frontiers | Frontiers in Ocean Sustainability

TYPE Original Research
PUBLISHED 04 April 2025
DOI 10.3389/focsu.2025.1575728

OPEN ACCESS

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RECEIVED 12 February 2025
ACCEPTED 18 March 2025
PUBLISHED 04 April 2025

CITATION
Alves AV, Gusso-Choueri PK, Altafim GL, Ferraz MA, Trevizani TH, Felix CSA, Figueira RCL, Abessa DMdS and Choueri RB (2025) Influence of CO_2-induced acidification and temperature increased on the toxicity of metals in sediment in the mussel *Mytella charruana*. *Front. Ocean Sustain.* 3:1575728. doi: 10.3389/focsu.2025.1575728

Influence of CO_2-induced acidification and temperature increased on the toxicity of metals in sediment in the mussel *Mytella charruana*

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Environmental and climate changes have placed increasing pressure on the resilience of marine ecosystems. In addition to these transformations, coastal environments are also affected by anthropogenic stressors, such as metal contamination. Bivalves play a crucial ecological role in marine and estuarine ecosystems. This study aimed to evaluate the effects of CO_2-induced acidification, warming, and mixed metals contamination on the mangrove mussel *Mytella charruana*. We evaluated DNA damage (strand breaks), lipid peroxidation (LPO) levels, and reduced glutathione (GSH) content, as well as the enzymatic activities of glutathione S-transferase (GST) and glutathione peroxidase (GPx) in the gills and digestive glands. Additionally, neurotoxicity was assessed in muscle tissues through acetylcholinesterase (AChE) activity. Laboratory experiments were conducted using sediments spiked with metals (Cu, Pb, Zn, and Hg), alongside a control group (non-spiked sediments), combining with three pH levels (7.5, 7.1, and 6.7) and two temperatures (25 and 27°C). Five mussels per treatment (four replicates) were exposed for 96 h. Two pools of two organisms each were separated per replicate ($n = 8$) and their gills, digestive glands, and muscles were dissected for biochemical biomarkers analyses. Temperature increase and metal contamination were the primary factors modulating antioxidant responses in the gills and digestive glands, as well as AChE activity in the muscle. However, when combined with CO_2-induced acidification, these stressors also affected DNA integrity and LPO. Acidification alone showed no effect for any biomarker analyzed. Higher IBR values indicated effects for combined metal exposure, even at concentrations below individual safety levels. Here, we provide insights from a short-term experiment on the complex interactions between predicted scenarios, in which climate change stressors influenced estuarine mussel responses when associated with a mixture of metals in sediments. These findings contribute to

understanding of organismal responses in complex scenarios of contamination and climate change, particularly in estuarine environments.

KEYWORDS

biomarkers, ocean acidification, ocean warming, antioxidants defenses, contamination, ecotoxicology

1 Introduction

Since the pre-industrial period, atmospheric CO_2 concentrations have increased from 277 to 414 ppm in 2021, and they have continued to increase by approximately 2.4 ppm per year over the last decade, reaching 1.070 ppm until the end of the century (Friedlingstein et al., 2020; IPCC Intergovernmental Panel on Climate Change, 2022). In addition, current surface seawater temperatures have been approximately 1.0 °C warmer since the beginning of the industrial period (Lough et al., 2018; Garcia-Soto et al., 2021; IPCC Intergovernmental Panel on Climate Change, 2022). The global ocean absorbs approximately one third of atmospheric CO_2 and heat (Caldeira and Wickett, 2003; Levitus et al., 2005) leading to an expected reduction of surface ocean water pHs of an average of 0.3–0.4 units and temperature elevation by 2.6–4.8°C by 2100 (IPCC Intergovernmental Panel on Climate Change, 2014).

Ocean acidification and warming are critical threats to coastal ecosystems (Boyd et al., 2018; Lu et al., 2018; Bahamon et al., 2020; Cotovicz Júnior et al., 2022). Furthermore, sediments from densely urbanized and industrialized coastal areas are frequently contaminated by metals and metalloids such as mercury, arsenic, cadmium, lead, zinc, and copper (Boyd, 2010; Kim et al., 2016; Alves et al., 2022). Under such conditions, estuarine and coastal organisms are commonly exposed to the metals present in sediments (Trevizani et al., 2023; Pinho et al., 2024). Previous studies have reported that both low pH/high pCO_2 and warming can affect the solubility and speciation of metals in the environment (Tsai et al., 2003; Sokolova and Lannig, 2008; Millero et al., 2009; Banaee et al., 2024) as well as their availability, increasing their potential toxic effects on biota (Roberts et al., 2013; Halsband et al., 2024).

Bivalves play a crucial ecological role in marine and estuarine ecosystems (Byrne and O'halloran, 2001). The benthic mangrove mussel *Mytella charruana* (Mollusca: Mytilidae) is commonly found in tropical estuarine regions (Yuan et al., 2016), adhering to hard substrates or forming dense aggregates in estuarine mud. Owing to its suspension-feeding mode, this species is exposed to sediment contamination through water and food ingestion. Bivalve molluscs are considered effective bioindicators because of their tolerance to fluctuations in abiotic factors (Belivermiş et al., 2016, 2023), including elevated pCO_2 (Range et al., 2012), and their capacity to accumulate contaminants throughout their life cycle, making them widely used in environmental risk assessments of metal pollution in marine systems (Zhang et al., 2010; Basallote et al., 2015; Campana et al., 2015; Chandurvelan et al., 2015; Rocha et al., 2016). Therefore, these organisms can be considered excellent test organisms for evaluating scenarios of marine acidification and warming in sediments contaminated by metals.

Although many organisms, such as bivalves, have evolved to resist daily or seasonal abiotic variations in coastal areas (Ross et al., 2016; Zhao et al., 2020; Trevisan and Mello, 2024), complex interactions in multiple potential stressors vary and can drive changes in physiological, biochemical, and ecological fitness tolerance thresholds (Pörtner et al., 2004; Noyes et al., 2009; Maulvault et al., 2017; Alves et al., 2024) as well as their metal accumulation potential (Lacoue-Labarthe et al., 2011; Belivermiş et al., 2016; Nardi et al., 2018), increasing their vulnerability.

Specifically, acidification directly affects mollusks by (i) alterations in shell structure and growth (Michaelidis et al., 2005; Beniash et al., 2010; Dickinson et al., 2013; Harvey et al., 2013; Thomsen et al., 2013; Sokolova et al., 2016), (ii) changes in the opening and closing of valves (Clements et al., 2018), (iii) changes in metabolic processes (heat tolerance and growth rates) (Duarte et al., 2014; Nikinmaa and Anttila, 2015; Wang et al., 2015; Freitas et al., 2017; Cao et al., 2022), and (iv) induction of oxidative stress (Matozzo et al., 2013; Regoli and Giuliani, 2014; Cao et al., 2018). In addition, temperature increases in coastal surface waters affect the physiological processes in bivalves, increasing metal uptake (Belivermiş et al., 2016; Maulvault et al., 2016; Sampaio et al., 2018), changing their energy balance (Sokolova and Lannig, 2008; Cherkasov et al., 2010), causing oxidative stress (Verlecar et al., 2007; Mangan et al., 2017), modifying the lysosomal system (Izagirre et al., 2014; Múgica et al., 2015), and mortality (Lannig et al., 2006).

The effects of exposure to toxic substances or other stressors can be assessed by using various biomarkers. These serve as monitoring tools that provide mechanistic insights into contaminant concentrations in tissues and their associated toxic effects and, by detecting sublethal changes, biomarkers offer an early warning of environmental quality degradation (Depledge et al., 1995; Monserrat et al., 2007; Hwang et al., 2008). This study aimed to evaluate the biomarker responses of *M. charruana* exposed to multiple stressors simultaneously, such as CO_2-induced acidification, warming, and sediment metal contamination. Considering the potential effects in multiple stressor scenarios, we hypothesized that the acidification and warming of coastal waters caused by the increase in atmospheric CO_2 can modify the toxicity of metals due to changes in the physiology of the mussel *M. charruana*. These results will allow for a better understanding of the mechanisms underlying organismal resilience, prediction of ecological impacts, and improvement of environmental risk assessments in a changing ocean.

2 Materials and methods

2.1 Experimental design

Biomarker responses of the gills, digestive glands, and muscles of *M. charruana* were assessed using a full factorial experimental design with three fixed factors (and their interactions): (i) CO_2-induced acidification, (ii) warming, and (iii) sediment contamination by metals (Cu, Pb, Zn, and Hg). The effects of changes in pH/pCO_2 were tested at three levels: pH 7.5 (control pH/pCO_2 – without CO_2 injection), pH 7.1 (medium pH/medium pCO_2), and pH 6.7 (low pH/low pCO_2). This range of tested pH/pCO_2 levels was established based on predictions of oceanic pH made using Representative Concentration Pathways (RCP) 8.5 (Bindoff et al., 2022) and the Coupled Model Intercomparison Project (CMIP6) of the World Climate Research Program (IPCC Intergovernmental Panel on Climate Change, 2021). Two temperatures were tested: 25°C (control temperature) and 27°C (increased temperature), based on the projections range for the end of the century by Intergovernmental Panel on Climate Change on the Representative Concentration Pathways (RCP) 4.5 model (IPCC Intergovernmental Panel on Climate Change, 2014). The metal contamination in sediment was assessed using sediment spiked with Cu, Pb, Zn, and Hg, metals commonly reported in estuarine sediments contaminated, and harmful to organisms (Alves et al., 2022; Zhang et al., 2024). According to a pilot assay, the concentrations of metals were established in two levels: control (cntrl) (no metal-spiked sediment) and a mild metal contamination, corresponding to 75% of the Interim Sediment Quality Guidelines (ISQG) and Probable Effects Level (PEL) [Canadian Council of Ministers of the Environment (CCME), 2002], simulating a reality commonly found in contaminated coastal regions.

