = 0.019$, in MHW and MHW + TOX, respectively, corresponding 40 % and 62 % decrease in relation to CTR; Fig. 5C).

Additionally, LDH activity was inhibited by MHW and toxins (alone or combined) in the muscle and gills ($p < 0.05$, in relation to Control; Fig. 5B and D). A different trend was observed in the digestive gland where LDH activity was increased by MHW + TOX ($p = 0.017$, 77 % increase in relation to Control; Fig. 5F).

3.2.2. Recovery from MHW and P. lima exposure (T10)

Bivalves' antioxidant responses upon recovery from the exposure to *P. lima* and MHW (T10) are shown in Fig. 3A-I and Table SM1. Starting with the muscle tissue, overall, exposure to toxins decreased SOD activity in relation to values found in the Control ($p = 0.028$, 17 % decrease; Table SM1) and MHW ($p = 0.027$, 17 % decrease) treatments (Fig. 3A). In addition, a decrease in SOD activity was observed between T5 and T10 in TOX treatment ($p = 0.028$, 15 % decrease; Fig. 3A). Moreover, muscle CAT activity was reduced in MHW + TOX at T10 ($p = 0.029$ 77 % decrease in relation to Control; Fig. 3B) and enhanced between T5 and T10 in MHW treatment ($p = 0.048$, >100 % increase against T5; Fig. 3B). As for LPO, values remained lower in mussels exposed to the interactive effects of MHW and toxins ($p = 0.026$, 78 % decrease in relation to Control), with MHW + TOX treatment showing a significant decrease between T5 and T10 ($p = 0.028$, 76 % decrease against MHW + TOX at T5; Fig. 4A; Table SM1). Upon recovery from the MHW (T10), antioxidant enzymes activities in mussels' gills exhibited a similar pattern to the one observed at T5 in this tissue, with CAT remaining significantly enhanced in MHW + TOX ($p = 0.029$, >100 % increase) in relation to all treatments, while GST activity was inhibited in treatments subjected to MHW (MHW: $p = 0.030$, 57 % decrease; MHW + TOX: $p = 0.030$, 42 % decrease, in relation to Control; Fig. 3E-F). A different trend was observed in the digestive gland, with CAT activity being significantly lower in MHW treatment (T10, $p = 0.030$, 43 % decrease in relation to Control, Fig. 3H), whereas GST activity was significantly higher in treatments previously exposed to toxins (T10, TOX: $p = 0.028$, 53 % increase; MHW + TOX: $p = 0.032$, 50 % increase in relation to Control), but was inhibited by MHW alone (T10, $p = 0.029$, 13 % decrease against Control, Fig. 3I).

Regarding heat shock proteins content, distinct patterns were observed in the 3 tissues studied (Fig. 4B, D, F; Tables SM1). A significant increase in HSP70/HSC70 content was observed in the muscle of bivalves from TOX values at T10 to TOX values at T5 (T10 versus T5, $p = 0.030$, >100 % increase against TOX at T5, Table SM1), as well as to values of MHW treatment at T10 ($p = 0.029$, >100 % increase in relation to TOX at T10; Fig. 4B). Both gills and digestive gland showed significantly higher HSP70/HSC70 levels in MHW treatment ($p = 0.028$ and $p = 0.026$, respectively, with >100 % increase in relation to Control; Fig. 4D and F; Table SM1). Noteworthy, between T5 and T10, a significant decrease was observed in the gills of mussels from MHW + TOX treatment (T10 versus T5, $p = 0.028$, 72 % decrease against MHW + TOX at T5; Fig. 4D; Table SM1).