Specimens of *M. charruana* were obtained from fishermen's farms in mangrove areas along the banks of the Bertioga Channel (Bertioga, northern coast of São Paulo State, Brazil, within a marine protected area), where this species is abundant. They were transported to the laboratory in thermal boxes for acclimatization. Before the experiments, the organisms underwent a 7-day acclimation period under controlled laboratory conditions. They were divided into three distinct groups of 60 individuals of homogeneous size and kept in 45 L aquariums containing natural seawater diluted with freshwater (reaching salinity 24), without sediments, to simulate estuarine condition (hereafter referred as experimental water), under constant aeration. Water pH and temperature were gradually adjusted over the 7-day period (±0.4 unit/day) to reach the target experimental conditions, minimizing osmotic and thermal stress on the organisms. During the acclimatization period, the organisms were fed every 48 h with a commercial suspension designed for filter-feeding organisms (Phyto-Plus B®), containing a mixture of microalgae species (2–23 μm), at a dosage of 50 μL per individual. Prior to the experiment, eight organisms were randomly collected from each acclimatization aquarium, and their gills, digestive gland, and muscle tissues were removed for analysis to determine the baseline physiological conditions (T0).

The bioassays were conducted in a hermetic box system adapted from Altafim et al. (2023). Each box contained glass bottles (3 L) with 500 mL of sediment and 2 L of filtered experimental water (salinity 24), with four replicates per treatment, each containing 5 individuals ($n = 5$). The organisms were fed once after 2 days of exposure, and the experiment lasted for 4 days. Physicochemical parameters (i.e., pH, dissolved oxygen (DO), and salinity) were measured at the beginning and at the end of the bioassay. At the end of exposure, the organisms were kept on ice, and the gills, digestive glands, and muscle tissues were collected for biomarker analyses, in pools of two organisms per replicate ($n = 8$ per experimental treatment). After the dissection of the organisms, the soft tissues were immediately stored in an ultra-freezer (−80°C).

2.2 Sediment collection, characterization and spiking

Sediment samples were collected from a reference area, in a mangrove portion located at the mouth of the Itaguaré River (23.763°S, 45.773°W), also in the municipality of Bertioga. This location is legally protected as the Restinga of Bertioga State Park (Decree N°. 53.526/2008). In the laboratory, sediment was stored at a constant temperature of 4°C and protected from light until the start of the experiment. The sediment was characterized for grain size fractions through dry sieving and classified based on the Wentworth (1922) scale. The $CaCO_3$ content was quantified by digestion using hydrochloric acid (HCL 5 N) (Hirota and Szyper, 1975), and the organic matter (OM) content was quantified using the loss by ignition method (Luczak et al., 1997).

For the sediment spiking process, the moisture content of the sediment was determined by oven-drying (for 4 d at 60 °C). The moisture was restored by adding distilled water and the spiked sediment was carried out using stock solutions of each metal of interest, prepared by the dissolution of inorganic salts of copper sulfate ($CuSO_4$), lead chloride ($PbCl_2$), zinc sulfate ($ZnSO_4$), and mercuric chloride ($HgCl_2$) (Merck®, reagent grade) in distilled water, following the protocols established by the United States Environmental Protection Agency (United States Environmental Protection Agency (USEPA), 2001) and the American Society for Testing and Materials (American Society for Testing Materials (ASTM), 2014). Quantities of each metal stock solution were spiked to an amount of the sediment, in order to reach nominal concentrations, in dry weight, of 14.03 mg kg^{-1} of Cu, 22.65 mg kg^{-1} of Pb, 93.0 mg kg^{-1} of Zn, and 0.1 mg kg^{-1} of Hg. This method has been described in detail by Alves et al. (2024). An amount of non-spiked sediment was subjected to the same spiking process as a negative contamination control, in which only distilled water was added to the same volume of the metal stock solution used.

2.3 CO_2 Injection system, pH, and temperature control

The CO_2-induced acidification was controlled by an automated series of solenoid valves connected to an Apex Fusion® software

that manipulated the CO_2 injection. The method employed was CO_2 indirect introduction, which manipulated the pCO_2 in the atmosphere of semi-closed boxes (Altafim et al., 2023; Alves et al., 2024). To achieve this, there is a direct introduction of CO_2 into a beaker containing distilled water, allowing CO_2 to disperse into the atmosphere inside the box, controlling the decrease and stabilization of pH in each experimental unit of each treatment. The pH of each experimental treatment box was continuously monitored throughout the exposure period using pH sensors connected to the Apex system, as when the pH increased or decreased the established value by 0.05, the system automatically released or opened CO_2 injection to restore the set pH.

The temperature at 25 ± 1°C was maintained by air conditioning the room, while the temperature of 27 ± 1°C was kept stable by aquarium thermostats within the respective treatment boxes during the entire exposure period. Additionally, before and during the experiment period, the temperature in each box was calibrated and constantly monitored using analog glass thermometers.

2.4 Carbonate system speciation analysis

Total alkalinity (TA) was measured in each replicate in all treatments, after the exposure time, through titration with HCl (0.1 M) (Sigma-Aldrich analytical grade) using an automatic titrator (Hanna HI901C) coupled to a meter (Hanna Instruments, ref. HI1131) for pH measurement. Then, data from pH (total pH scale/mol kg^{-1} H_2O), TA, measured salinity and temperature were used to estimate the parameters of the carbonate system speciation (partial pressure of CO_2 (pCO_2), inorganic carbon (TIC), HCO_{-3}, CO_3^{-2}, Ω aragonite and Ω calcite saturation states), using the CO2SYS program (Pierrot et al., 2006), employing the dissociation constants of carbonic acid in seawater (Mehrbach et al., 1973; Dickson and Millero, 1987; Dickson, 1990).

2.5 Sediment metal analysis

The concentrations of metals (Cu, Pb, Zn, and Hg) in the sediments from each contamination treatment (control and metal contamination) were measured before the beginning of the experiments (T0) and at the end of exposure (T1) from each treatment. All laboratory instruments and non-volumetric glassware were decontaminated with detergent, ultrapure water, and 5% HNO_3.

The sediment samples were lyophilized and subjected to partial acid digestion following a modification of the USEPA method 3050B (United States Environmental Protection Agency (USEPA), 1996). The digestion methods for Cu, Pb, and Zn were detailed by Altafim et al. (2023) and the acid extracts were quantified using Inductively Coupled Plasma Optical Emission spectroscopy (ICP-OES) (Varian equipment, model 710ES), following USEPA 6010C method (United States Environmental Protection Agency (USEPA), 2007). To control method accuracy and precision, certified reference material (CRM) was analyzed using the same analytical procedure for the respective analyses. The certified reference material SS-2 (EnviroMAT Contaminated Soil, SCP Science) ($n = 3$) was used for Cu, Pb, and Zn quantification, with recoveries of 87–97% (Supplementary Table 1). The analytical limits of quantification (LOQ) were calculated for each element of interest for an uncertainty of 20% (m = 1.0 g; v = 50 mL) (Supplementary Table 2).

For the determination of mercury (Hg) in sediments, the Direct Mercury Analyzer DMA-80 Tri Cell (Milestone, Italy) was used. This method eliminates the need of sample preparation, enabling direct analysis through a simplified process involving drying, decomposition, amalgamation, and detection by atomic absorption spectrometry (AAS). The main advantage lies in its efficiency, bypassing time-consuming steps. The samples were weighed (50 mg) directly into nickel boats and subjected to a heating cycle: drying at 200 °C for 60 seconds, decomposition at 650°C for 120 s and amalgamation for 12 s. Mercury vapor was transported by 99.9% pure oxygen (White Martins, Brazil) at 4 kg cm^1, with detection based on absorbance at 253.7 nm. Each analysis was completed in approximately 5 min. Method accuracy was validated using CRM MESS-3 and DORM-4 (National Research Council of Canada, NRC – CNRC) ($n = 3$), yielding results of 0.088 ± 0.004 μ g^{-1} (MESS-3) and 0.457 ± 0.04 μg g^{-1} (DORM-4), consistent with certified values of 0.091 ± 0.009 μg g^{-1} and 0.412 ± 0.036 μg g^{-1}, with recovery result of 97 and 111%, respectively (Supplementary Table 1). Calibration curves (0.01–100 ng Hg) showed determination coefficients > 0.999, with detection and quantification limits of 4.0 pg and 12.0 pg, calculated as (3σ/S) and (10σ/S), where σ is the standard deviation of 10 blank measurements and S is the calibration curve slope.

2.6 Biomarkers responses

Aliquots of the samples of gills and digestive glands of mussels were separated for quantification of levels of non-protein thiols (reduced glutathione, GSH), and DNA damage (strand-breaks). In addition, glutathione-S-transferase (GST) and glutathione peroxidase (GPx) enzymatic activities were analyzed. The levels of LPO in gills were also quantified. Adductor muscle tissues were separated for acetylcholinesterase (AChE) enzymatic activity analysis. The tissues were homogenized (in a pool of 2 organisms) to 5% w/v in a Tris-HCl buffer in pH 7.6 in Tris-HCl buffer (50 mM Tris; 1 mM EDTA; 1 mM DTT; 50 M Sucrose; KCl a 150 mM, 1 mM PMSF, pH 7.6) in a cell disruptor (Loccus L-beader 24). Aliquots of gill and digestive gland homogenates were separated for total protein quantification, DNA damage, and LPO (150 μl). Then, the remained samples were centrifuged for 20 min at 12,000 G in 4°C and the supernatant was separated for analysis of GST and GPx enzymatic activities, and GSH levels. In the case of muscle tissue, the supernatant fraction was used to determine AChE. Total protein contents were quantified in the homogenized and centrifuged fractions using the dye-binding method by Bradford (1976) using bovine serum albumin protein (BSA) as standard. All analyzes were performed on a microplate reader (Biotek-Synergy ™ HT).

For DNA damage analysis, the alkaline precipitation method described by Olive (1988) was used, with DNA quantification

performed through fluorescence (Gagné and Blaise, 1995). DNA damage was expressed as μg of DNA mg^{-1} of protein. Oxidative stress-induced changes in LPO levels were assessed indirectly using the thiobarbituric acid reactive substances (TBARs) method, following Wills (1987), with results expressed as μM TBARs mg^{-1} of total protein. GSH levels were determined using the method of Sedlak and Lindsay (1968), measured spectrophotometrically at 415 nm, and expressed as nmol min^{-1} mg^{-1} of total protein. GST enzymatic activity was determined by incubating supernatants with 42 mM 1-chloro-2,4-dinitrobenzene (CDNB), 1 mM glutathione, and 80 mM K_2HPO4 (pH 6.5). The reaction was monitored spectrophotometrically at 340 nm for 5 min, with measurements taken at 30-s intervals (Keen et al., 1976). GST enzymatic activity was expressed as nmol GSH-CDNB min^{-1} mg^{-1} of protein. GPx enzymatic activity was assessed following the method of Sies et al. (1979), using a reaction mixture containing 1 mM hydroperoxide in sodium phosphate buffer (pH 7.0), with NADPH added as a cofactor. Absorbance was measured spectrophotometrically at 340 nm, and GPx enzymatic activity was expressed as nmol min^{-1} mg^{-1} of total protein. Neurotoxicity effects were assessed by measuring AChE activity following the method of Ellman et al. (1961). A reaction mixture containing 10 mmol L^{-1} DTNB and 0.075 mol L^{-1} acetylcholine iodide in potassium phosphate buffer (pH 7.6) was added to the samples. Absorbance was immediately recorded at 405 nm for 2 min, with measurements taken at 30-s intervals. AChE activity was expressed as nmol NTB min^{1} mg^{1} of protein.

2.7 Data treatment

Mean sediment quality guideline quotients (mSQGQs) were calculated for each experimental treatment based on the Probable Effects Level (PEL) [Canadian Council of Ministers of the Environment (CCME), 2002] to estimate the relative impact of sediment metal contamination in sediments on biomarker responses in *Mytella charruana*. The mSQGQ was determined by normalizing the concentration of each metal in the sediments to its respective sediment quality guideline (SQG) and then averaging the individual metal quotients into a single value (Long, 2006). The means Probable Effects Level quotient (mPELq) was determined according to Equation 1:

$$\mathrm{mPELq} = \Sigma(\mathrm{Ci/PELi})\mathrm{n}^{-1} \quad (1)$$

where "Ci" represents the investigated content of metal "i", "PELi" represents the PEL of metal "i" and n represents the number of metal species. Categories of mPELq was classified according to ecotoxicological risk with 10, 25, 50, and 75% probability of toxicity, respectively: low risk level (mPELq $\leq$ 0.1); low to medium risk level (0.11 < mPELq < 1.5); medium to high risk level (1.51 < mPELq < 2.3), and high ecotoxicological risk level (mPELq > 2.3) (Long and MacDonald, 1998).

Biological data were analyzed by first assessing potential laboratory effects of acclimation to pH/pCO_2 and warming on the general health condition of the tested organisms. This was done by comparing biomarker data before (T0) and after the bioassays (control treatment for each pH and temperature). Then the effects of CO_2-induced acidification, temperature, and metal contamination on biomarker responses were assessed. For both comparisons, a General Linear Model (GLM) analysis with an identity link function were built using the statistical software (Jamovi Project, 2022) (version 2.3) Gallucci (2019) for each biomarker analyzed. A confidence level of $p \leq 0.05$ and the distribution was determined according to the best fit of the model to the data distribution, which are the lowest Akaike Information Criterion (AIC) (Quinn and Keough, 2002) and best normality of residuals were adopted for all statistical analyses. Tukey's *post hoc* test was performed, when necessary, to detect significant differences between levels.

2.7.1 Integrative approach

Mean values of each biomarker as variables was transformed by Z-score. After, PCA was performed using the software PAST (version 5.0.2) (Hammer et al., 2001), and values greater than $\geq$ 0.4 were considered relevant associations.

A more general view of the biomarker responses was obtained by means of the calculation of the "Integrated Biomarker Response" – version 2 individual (IBRv2i) for each treatment, following the methodology proposed by Sanchez et al. (2013) and adapted by Mattos et al. (2024). This IBR approach compares the average of biomarker responses of mussels exposed to stressors (and their interactions) with the average of those of animals under control conditions (at normal or basal levels of each biomarker), maintaining biomarker data for each individual in order to enabling the use of statistical analysis. For this study, the respective control groups of each pH at each temperature were used as reference. The index was calculated as: (i) log transformation of the data; (ii) Z-score; (iii) subtraction of the mean Z-score of the control or reference group from the Z-score of all groups; (iv) modulus of all Z-scores to obtain absolute values; (v) sum of the absolute Z-scores of the biomarkers of each experimental group with the weight attributed to each biomarker according to its systemic importance (GSH, GST, and GPx = 1; DNA and LPO = 2) to obtain the IBRv2i. In general, lower values of the IBR index are indicative of a better health status (or greater animal fitness), and higher scores may indicate a worsening of the physiological condition of the organisms. IBRv2i of the gills and digestive gland were compared to the control using the same GLM approach described to detect significant differences between levels.

3 Results

3.1 Physicochemical analysis of sediment

The grain size analysis demonstrated a predominance of very fine sands (54.74%), followed by fine sands (41.38%), with 0.87% silt and clay. The sediment had $CaCO_3$ levels of 2.23% and OM of 1.90%.

Sediment metal spiking was effective, with sediment concentrations at the beginning of exposure approaching the pre-established nominal concentrations set for this study. The concentrations of Cu, Pb, Zn, and Hg in sediment are in Table 1. The final exposure period (T1) concentrations of

trace metals added to the sediment did not show substantial differences among pH and temperature levels comparing with the beginning (T0). In addition, the mPELq values of metals spiked sediment in the control and metal contamination treatments at 25 and 27°C exhibited low to medium ecotoxicological risk classification at the beginning and end of the experiment (Table 1).

3.2 Carbonate chemistry speciation

In general, the physical-chemical parameters of the seawater measured at the beginning and the end of the bioassay were maintained within acceptable limits for the experiment and low variations regarding at T0. Salinity levels were maintained around 25 ± 1, while dissolved oxygen concentrations remained 5.95 ± 0.68 mg L^{-1}, and non-ionized ammonia were 0.02 ± 0.01 mg L^{-1}. The pH and temperature exhibited minimal variation within the same treatment but were well different between the tested levels, with 7.46 ± 0.03 for the control, 7.14 ± 0.04 for the medium pH and 6.8 ± 0.03 to low pH, and 24.42 ± 0.63 °C / 27.09 ± 0.42 °C, respectively (Supplementary Table 3).

The carbonate system parameters analyzed did not vary regarding the increase in temperature, validating the manipulations by CO_2 injection (Table 2). As expected, the CO_2-induced acidification treatments (pH 7.1 and 6.7) had increasing in total alkalinity, total inorganic carbon, pCO_2, and CO_2 concentrations. An inverse relationship with TIC, ΩCa and ΩAr saturations and carbonate concentrations were reduced. These analyses confirmed the reduction of pH by manipulations with CO_2 injection and are in line with other studies (Ferraz et al., 2022; Altafim et al., 2023; Alves et al., 2024; Portugal et al., 2025).

3.3 Biomarkers responses

3.3.1 Interactive effects of pH, temperature, and contamination on gills tissue

The results of GLM analysis of biomarker responses on gills tissue are presented in Figure 1, and QQ Plot residuals are detailed in Supplementary Figure 1. In the comparison between the condition after 7 days of organisms acclimatization to the laboratory conditions (T0) with their respective control treatments for each pH and temperature, there was a significant difference only at pH 6.7 at the highest temperature for DNA damage ($p < 0.001$), and for GSH at pH 7.5 ($p < 0.001$) and 6.7 ($p = 0.004$) at 25°C, which indicates low or no "laboratory effect" on biomarkers in the gills of *M. charruana*.