As for metabolic enzymes activities, CS decreased in the muscle of MHW treatment in relation to the other ones (T10, $p < 0.05$), while higher LDH values were found in MHW treatment in relation to Control (T10, $p = 0.028$, 20 % increase; Fig. 5A and B, Table SM1). CS activity was enhanced at T10 in the gills of bivalves from TOX in relation to non-toxic treatments ($p < 0.05$, Fig. 5C; Table SM1) and from MHW + TOX in relation to MHW treatment at T10 ($p = 0.023$, Fig. 5D). In the digestive gland, between T5 and T10, a significant decrease in CS activity was observed for TOX treatment (T10 versus T5, $p = 0.038$, 31 % decrease against TOX at T5; Fig. 5E; Table SM1).

Contrasting CS results, LDH activity was diminished in the gills of bivalves at T10 from both TOX and MHW + TOX treatments in relation to non-toxic treatments ($p < 0.05$; Fig. 5D, Table SM1). Also, between T5 and T10 in bivalves' gills, whereas a significant increase in LDH activity was observed for MHW treatment (T10 versus T5, $p = 0.030$, 25 % increase against MHW at T5), the opposite pattern was observed for MHW + TOX treatment (T10 versus T5, $p = 0.027$, 40 % decrease against MHW + TOX at T5, Fig. 5D, Table SM1). LDH activity presented a different response pattern in the digestive gland, as it was significantly higher in TOX and MHW + TOX (against non-toxic treatments; $p < 0.05$; Fig. 5F, Table SM1). Noteworthy, between T5 and T10 in bivalves' digestive gland, a significant increase in LDH activity was observed for TOX treatment (T10 versus T5, $p = 0.028$, 66 % increase against TOX at T5; Fig. 5E; Table SM1).

4. Discussion

4.1. Effects of MHW on toxins' accumulation, biotransformation and elimination dynamics

Upon 5 days of exposure trial (T5), detectable levels of the toxins okadaic acid (OA) and dinophysistoxin-1 (DTX1) were found in mussels exposed to both temperature scenarios, confirming toxins accumulation via *P. lima* ingestion/filtration. Yet, toxins concentration in *M. galloprovincialis* did not exceed the maximum permitted levels for human consumption set by EU (i.e., 160 μg toxin equivalent per kg shellfish; European Commission, 2004), regardless of temperature scenario. It should be noted, though, that these results might have been somewhat influenced by the fact that toxins exposure was simulated by feeding mussels with freeze-dried (dead) algae cells (and not alive, as happens in the wild) and this can, perhaps, affect bivalves' digestive process and contaminant/nutrient metabolization. The duration of toxins exposure period is another factor that deserves attention, as more prolonged HAB events (beyond 5 days) may certainly have different outcomes, such as an exacerbation of DSTs accumulation and toxicity (e.g., Funesto et al., 2023), which can be problematic from the ecological, economic and public health standpoints. Recent studies have provided compelling evidence that increased water temperatures enhance animal feeding and ventilation rates, in response to higher metabolism and energy demands (Masanja et al., 2023). This, in turn, often translates into higher contaminant accumulation (Maulvault et al., 2018). However, our results did not match this trend, as an overall significant decrease in toxins content was observed in mussels exposed to the MHW + TOX when compared to TOX treatment. Yet, it has been observed that *M. galloprovincialis* acclimated to 24 °C increased the duration of valve closure by about sixfold when compared to mussels acclimated to 17 °C (Anestis et al., 2007). This behavior may cause a metabolic depression, to balance the increase in energy demand associated with warmer water temperatures, and a shift from aerobic to anaerobic metabolism (Anestis et al., 2007). The response pattern of the metabolic enzymes' activities analysed in the digestive gland (i.e., the organ responsible for food digestion and nutrients/contaminants metabolization) of *M. galloprovincialis* support this observation, i.e., decreased CS and increased LDH activities at higher temperatures (24 °C, MHW + TOX) than at lower temperatures (20 °C, TOX). Moreover, this mussel behavior related to water temperature may also explain the lower levels of toxins accumulation observed at higher temperatures (MHW + TOX). Corroborating Anestis et al. (2007) findings, our results suggest that *M. galloprovincialis* metabolism was less active at higher temperatures (MHW + TOX) as the content of esterified OA and DTX1 is lower in this treatment than at lower temperatures (TOX), indicating that toxins biotransformation/metabolization was less efficient at higher temperatures. Alongside, a significant decrease in toxin concentrations was observed between sampling points T5 and T10 for the TOX treatment but was not observed under the MHW effect (MHW + TOX). This is probably the result of metabolic depression when facing a drastic change