For DNA damage in gills, GLM analysis showed that the variation in pH/pCO_2 did not show a significant individual effect, but only when combined with contamination (AIC = 1,245.23, $F = 4.52$, df = 2, $p = 0.014$), causing a decrease of DNA damage in pH 6.7, compared with pH 7.5, at 25 °C ($p_{tukey} = 0.007$). Also, there was an increase in DNA damage due to the increase in temperature in the control treatments (no metal contamination) ($p_{tukey} = 0.015$). Interactions among the three factors were found for levels of LPO (AIC = 638.23, F = 12.50, df = 2, $p < 0.001$), with reduced effects on metal-contaminated treatments in pHs 7.5 and 7.1 at the control temperature (25°C) compared with their respective controls ($p_{tukey} = 0.049$ and $p_{tukey} = 0.014$, respectively), however, high pCO_2 decreasing LPO levels in control treatment ($p_{tukey} = 0.020$) and increased in metal contamination ($p_{tukey} = 0.002$). In addition, temperature induced LPO levels in pH control (7.5) at metal contamination while inhibiting them in control at pH 7.1 compared to the same treatments in temperature control ($p_{tukey} = 0.005$ and 0.004, respectively). Regarding GSH levels, significant difference in the interaction between the three factors were also observed (AIC = 964.77, $F = 3.13$, $df = 2$, $p = 0.049$), with an increase only in the pH 7.5 with metal exposure at 27°C when comparing on all pHs at 25°C ($p_{tukey} = 0.043$; 0.005; 0.007), but when combining low pH (6.7) and warming, the GSH levels were inhibited in metal-contaminated treatment ($p_{tukey} = 0.048$). As for GST enzymatic activity, significant difference was found only among the temperatures tested (AIC = 973.46, $F = 23.14$, $df = 1$, $p < 0.001$), which increased in warming scenario ($p_{tukey} < 0.001$). GPx enzymatic activity showed interaction between temperature and metal contamination (AIC = 1,296.88, $F = 3.38$, $df = 1$, $p = 0.05$), with an increase in GPx activity related with metal contamination in control temperature (25°C) ($p_{tukey} < 0.001$), but decrease associated temperature increase (27°C) ($p_{tukey} = 0.014$).

3.3.2 Interactive effects of pH, temperature, and contamination on digestive gland tissue

The results of GLM analysis of biomarker responses on digestive gland tissue of *M. charruana* are presented in Figure 2, and QQ Plot residuals are detailed in Supplementary Figure 2. In the comparison between the acclimatization period (T0) and the respective control treatments, there was a significant difference only in the pH 7.5 at 27°C for GPX enzymatic activity ($p = 0.017$). On the exposure comparisons between treatments, DNA damage exhibited a slight increase when the temperature increased (AIC = 598.71, F = 4.48, $df = 1$, $p = 0.043$). For GSH, the interaction between temperature and metal contamination was significant (AIC = 309.71, $F = 13.64$, $df = 1$, $p < 0.001$), showing the induction of GSH after metal exposure at 27°C, compared to 25°C ($p_{tukey} < 0.001$), and among control and metal contamination treatment at the same temperature ($p_{tukey} = 0.002$). Effects related with sediment contamination by metal were observed (AIC = 500.27, $F = 5.26$, $df = 1$, $p = 0.029$), with decreased GST enzymatic activity in metal-contaminated treatments compared to control in 25°C ($p_{tukey} = 0.013$). As for GPx enzymatic activity, significant interactions between temperature and metal contamination were observed (AIC = 684.08, $F = 9.77$, $df = 1$, $p = 0.004$), with a decrease in metal-contaminated treatments at 25°C when compared with controls (no-spiked sediment) ($p_{tukey} = 0.013$), and between control treatments (no spiked-metal sediment) at 25 and 27°C ($p_{tukey} < 0.001$). Although not statistically different, there is a tendency of reduction of the GPx enzymatic activity associated with metal contamination in the more acidic treatment (pH 6.7) at 27°C compared to the same treatment at control temperature.

TABLE 1 Analytical concentrations (mg kg^{-1}) and calculated mPELq for the spiked trace metals, other trace elements, and all trace elements in the tested sediments, at the beginning and end of the tests, in all experimental treatments.

Factors				Metals spiked (mg kg^{-1})				mPELqespcific (metals spiked)		
T °C	Time	pH	Cont. level	Cu	Pb	Zn	Hg	Mean	SD	Risk classification
25°C	T0	-	Cntrl	1.31	5.81	4.61	0.03	0.15	2.35	Low to Medium
			MC	15.71	26.00	96.00	0.12	0.89	36.70	Low to Medium
	T1	7.5	Cntrl	0.96	5.00	6.01	0.04	0.14	2.54	Low to Medium
			MC	13.00	23.03	79.00	0.12	0.78	30.10	Low to Medium
		7.1	Cntrl	0.97	5.82	5.24	0.03	0.14	2.54	Low to Medium
			MC	15.73	25.60	89.98	0.12	0.87	34.20	Low to Medium
		6.7	Cntrl	0.96	6.00	4.62	0.05	0.17	2.47	Low to Medium
			MC	15.06	25.78	84.20	0.15	0.90	31.87	Low to Medium
27°C	T0	-	Cntrl	0.92	5.83	5.00	0.04	0.15	2.50	Low to Medium
			MC	15.49	25.34	91.26	0.10	0.84	34.79	Low to Medium
	T1	7.5	Cntrl	1.40	4.92	5.18	0.05	0.16	2.21	Low to Medium
			MC	15.79	26.54	95.04	0.12	0.89	36.26	Low to Medium
		7.1	Cntrl	0.90	5.27	5.49	0.05	0.16	2.47	Low to Medium
			MC	13.72	19.30	70.12	0.11	0.71	26.51	Low to Medium
		6.7	Cntrl	0.88	13.61	5.50	0.05	0.28	5.38	Low to Medium
			MC	15.08	24.45	80.70	0.12	0.84	30.48	Low to Medium

3.3.3 Interactive effects of pH, temperature, and contamination on adductor muscle tissue

For the AChE enzymatic activity in muscle tissues, there were differences between the acclimatization period (T0) and the respective control treatments only in the lowest pH tested (6.7) at warm temperature (27°C) ($p < 0.001$). There was a significant difference among the metal contamination and temperatures (AIC = 952.60, $F = 5.16$, $df = 1$, $p = 0.02$), showing that the increase in enzyme cholinesterase levels in metal contaminated exposure at 27°C treatments compared to 25°C ($p_{tukey} = 0.037$) (Figure 3 and QQ Plot residuals are detailed in Supplementary Figure 3).

3.3.4 PCA integration of biomarkers responses

The results of the PCA integrated all data from biomarkers responses for gills, digestive glands, and muscle tissues, and the factors tested: CO_2-induced acidification, warming, and metal contamination. Three principal components (PCs) explained 74.9% of the original data variance, with coefficients $\geq |0.4|$ (Table 3, Figure 4). PC1 explained 30.1% of the data variance and showed positive associations among GSH and GST activity in gills, GSH in digestive gland, and AChE activity in muscle. PC2 explained 24.4% of the data variance, showing a positive association for GST enzymatic activity in digestive gland and a negative association for DNA damage and GPx enzymatic activity in gills. For PC3 there was a strong association between LPO levels in gills and GPx enzymatic activity in digestive glands, explaining 20.4% of variances. Overall, the observed PCA results suggest that temperature and metal contamination and temperature are the factors most responsible for the effects. In addition, when the three factors occur simultaneously, they may influence the effects of antioxidant enzymes and neurotoxicity.

3.4 IBRv2i index

3.4.1 IBRv2i in gills tissue

The integration of biomarkers responses showed interaction among temperature and metal contamination (AIC = 424.82, $F = 15.67$, $df = 1$, $p = 0.002$), increasing the IBRv2i index in the control treatments at 27°C compared with 25°C ($p_{tukey} = 0.05$). In addition, highest alteration IBRv2i index was observed between the control and metal-contaminated treatments at 25°C ($p_{tukey} < 0.001$) and 27 °C ($p_{tukey} = 0.013$) (Figure 5A and QQ Plot residuals are detailed in Supplementary Figure 4A). Therefore, these results suggest that temperature and metal contamination were the factors that most influenced the effects in gills of *M. charruana*.

3.4.2 IBRv2i in digestive gland tissue

The IBRv2i index for the digestive gland showed a significant difference in the interaction among pH/pCO_2 and metal contamination (AIC = 195.65, $F = 5.78$, $df = 2$, $p = 0.002$), showed an increasing in IBRv2i index along with the metal-contaminated treatment in pH 7.5 compared to the respective control ($p_{tukey} < 0.001$), followed of a decrease in the lowest pH tested (6.7) in the metal-contaminated treatment ($p_{tukey} = 0.04$) (Figure 5B and QQ Plot residuals are detailed in Supplementary Figure 5B).

TABLE 2 Values of carbonate system speciation calculated at the end of the experiment with metals in sediment under different warming scenarios. Quantified values of total alkalinity (TA) in water samples and estimated concentrations of total inorganic carbon (TIC), bicarbonate (HCO_3^-), carbonate (CO_3^{-2}); partial pressure of CO_2 (pCO_2); saturation state for calcite (ΩCa) and aragonite (ΩAr).