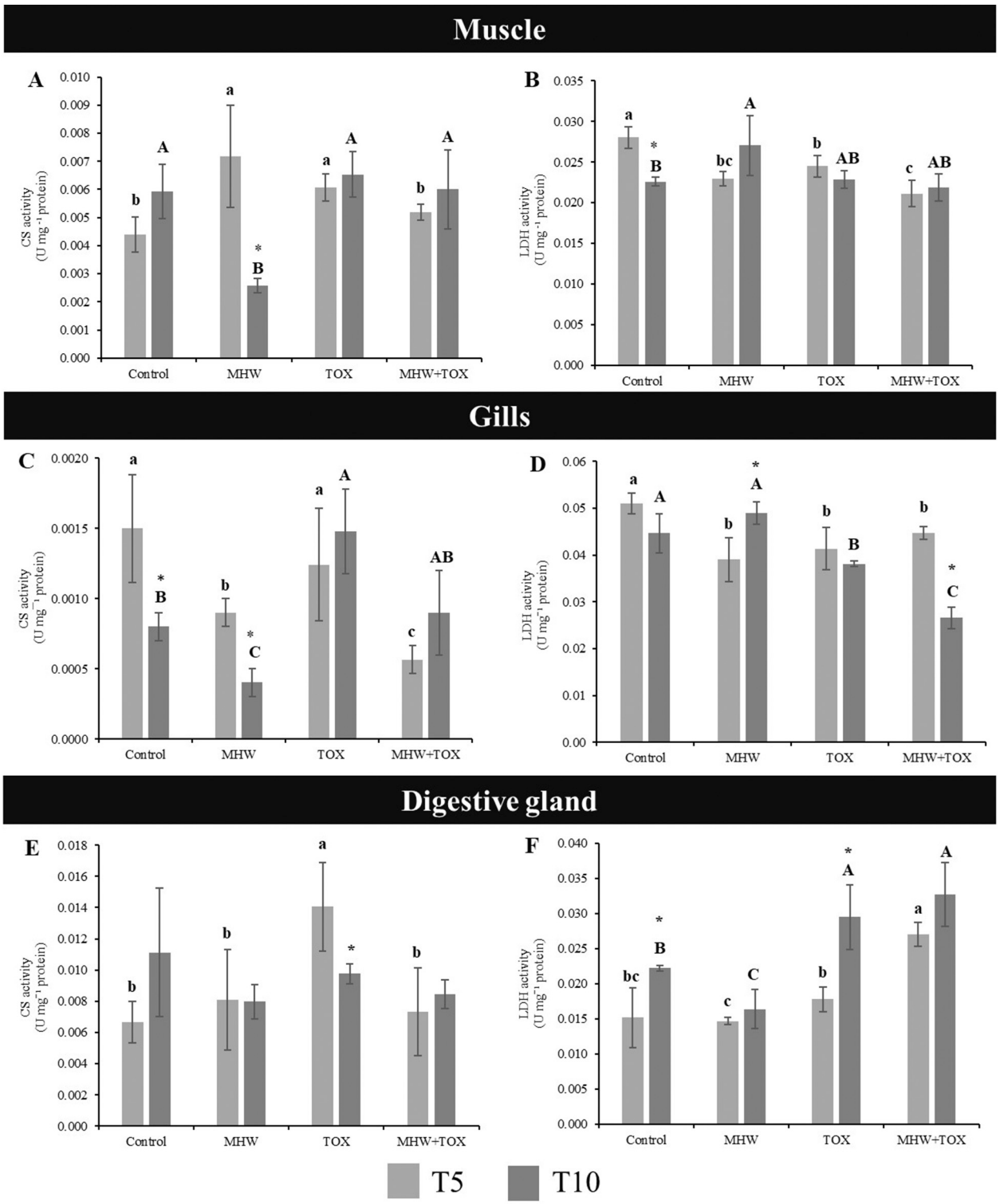

Fig. 5. Metabolic enzymes activity (A, C, E – CS, U mg^{-1} protein; B, D, F – LDH, U mg^{-1} protein; mean ± standard deviation; $n = 6$) in *M. galloprovincialis* muscle (A-B), gills (C—D) and digestive gland (E-F), after 5 days of exposure to MHW and *P. lima* (T5) and after 5 days of recovery period from both stressors (T10). Different letters denote significant differences between treatments, at the same sampling point (T5: lower-case; T10: upper-case; $p < 0.05$). Asterisks indicate significant differences between T5 and T10 for the same tissue ($p < 0.05$). Abbreviations: Control - Mussels subjected to average seawater temperature in Porto Brandão (20 °C); MHW- Mussels subjected to a category II marine heatwave typically registered in the 24 °C); TOX- Mussels fed *P. lima*; and subjected to average seawater temperature in Porto Brandão (20 °C); MHW + TOX- Mussels fed *P. lima* and subjected to a category II marine heatwave (24 °C); CS – citrate synthase; LDH – lactate dehydrogenase.

in temperature as simulated in this study with a category II MHW, which, in turn, culminated in a lower ability to eliminate toxins.

Hence, based on the results observed at T5 (*P. lima* exposure phase) and T10 (*P. lima* depuration phase) it can be argued that during the acute exposure to +4 °C (MHW + TOX), *M. galloprovincialis* mussels presented lower accumulation, biotransformation/metabolization and elimination

of *P. lima* DSTs than at lower temperature (20 °C, TOX). In accordance with our results, reduced toxins accumulation/elimination mechanisms have been observed in bivalves exposed to warmer temperatures (e.g., paralytic shellfish toxins, PSTs; Braga et al., 2018; Tang et al., 2021).

The interactive effects between climate change-related stressors and marine biotoxins is a novel topic and, to the best of our knowledge, only three other recent studies (Braga et al., 2018; Tang et al., 2021; Funesto et al., 2023) were conducted on this direction so far and, thus, interpreting and drawing comparisons based on the currently available literature is rather