Factors			pH final (total scale)	TA (μmol kg^{-1})	TIC (μmol kg^{-1})	pCO_2 (μatm)	HCO_3^- (μmol kg^{-1})	CO_3^{-2} (μmol kg^{-1})	CO_2 (μmol kg^{-1})	ΩCa	ΩAr
T °C	pH	Cont. level	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
25 °C	7.5	Cntrl	7.53 ± 0.07	1,175.15 ± 59.45	1,147.42 ± 63.56	855.96 ± 161.64	1,090.65 ± 60.63	31.37 ± 4.69	25.40 ± 4.86	0.82 ± 0.12	0.52 ± 0.08
		MC	7.45 ± 0.04	1,212.78 ± 47.45	1,201.19 ± 53.52	1062.70 ± 145.92	1,142.62 ± 50.30	26.13 ± 1.35	32.45 ± 4.27	0.68 ± 0.04	0.43 ± 0.02
	7.1	Cntrl	7.13 ± 0.04	1,046.20 ± 54.26	1,460.86 ± 63.04	2,619.67 ± 335.77	1,366.16 ± 54.94	15.47 ± 1.39	79.23 ± 10.21	0.40 ± 0.04	0.26 ± 0.02
		MC	7.14 ± 0.08	1,559.40 ± 121.26	1,619.85 ± 145.77	2,885.09 ± 728.69	1,514.61 ± 125.64	17.61 ± 1.69	87.62 ± 22.29	0.46 ± 0.05	0.29 ± 0.03
	6.7	Cntrl	6.85 ± 0.05	2,551.03 ± 70.23	2,804.96 ± 54.76	9,095.08 ± 828.13	2,515.60 ± 65.82	15.29 ± 2.13	274.07 ± 25.73	0.40 ± 0.06	0.25 ± 0.04
		MC	6.82 ± 0.08	2,538.70 ± 163.95	2,825.29 ± 227.73	9,898.17 ± 2317.77	2,506.81 ± 166.22	13.75 ± 2.34	304.73 ± 70.43	0.36 ± 0.06	0.23 ± 0.04
27 °C	7.5	Cntrl	7.40 ± 0.04	1,171.14 ± 231.30	1,162.02 ± 238.99	1,170.64 ± 333.59	1,103.89 ± 226.47	24.87 ± 3.21	33.27 ± 9.47	0.65 ± 0.08	0.42 ± 0.05
		MC	7.47 ± 0.03	1,146.42 ± 88.77	1,126.30 ± 88.31	965.62 ± 107.99	1,070.75 ± 83.92	28.04 ± 3.23	27.51 ± 3.08	0.74 ± 0.09	0.47 ± 0.05
	7.1	Cntrl	7.17 ± 0.01	1,551.02 ± 70.23	1,597.09 ± 73.25	2,675.98 ± 135.87	1,501.07 ± 68.72	19.86 ± 0.93	76.16 ± 3.97	0.52 ± 0.02	0.33 ± 0.02
		MC	7.18 ± 0.03	1,457.57 ± 32.37	1,498.14 ± 28.36	2,464.84 ± 173.01	1,408.88 ± 28.91	19.12 ± 1.78	70.14 ± 4.98	0.50 ± 0.05	0.32 ± 0.03
	6.7	Cntrl	6.73 ± 0.07	2,352.40 ± 51.55	2,665.60 ± 96.90	1,1517.79 ± 2145.85	2,326.09 ± 52.17	11.33 ± 1.74	328.18 ± 61.14	0.30 ± 0.05	0.19 ± 0.03
		MC	6.80 ± 0.02	2,484.27 ± 92.24	2,824.86 ± 83.27	12,543.89 ± 3,603.42	2,455.90 ± 86.47	12.30 ± 3.16	356.66 ± 103.13	0.32 ± 0.08	0.21 ± 0.05

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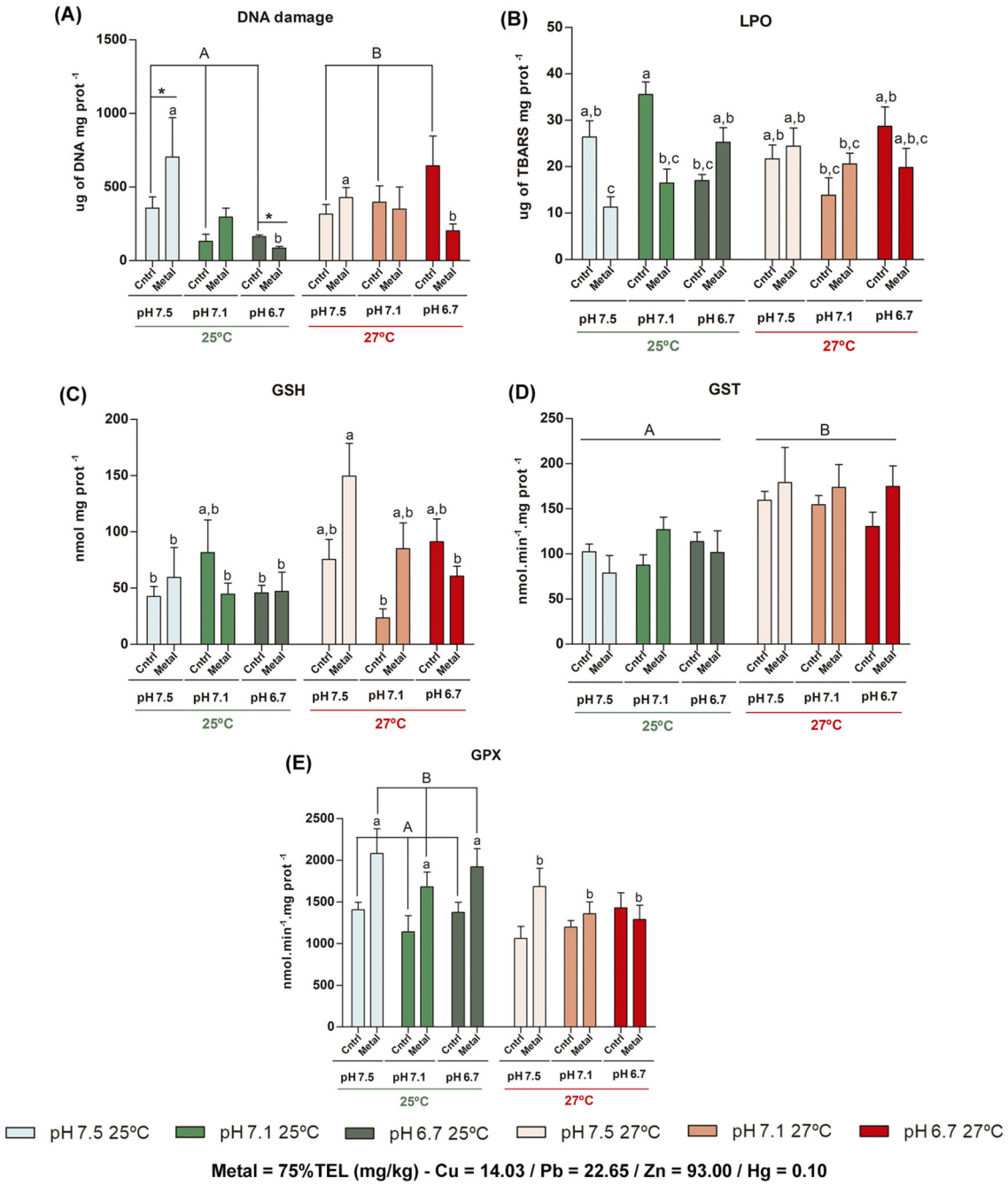

FIGURE 1
Means and standard deviations ($n = 8$) of biomarkers responses on gills tissue of *Mytella charruana*. **(A)** DNA damage, **(B)** Lipid peroxidation (LPO), **(C)** Reduced glutathione (GSH), **(D)** Glutathione s-transferase (GST), and **(E)** Glutathione peroxidase (GPx). Green-toned bars represent treatments at 25°C, and red-toned bars represent treatments at 27°C. Light green and light red bars represent treatments at pH 7.5, green and salmon red bars represent treatments at pH 7.1, and dark green and red bars represent treatments at pH 6.7. C = control treatments (without metal exposure) and Metal = Cu, Pb, Zn, and Hg sediment contaminated treatments. Capital letters represent significant differences between factors (considering the entire factor group), and lowercase letters represent significant differences between treatments (each single bar).

4 Discussion

This study demonstrated that the interaction of warming and/or CO_2-induced acidification with metal-contaminated sediments, even at concentrations below the threshold effect levels, could alter the antioxidant defense mechanisms of *M. charruana* within a short exposure period. Overall, significant effects of stressors and their interactions were observed across different soft tissues. The gills and digestive glands were particularly influenced by increased temperature and metal contamination in the sediments. Acidification is recognized as a strong pro-oxidant stressor in bivalves (Dean, 2010; Munari et al., 2018; Romero-Freire