challenging. Somewhat matching our results: i) *M. galloprovincialis* chronically exposed to warming and *Gymnodinium catenatum* not only accumulated lower levels of PSTs, but also exhibited lower ability to depurate them (Braga et al., 2018) and ii) *M. coruscus* acutely exposed to warming and *Alexandrium tamarense* presented a significant decline in PSTs levels but, contrary to our results, rapid elimination rates (Tang et al., 2021). Contrasting the trends observed in our study, as well as, in the ones carried out by Braga et al. (2018) and Tang et al. (2021), Funesto et al. (2023) reported an increase in DSTs concentration in Pacific rock oyster (*Magallana gigas*) specimens exposed to *P. lima* and a MHW ($\Delta T = +5$ °C; duration of exposure to peak temperature = 5 days), compared to the average values observed in bivalves subjected to the control temperature (20 °C). Moreover, DSTs concentration in the treatment combining MHW and *P. lima* exposure exceeded the maximum permitted levels for human consumption (173.3 ± 19.78 μg kg^{-1} soft tissue) (Funesto et al., 2023). Considering the similarities of the experimental design followed in Funesto et al. (2023) (i.e., similar temperature conditions, exposure durations and toxic microalgae species), the inconsistence of results can be most likely attributed to: i) the distinct physiological traits of the two model bivalve species (mussels versus oysters), namely, their filtration rates (e.g., Comeau et al., 2008), toxins uptake kinetics (Mafra Jr et al., 2010; Haberkorn et al., 2011) and thermal plasticity (or tolerance to thermal stress). Indeed, it has been previously reported that the *M. gigas* (formerly *Crassostrea gigas*) has an outstanding upper temperature tolerance, compared to other bivalve species (e.g., *Mytilus edulis*; Rajagopal et al., 2005); ii) species thermal history and habitat (i.e., intertidal versus subtidal) can also play an important role in bivalves' response to thermal stress (Collins et al., 2020); iii) toxins vehicle (dead versus live *P. lima* cells); and iv) depuration duration (5 days versus 21 days).