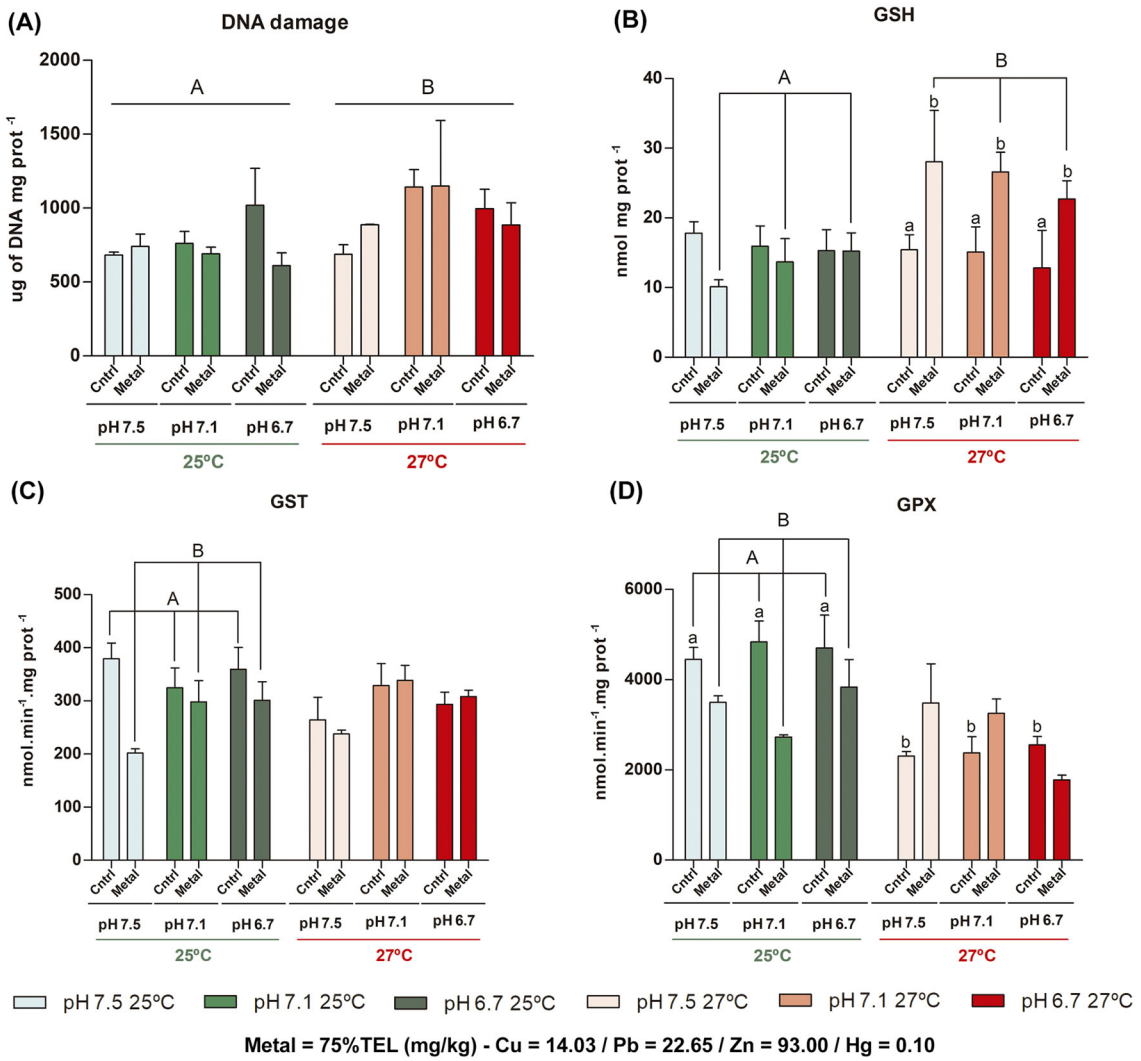

FIGURE 2
Means and standard deviations ($n = 8$) of biomarker responses on digestive gland tissue of *Mytella charruana*. **(A)** DNA damage, **(B)** Reduced glutathione (GSH), **(C)** Glutathione s-transferase (GST), and **(D)** Glutathione peroxidase (GPx). Green-toned bars represent treatments at 25°C, and red-toned bars represent treatments at 27°C. Light green and light red bars represent treatments at pH 7.5, green and salmon red bars represent treatments at pH 7.1, and dark green and red bars represent treatments at pH 6.7. Cntrl = control treatments (without metal exposure), and Metal = Cu, Pb, Zn, and Hg sediment contaminated treatments. Capital letters represent significant differences between factors (considering the entire factor group), and lowercase letters represent significant differences between treatments (each single bar).

et al., 2024; Sun et al., 2025). In this study, elevated pCO_2 primarily exerted an effect when combined with a temperature increase and/or metal exposure. In scenarios involving multiple stressors, including global environmental changes, it is crucial to consider (eco)toxicological interactions at cellular and physiological levels, as they may influence the overall toxicity of metals (Ivanina and Sokolova, 2015; Zeng et al., 2015).

The data showed that warming alone inhibited glutathione peroxidase (GPx) enzymatic activity in the digestive gland (without interaction with metals or increase in pCO_2). GPx plays a role in the defense mechanism by catalyzing hydrogen peroxide degradation, thus preventing the accumulation of these radicals in cells (Storey, 1996; Reed, 2013). GPx and some isoforms of glutathione S-transferase (GST) reduce lipid hydroperoxides to alcohol, with the concomitant oxidation of GSH to GSSG (Regoli and Giuliani, 2014). Higher temperatures are likely to increase the release of ROS, thus increasing the risk of oxidative damage, thus an overload of the antioxidant system may occur (Benedetti et al., 2022). Therefore, defenses related to catalyzing hydrogen peroxide degradation were likely compromised, which may have caused the inhibition of GPx activity in this study, probably due to an increase in metabolic processes (Matoo et al., 2021) and/or damage in the defense mechanism. In contrast, exposure to increased temperatures induced GST enzymatic activity in the gills. To neutralize the effects of increased reactive oxygen species (ROS), bivalves can increase antioxidant defenses in cells (Jeeva Priya et al., 2017). GST contributes to detoxification by neutralizing ROS by conjugating them with glutathione so that they can be excreted (Stegeman et al., 1992). Therefore, this induction may indicate the activation of a detoxification mechanism as a result of

TABLE 3 Results of principal component analysis (PCA) integrating all data from biomarkers responses for gills (G), digestive gland (DG), and adductor muscle tissues (AM) of the mussel *M. charruana* exposed to CO_2-induced acidification, warming, and metal contamination experimental treatments.

Eigenvectors			
Variables	Component 1	Component 2	Component 3
DNA damage_G	0.11	**−0.48**	−0.11
LPO_G	0.09	0.21	**0.56**
GSH_G	**0.45**	−0.18	0.34
GST_G	**0.47**	0.14	−0.30
GPX_G	−0.14	**−0.48**	0.10
DNA damage_DG	0.30	0.18	−0.28
GSH_DG	**0.45**	0.19	0.09
GST_DG	−0.12	**0.56**	0.01
GPX_DG	−0.22	0.12	**0.51**
AChE_AM	**0.40**	−0.16	0.32
Eingenvalues	3.01	2.44	2.04
% of variance	30.11	24.47	20.40

Relevant correlations highlighted in bold (≥ |0.4|).

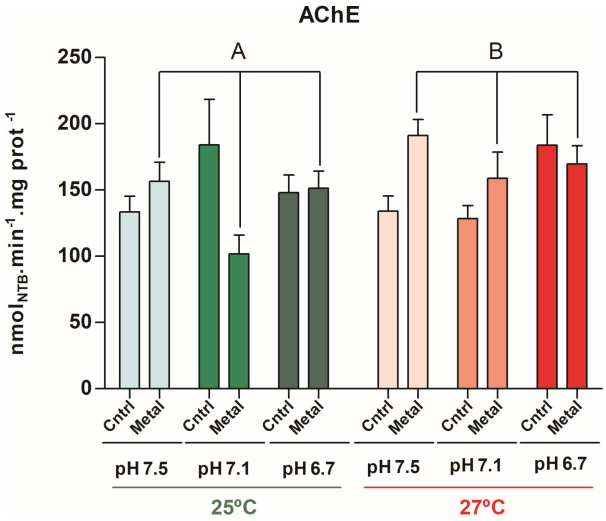

FIGURE 3
Means and standard deviations ($n = 8$) of biomarker response of neurotoxicity effects (Acetylcholine - AChE) on adductor muscle tissue of *Mytella charruana*. Green-toned bars represent treatments at 25°C, and red-toned bars represent treatments at 27°C. Light green and light red bars represent treatments at pH 7.5, green and salmon red bars represent treatments at pH 7.1, and dark green and red bars represent treatments at pH 6.7. Cntrl = control treatments (without metal exposure), and Metal = Cu, Pb, Zn, and Hg sediment contaminated treatments. Capital letters represent significant differences between factors.

heat stress, since GST may be one of the most active antioxidant enzymes in mussels and its increased activity could represent a compensatory mechanism for the simultaneous inhibition of other antioxidant enzymes (Vidal-Liñán et al., 2010; Cui et al., 2020), such as the GPx in this study. These findings are supported by the literature, which indicates that temperature is a dominant factor in the physiological regulation of bivalves (Matoo et al., 2021). Although mussels are generally tolerant to temperature stress, when optimum physiological ranges are extrapolated and/or when co-exposure to other stressors occurs, one of the consequences of heat stress may be increased oxidative stress due to the imbalance between the production and detoxification of ROS, such as superoxide, hydroxyl, and hydrogen peroxide, overloading antioxidant enzymes (Abele et al., 2002; Matoo et al., 2013). In line with our results, temperature was the main variable that induced oxidative stress, such as GST in the gills of the mangrove oyster *Crassostrea rhizophorae* in estuaries from Brazilian coast (Zanette et al., 2006).

In addition to the direct effects on organisms, temperature can also influence susceptibility to metals (Banni et al., 2014; Nardi et al., 2017; Morosetti et al., 2020), as observed in this study, owing to changes in chemical speciation. However, this influence may be limited (Sokolova and Lannig, 2008). The interactions between heat stress and metal exposure are likely driven by additive or synergistic mechanisms, as metal uptake is temperature-dependent owing to increased metabolic demand and membrane permeability (Quinn, 1988; Foulkes, 2000). Additionally, metals can disrupt regulatory mechanisms for essential redox elements and affect the thermal tolerance of organisms (Cosson et al., 2008; Sokolova and Lannig, 2008). Moreover, metals can specifically contribute to the imbalance of antioxidant enzymes by ROS production, either by donating electrons to stress markers or by catalyzing Fenton-type reactions, thereby increasing intracellular ROS levels (Yakovleva et al., 2004; Varotto et al., 2013; Regoli and Giuliani, 2014; Benedetti et al., 2016). This, in turn, can affect the capacity of antioxidant defenses (Regoli and Principato, 1995), further exacerbating oxidative stress in the exposed organisms.

In this study, metal exposure in sediment inhibited GPx and GST enzymatic activities, while increasing GSH levels in the digestive gland of *M. charruana*, regardless of acidification or warming. This response may indicate a shift in the cellular redox state, which contributes to the elimination of oxyradicals (Viarengo et al., 1990). Mussels can counteract the initial oxidative stress by enhancing antioxidant defense mechanisms (Mocan et al., 2010; Coppola et al., 2018). Consistent with our findings, previous studies have reported the activation of antioxidant responses following exposure to various metals (Verlecar et al., 2007; Coppola et al., 2017; Morais et al., 2023). Analysis of the antioxidant responses in the gills revealed that metal contamination significantly increased GPx enzymatic activity under controlled temperature conditions (25°C). However, when the temperature was raised to 27°C, individuals from the same treatments exhibited significant inhibition of enzymatic activity, suggesting a reduced capacity for hydroperoxide degradation in a warmed environment. These findings are consistent with those of previous studies, such as Benedetti et al. (2016), who reported that the interaction between temperature and Cd reduced GPx enzymatic activity in the digestive gland of the scallop, *Adamussium colbecki*.

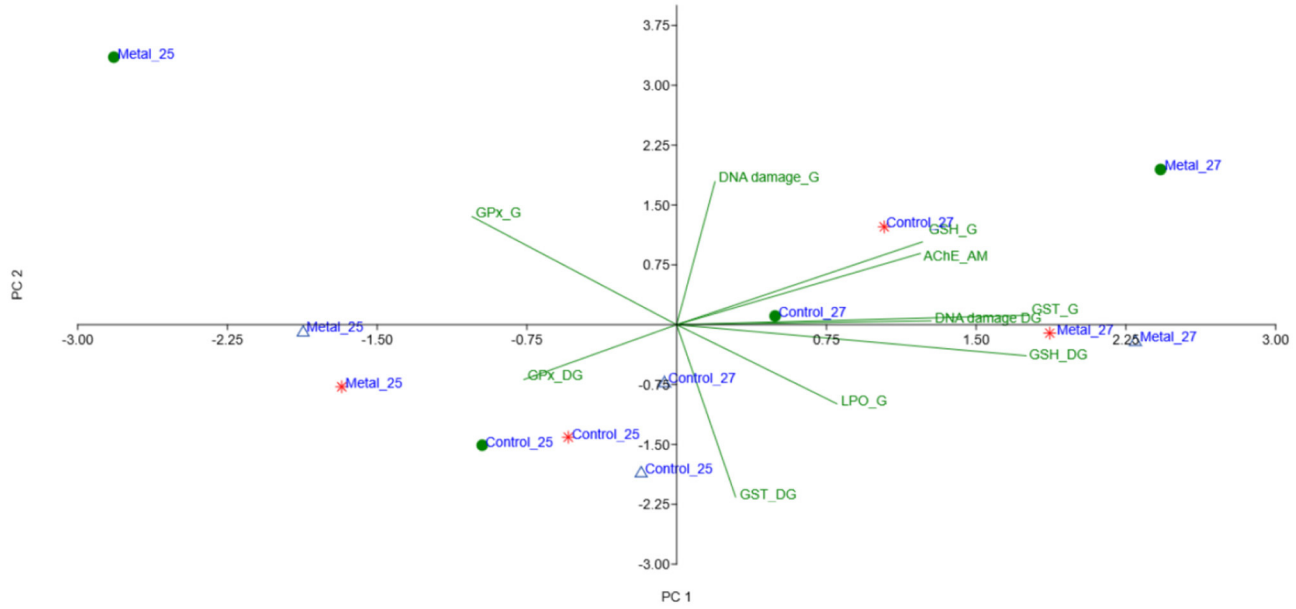

FIGURE 4
Biplot of PC1 and PC2 based on PCA results, integrating all data from biomarkers responses in gills, digestive glands, and muscle tissues of the mussel *Mytella charruana* exposed to CO_2-induced acidification, warming, and metal contamination experimental treatments. G, gills; DG, digestive gland; AM, adductor muscle. Green circles = pH 7.5; Blue triangles = pH 7.1; Red asterisk = pH 6.7.

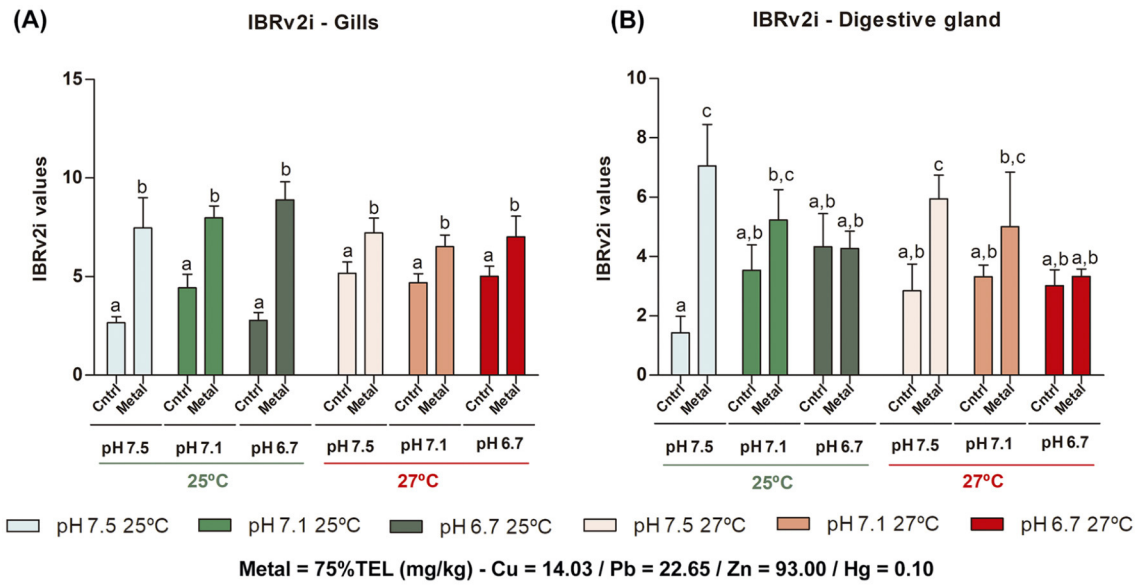

FIGURE 5
The Integrated Biomarker Response – version 2 individual (IBRv2i) index for **(A)** gills and **(B)** the digestive gland tissues of *Mytella charruana*. Green-toned bars represent treatments at 25°C, and red-toned bars represent treatments at 27°C. Light green and light red bars represent treatments at pH 7.5, green and salmon red bars represent treatments at pH 7.1, and dark green and red bars represent treatments at pH 6.7. Cntrl = control treatments (without metal exposure), and Metal = Cu, Pb, Zn, and Hg sediment contaminated treatments. Capital letters represent significant differences between factors (considering the entire treatment group within the same factor), and lowercase letters represent significant differences between treatments.

Similarly, Coppola et al. (2018) observed limited activation of antioxidant defenses in *Mytillus galloprovincialis* exposed to Hg at elevated temperatures (21°C), attributing this effect to metabolic downregulation mechanisms. At higher temperatures, organisms may employ strategies, such as valve closure, to reduce metabolism and serve as a defense against thermal stress. Adaptive responses have also been documented in other studies (Anestis et al., 2007; Sokolova et al., 2012; Morosetti et al., 2020). Valve closure is also a common response to other types of stress such as metal exposure (Liao et al., 2005) and low pH (Pynnönen and Huebner, 1995).

Exposure to CO_2-induced acidification affects the gills of *M. charruana* only when combined with warming and/or metal contamination, leading to distinct response patterns. In the case of GSH levels in the gills, although GSH activity increased in the presence of metal contamination at elevated temperatures, a decrease was observed as the pH decreased. This suggests that GSH is rapidly consumed by binding to metals (or metal-induced ROS) for excretion at a rate exceeding its replenishment capacity. Such an imbalance in the antioxidant system could compromise cellular defense against oxidative stress (Franco et al., 2009; Morosetti et al., 2020). In bivalves, exposure to high pCO_2 levels has been widely associated with a reduced antioxidant capacity (Wang et al., 2016; Zhang et al., 2022). However, there is no clear consensus in the literature, as some studies have reported an increase in GSH levels under high pCO_2 and warming in the gills of *Mytilus coruscus* (Hu et al., 2015), and in combination with metal contamination and warming also in gills of *A. colbecki* (Benedetti et al., 2016) and *Perna viridis* (Verlecar et al., 2007). Conversely, Wang et al. (2016) found that elevated pCO_2 (~2,000 ppm) inhibited GSH levels in the digestive gland of *Crassostrea gigas*, with no observed interaction with other stressors.