4.2. Interactive effects of MHWs and P. lima on M. galloprovincialis ecotoxicological responses

As ectothermic organisms, bivalves rely on the temperature conditions from the surrounding environment to perform their internal regulation. As fast-developing weather events, the occurrence of MHWs usually does not leave much time for marine bivalves to acclimate and reestablish their internal homeostasis, thus, being frequently correlated with depressed physiological and immunological pathways (e.g., Matozzo et al., 2012; Rahman et al., 2019; Feidantsis et al., 2020), increased vulnerability to concomitant stressors (e.g., pathogens or toxins from HABs; Boukadida et al., 2019; Funesto et al., 2023) or even, in most severe cases, mass mortality phenomena (Lattos et al., 2022). Hence, the present study provides novel insights on the extent to which MHWs can shape bivalves' antioxidant, heat shock and metabolic responses, confirming that, indeed, these extreme events alter their ecotoxicological responses to biotoxins (from *P. lima* HAB).

When it comes to the impacts of climate change-related stressors in marine bivalves, chronic exposure to ocean warming (which, by definition, has gradual and milder features that allow species' acclimation throughout time, to some extent) is, by far, one of the best studied climate change drivers (review in Liu et al., 2024). In contrast, information about the effects of unpredictable, fast-developing and extremely intense weather events, such as MHWs, is far less investigated, thus hampering appropriate comparisons between our study and previous research. In general, thermal stress has been associated with increased oxidative stress (Abele et al., 2001) and HSP70/HSC70 content (Feidantsis et al., 2020) in marine organisms' tissues, as well as metabolic deprivation and anaerobiosis (Anestis et al., 2010; Sokolova et al., 2012). Despite the results observed in mussels exposed to the MHW somewhat matched these patterns, differential responses were observed in muscle, gills and digestive gland, most likely, related with the distinct functionality, protein content (and basal enzyme activity) and sensitivity of the three studied tissues to environmental stressors (Snyder et al., 2001; Lyons et al., 2003; Piano et al., 2004; Alves de Almeida et al., 2007; Pernet et al., 2007). Overall, antioxidant scavengers' activity (CAT, SOD and GST) was not affected by the MHW and/or toxins in the muscle and digestive gland, whereas gills were much more responsive to stressors during the 5 days of exposure phase (T5). The increased sensitivity to toxins exposure observed in the gills could result from the fact that this tissue has direct contact with the water (and whatever toxins/contaminants it may contain) being responsible for filtration/ingestion of food (in this case, *P. lima*). Interestingly, while interaction of MHW and toxins exposure seemed to trigger increased damage to the lipids of cell membranes (i.e., LPO) in one of the aerobic tissues (i.e., muscles), toxins exposure had a more preponderant role in the organ responsible for food digestion and nutrients/contaminants metabolization (i.e., digestive gland) regardless of seawater temperature. This differential tissue pattern has been also reported in previous studies focused on thermal stress responses in bivalves (e.g., Piano et al., 2004; Meistertzheim et al., 2007; Zhang et al., 2014; Feidantsis et al., 2020). The fact that the combination of MHW and toxins did not exacerbate the effects of these two variables acting alone suggests that stressors interaction is a complex (and not straight forward) matter, as the final outcomes are not necessarily synergistic or additive. In other words, matching the findings of Funesto et al. (2023), the co-exposure to MHW and toxins did not seem to have a cumulative (and negative) effect on bivalves' antioxidant machinery.