Lipid peroxidation is commonly used as an indicator of cellular damage to oxidative stress by the interaction of ROs with cell membrane structures or through the interaction of membranes with electrophiles (Pisoschi et al., 2021). Increased levels of LPO in co-exposure to high pCO_2 and metal contamination were observed in the gills. Increased LPO levels were attributed to failures in detoxification mechanisms to prevent cellular damage in the gills of the oyster *C. gigas* exposed to Cu and acidification (pH 7.6) (Cao et al., 2019). Therefore, it can be attributed that the increase in LPO levels in the current study may be due to effects on antioxidant mechanisms, because despite the increase in GST enzymatic activity in the gills, there was a reduction in GSH levels and GPx enzymatic activity, which probably contributed to a lower cellular protective capacity.

Another harmful effect observed in *M. charruana* in this study was increased DNA damage in the digestive gland, particularly at 27°C. As discussed earlier, climate change stressors, such as warming and acidification, combined with metal contamination can elevate ROS production. This leads to the induction or inhibition of oxidative enzymes, which can have a range of detrimental effects on the bivalve physiological functions, including lipid peroxidation and DNA damage (Gagné et al., 2008; Pöhlmann et al., 2011; Tomanek, 2011; Izagirre et al., 2014; Múgica et al., 2015; Munari et al., 2018). These findings are consistent with the results of the present study, in which higher levels of LPO were observed in the gills of *M. charruana* exposed to metal at the lowest pH. An interesting response was observed regarding DNA damage in gills (at 25 and 27°C). Under acidification scenarios (high pCO_2 levels and metal contamination), a reduction in DNA damage was observed in organisms exposed to metals. This effect, combined with the LPO results, suggests that despite the occurrence of oxidative damage, it does not reflect DNA fragmentation. Although this response may vary depending on the species and tissue analyzed. Previous studies have correlated this reduction with the activation of antioxidant defenses, reporting an increase of DNA strand breaks in gills and an inhibition in the digestive gland of *M. galloprovincialis* at low pH (7.0) (Munari et al., 2018). Metals can increase DNA strand breaks by producing ROS through the Fenton reaction (Stohs and Bagchi, 1995), but at the same time, the oxidative stress induced by them can be alleviated in hypercapnia (Ivanina et al., 2013). An increase in pCO_2 levels may attenuate the response to oxidative stress caused by metals and warming. The relief of hypercapnia in oxidative stress is attributed to the reaction of H^+ ions introduced into the medium with free radicals produced by other stressors, such as temperature and metal contamination, generating water and oxygen, decreasing the availability of ROS, and alleviating oxidative stress (Sampaio et al., 2017; Santos et al., 2022).

AChE is commonly found in nervous tissues, such as neuromuscular junctions (Silman and Sussman, 2005). The current results showed that the presence of a mixture of metals in a heated environment resulted in the significant activation of AChE in the muscle of *M. charruana*. The available information on the impact of co-exposure of metals and/or warming upon AChE activity is very inconsistent, varying according to the species, tissue and metal chosen. First, a recent study showed that metal ions inhibited AChE activity in *M. charruana* in the order $Hg^{2+} > Pb^{2+} > Cd^{2+} > As^{2+} > Cu^{2+} > Zn^{2+}$ (Santos et al., 2022). AChE activity in bivalves can be influenced by several abiotic factors, especially temperature (Dellali et al., 2001). In the gills of *Mytilus sp*. AChE showed higher activity at higher temperatures (~23°C) (Pfeifer et al., 2005), whereas Kamel et al. (2012) observed inhibition of AChE in *M. galloprovincialis* adults exposed to 18–26°C. Regarding co-exposure to stressors simultaneously, the results also vary. Attig et al. (2014) revealed that the combination of warming (26°C) and metal (Ni) caused inhibition of the AChE enzymatic activity in the digestive gland of *M. gallaprovincialis*. However, according Boukadida et al. (2022) thermal stress combined with Cu and Ag significantly increased the AChE activity in the larvae of the mussel *M. galloprovincialis*, corroborating our results. Furthermore, the effects on AChE activity were not influenced by pCO_2 in *M. charruana* in the present study. Qu et al. (2022) observed that the effect of CO_2 and Cu on *M. galloprovincialis* was tissue-dependent, with a greater effect on the gills.

Exposure of multiple stressors simultaneously can affect the physiological response and stress tolerance of marine organisms, especially bivalves (Cherkasov et al., 2007; Nardi et al., 2018; Maar et al., 2018; Maulvault et al., 2019; Khan et al., 2020; Chahouri et al., 2023). In this study, the results of biomarkers in the gills and digestive gland showed a dependence of stressor interaction and the biomarker analyzed, in which the interaction of the effects can induce or inhibit oxidative stress in mussels. For instance, PCA integration showed that temperature modulated most of the antioxidant enzyme responses in all the tissues analyzed. In addition, CO_2-induced acidification appears to be a stressor that affects biological responses only in interactions with temperature and/or metal contamination. In a general context, the IBR index results reinforce the biological effects of temperature and/or acidification on metal contamination in both tissues analyzed. Higher IBR values for contamination by a mixture of metals can indicate a significant effect (as seen in gills and digestive glands in the current study), even at concentrations below individual safety levels. Higher IBR values have been associated to

contamination exposure (Marigómez et al., 2013; Bertrand et al., 2018) and climate changes (Maulvault et al., 2018; Marques et al., 2020). Interactions between warming and metal contamination may be associated with increased metal absorption through increased metabolism and membrane permeability, affecting the organism's susceptibility to metal contaminants (Lannig et al., 2008; Sokolova and Lannig, 2008; Banni et al., 2014; Coppola et al., 2017; Lan et al., 2020). Previous studies showed that co-exposure to metals and elevated pCO_2 can regulate antioxidant mechanisms and attenuate metal-induced ROS generation, in which acidification may antagonize the negative physiological effects of metals on energy metabolism and oxidative stress in bivalves (Tomanek, 2011; Ivanina et al., 2013). Jin et al. (2021) revealed that the negative effects of metals on marine organisms can be alleviated by acidification, leading to a neutral impact of heavy metals in combination with high pCO_2. Antagonistic relationships between Cu and pCO_2 have also been reported (Marangoni et al., 2019; Marques et al., 2020), and it can also be explained by a CO_2-related increase in H^+ ions concentration in the cellular environment, which is counterbalanced by acid-base compensation to normalize the intracellular pH. This is because the presence of excess H^+ ions due to acidification activates antioxidant defenses to combat ROS (Michaelidis et al., 2007; Tiedke et al., 2013; Heuer and Grosell, 2014; Sampaio et al., 2017, 2018). On the other hand, when bivalves are simultaneously exposed to multiple stressors, intracellular ROS formation can increase, leading to alterations in antioxidant mechanisms and cellular damage (Regoli and Giuliani, 2014; Freitas et al., 2019). Figueiredo et al. (2022) were observed high LPO levels for the mollusk *Spisula solida* exposed to La metal, warming, and acidification.

Although studies with multiple stressors are important to achieve greater comparability to natural conditions, identifying the factors responsible for this effect is a complex challenge. As discussed previously, a combination of factors can result in synergistic or antagonistic effects depending on the biological response analyzed. Understanding the effects of co-exposure to multiple stressors is essential for predicting their impacts in future scenarios; however, we strongly encourage studies that also test isolated factors and their effects beyond the interaction between them.

5 Conclusion

The results of this study suggest that climate change stressors can influence mussel responses to contamination by a mixture of metals in sediments, even at levels below the TEL; therefore, estuarine organisms tend to be more vulnerable to contamination under climate change scenarios. These findings revealed that warming and metal contamination were the main factors modulating antioxidant responses in the gills and digestive glands, as well as AChE activity in the muscle. When combined with CO_2-induced acidification, these stressors also affect DNA integrity and LPO levels. Here, we provide insights into a short-term experiment on the complex interactions between predicted climate change scenarios and exposure to a mixture of metals, suggesting that estuarine organisms tend to be more vulnerable to contamination in climate change scenarios. Similar studies conducted over longer exposure periods and with more specific biological analyses are needed to better understand the mechanisms of the potential effects or adaptations of mussels to climate change scenarios in co-exposure to metal contamination. However, this study suggested that the effects of metal pollution tend to be more severe when combined with ocean warming and acidification. Understanding the effects of climate change in future scenarios on estuarine ecosystems and their biota is essential for guiding decision makers' actions and public conservation policies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Declaration of Research Project without Human Involvement/Vertebs no. 2041787/2024/Postgraduate Program in Bioproducts and Bioprocesses – declaration of responsibility for the research project. Federal University of São Paulo. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AA: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. PG-C: Investigation, Methodology, Writing – review & editing. GA: Investigation, Methodology, Writing – review & editing. MF: Investigation, Methodology, Writing – review & editing. TT: Investigation, Methodology, Writing – review & editing. CF: Investigation, Methodology, Writing – review & editing. RF: Investigation, Resources, Supervision, Writing – review & editing. DA: Methodology, Resources, Writing – review & editing. RC: Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. The authors thank the São Paulo Research Foundation – FAPESP (Grants #2017/07351-4 and #2023/11496-9) and National Council for Scientific and Technological Development – CNPq (Grant #409498/2021-1) for funding the study. AA (#88887.501096/2020-00) and GA (#88887.000767/2024-00) thank Coordination for the Improvement of Higher Education Personnel (CAPES) for their doctoral scholarships. RC (#304844/2022-5), PGC (#304855/2022-7), RF (#313633/2021-5), and DA

(#308533/2018-6 and #313420/2023-8) thank CNPq for their research productivity fellowships.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that no Generative AI was used in the creation of this manuscript.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/focsu.2025.1575728/full#supplementary-material

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