Previous research has revealed that exposure to mild/moderate stress conditions (e.g., warming, contaminants) can enhance the synthesis of HSP70/HSC70 proteins (Monari et al., 2011; Aleng et al., 2015; Rahman and Rahman, 2021). These chaperones mediate various cellular processes including protein homeostasis, folding and degradation, playing a vital role on protein "quality control mechanisms" on the protection from stress-induced misfolding and aggregation of polypeptides. Still, as protein synthesis is an energetically demanding process that requires adequate metabolic functioning (i.e., favoring aerobic pathways), HSP70/HSC70 protein repairing mechanisms can only be maintained to a certain point. Hence, whenever organisms are subjected to severe (or long lasting) levels of stress, one can rather observe an inhibition of HSP70/HSC70 synthesis, instead of an activation (Fabbri et al., 2008; Madeira et al., 2012; Yang et al., 2021). As aforementioned, apart from the degree and duration of the inflicted stress, organisms' responses will be also highly dependent on the sensitivity and basal protein level of a given tissue (Fabbri et al., 2008; Madeira et al., 2012).

Studies on bivalve species subjected to thermal stress have reported increased HSP70/HSC70 concentrations, particularly in gills, even after short-term exposure (Park et al., 2007; Nie et al., 2017; Chan et al., 2019). Interestingly, in the present study, HPS70/HSC70 concentrations were only significantly enhanced in the gills under the interaction of MHW and toxins, while the muscle and digestive gland tended to exhibit lower values, especially when bivalves were exposed to MHWs alone. This trend could be somewhat attributed to the fact that 24 °C of seawater temperature (i.e., MHW conditions herein simulated) is still within the physiological plasticity thresholds of *M. galloprovincialis* (upper thermal limit set at 26 °C; Feidantsis et al., 2020). Noteworthy, the presence of toxins, on its own (at the concentration and exposure duration defined in the experiment) only had a significant effect on protein chaperoning/damage in *M. galloprovincialis* muscle.

Effectively managing the energy budget (i.e., the amount of energy that is spent on internal regulation, locomotion, reproduction or growth versus the amount of energy that is acquired from food) is essential to

assure the livelihood of organisms (Sokolova et al., 2012; Sokolova, 2013). Coping with the exposure to environmental stress (including thermal stress and/or contaminants) has substantial impacts on organisms' physiology and resilience, as it generally comes with high energy costs and implies an energy reallocation towards vital processes (Sokolova et al., 2012; Chovatiya and Medzhitov, 2014). Matching the results reported elsewhere (Peck et al., 2002; Stevens and Gobler, 2018), organisms' aerobic metabolism was significantly elevated by the exposure to MHWs and/or toxins (i.e., higher CS activity followed by lower LDH activity in muscle), confirming that both stressors (alone or combined) can, indeed, affect bivalves' metabolic rates. Once again (and in agreement with previous studies), the gills exhibited increased sensitivity to stressors (i.e., decreased CS and LDH activities) most likely due to their involvement in water filtration, which is crucial to perform internal temperature adjustments and increase oxygen sequestration in face of higher metabolic demands (Tripp-Valdez et al., 2019; Feidantsis et al., 2020).

Upon 5 days of recovery period (from the two stressors), bivalves' biomarker responses were, in some cases, significantly altered, though, a full return to basal levels (i.e., to values similar to those of the control treatment) was not necessarily observed. For instance, the significant increase in CAT activity observed between T5 and T10 in bivalves' muscles when exposed to MHW alone may indicate that CAT returned to the basal levels once seawater temperature returned to 20 °C. From the oxidative damage standpoint, muscle and gills showed increased ability to recover from the stress induced by toxins compared to the digestive gland, considering the significant decrease of LPO in TOX (gills) MHW + TOX (muscle and gills) treatments between T5 and T10. The heterogeneous distribution of toxins across mussels' tissues, together with digestive gland involvement in contaminants' elimination may explain the persistence of cellular stress in this tissue beyond the recovery period (Blanco et al., 2007). As for protein damage, a consistent pattern was not observed among the different tissues, as both inductions and inhibitions of HSP70/HSC70 synthesis were found upon 5 days of recovery from the MHW and/or toxins exposure. This inconsistency in tissue responses constitutes a main (though, common) challenge when interpreting multi-biomarkers data, and reflects the fact that tissues/organs can activate distinct biological pathways and have different paces to cope/recover from stress (Piano et al., 2004; Lattos et al., 2023).

From the metabolic perspective, noticeable differences between T5 and T10 were detected in bivalves' i) muscle (i.e., decreased CS activity), in animals previously exposed to the MHW, which indicates a recovery from 24 °C to 20 °C in this tissue, probably linked to a decrease in the metabolic demand associated to the previous exposure to elevated temperatures (Leal et al., 2019; George et al., 2023); ii) gills (i.e., decreased CS activity and increased LDH activity), in animals previously exposed to MHW, which can indicate that temperature exposure can indeed modulate mussels' metabolism; and iii) digestive gland (i.e., increased LDH and decreased CS activities), in animals previously exposed to toxins, but the absence of significant differences in the mussels previously exposed to the combined effects of MHW and toxins corroborates the fact that, indeed, toxins exposure can modulate mussels' metabolism, which, in turn, can be affected by higher temperatures, a result also reported by Funesto et al. (2023) in *M. gigas* exposed to *P. lima*.

5. Conclusions

The present study confirmed the influence of seawater temperature on OA and DTX1 accumulation and elimination mechanisms in the marine bivalve *M. galloprovincialis*. Overall, the exposure to +4 °C associated with a category II MHW event led to lower accumulation, biotransformation and elimination of *P. lima* DSTs, thus, resulting in lower toxins concentration during the exposure period and no significant changes in toxins concentration between exposure (T5) and elimination (T10) periods in MHW + TOX treatment. Data also underscored *M. galloprovincialis* susceptibility to MHWs and toxins from *P. lima*, revealing that the exposure to these two stressors can affect tissue antioxidant, heat shock and metabolic responses in distinct ways whenever stressors are acting alone or combined. However, it should be noticed that this finding may be limited to the presently studied model, as thermal tolerance, physiological plasticity and metabolic rates are highly variable among bivalve species. In addition, not all MHWs are felt in the same way across the planet and, as such, the magnitude and duration of these climate phenomena is also a parameter that needs to be carefully considered when interpreting results. Further environmental monitoring and modelling of both MHWs and HABs is, therefore, utmost required as to better understand the impacts that these events can have at the ecological, seafood safety and economical levels, as well as, to improve the readiness and resilience of the fisheries and aquaculture sectors against climate change effects. All in all, the interactive effects of climate change-related stressors (e.g., MHWs) on the occurrence of HABs is, thus, a poorly studied but most important topic that should be prioritized in future research, particularly, considering regional specificities and sensitivities, such as severity of climate stressor, type of toxic microalgae blooms and predominantly affected bivalve species.

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CRediT authorship contribution statement

Marta Dias: Visualization, Software, Investigation, Formal analysis, Data curation, Writing – review & editing, Writing – original draft. **Busenur Özkan:** Investigation, Writing – review & editing, Writing – original draft. **João Ramos:** Investigation. **Pedro Reis Costa:** Validation, Supervision, Conceptualization, Writing – review & editing. **Ana Luísa Maulvault:** Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization, Writing – review & editing, Writing – original draft. **António Marques:** Writing – review & editing. **Rui Rosa:** 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.

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Data availability

Data will be made available on request.

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