The influence of temperature and salinity on the impacts of Lead in *Mytilus galloprovincialis*

Rosa Freitas[a*], Carla Leite[a], João Pinto[b], Marcelo Costa[b], Rui Monteiro[b], Bruno Henriques[b], Francesco Di Martino[a], Francesca Coppola[a], Amadeu M.V.M. Soares[a], Montserrat Solé[c], Eduarda Pereira[b]

[a]Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[b]Departamento de Química & CESAM & LAQV-REQUIMTE, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[c]Instituto de Ciencias del Mar ICM-CSIC, E-08003 Barcelona, Spain

*Corresponding Author: Rosa Freitas, Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal

Abstract

Mussels, such as the marine bivalve *Mytilus galloprovincialis* are sentinels for marine pollution but they are also excellent bioindicators under laboratory conditions. For that, in this study we tested the modulation of biochemical responses under realistic concentrations of the toxic metal Lead (Pb) in water for 28 days under different conditions of salinity and temperature, including control condition (temperature 17±1.0 ºC and salinity 30±1.0) as well as those within the range expected to occur due to climate change predictions (±5 in salinity and +4ºC in temperature). A comprehensive set of biomarkers was applied to search on modulation of biochemical responses in terms of energy metabolism, energy reserves, oxidative stress and damage occurrence in lipids, proteins as well as neurotoxicity signs. The application of an integrative Principal Coordinates Ordination (PCO) tool was successful and demonstrated that Pb caused an increased in the detoxification activity mainly evidenced by glutathione S-transferases and that the salinities 25 and 35 were, even in un-exposed mussels, responsible for cell damage seen as increased levels of lipid peroxidation (at salinity 25) and oxidised proteins (at salinity 35).

Keywords: climate change; metal(oid)s; bioaccumulation; mussels; oxidative stress; metabolism

1. INTRODUCTION

Environmental pollution by potential toxic elements, such as metals, has been a topic of concern over the last decades, with several studies highlighting not only the accumulation of these elements in different aquatic compartments but also their impacts on freshwater and marine organisms (among others, Al Naggar et al., 2018; Ansari et al., 2004; Bielen et al., 2015). As a result of their persistence and ability to bioaccumulate metals are reported to exert toxic effects in bivalves through interference on their redox pathways, resulting in the overproduction of reactive oxygen species (ROS) that may react with cellular targets including lipids and proteins and alter the activity of antioxidant and biotransformation enzymes (Freitas et al., 2018; Monteiro et al., 2019; Regoli and Giuliani 2014). Studies conducted with Mercury (Hg), Arsenic (As), Copper (Cu) and Cadmium (Cd), all elements on the top list of the most hazardous materials, already showed the capacity of these elements to interfere on bivalve's biochemical performance (Company et al., 2004; Coppola et al., 2018ab; Freitas et al., 2018; Gagnaire et al., 2004; Nardi et al., 2017; Samuel et al., 2005; Zhang et al., 2010). In what regards to Lead (Pb), one of the most widely distributed metals in marine and estuarine systems (Chakraborty et al., 2012; de Souza Machado et al., 2016; Singh et al., 2011), recent information has highlighted the impacts of this metal towards bivalves inhabiting these areas (e.g., Marques et al., 2018). Under laboratory conditions, the impacts induced by Pb in bivalves were also demonstrated, evidencing the capacity of this metal to disturb organism's oxidative status. For example, Zhang et al. (2010) demonstrated that in the bivalve *Chlamys farreri* exposed to Pb the antioxidant capacity was compromised resulting in increased levels of lipid peroxidation. Also, Wadige et al. (2014) revealed that in the freshwater bivalve *Hyridella australis* the total antioxidant capacity decreased while lipid peroxidation and lysosomal membrane destabilization increased alongside to Pb exposure. Nonetheless, Freitas et al. (2014) demonstrated that when exposed to an increasing gradient of Pb the clam *Ruditapes decussatus* activated their defence mechanisms (e.g. antioxidant enzymes and metallothionein content) preventing the occurrence of cellular damage. Such former evidences indicate that impacts by Pb may vary according to species but also on metal concentration and length of exposure.

Besides exposure to pollutants, aquatic systems are currently subjected to increased atmospheric carbon dioxide (CO_2) concentrations, at present already above ~400 ppm compared to pre-industrial revolution levels (Pörtner et al., 2014). Such CO_2 increase is responsible for the rise in global temperature, with a concomitant increase in mean seawater values of about 0.7°C since pre-industrial times, and a further rise of 3-4 ºC is foreseen at the end of this century (Collins et al., 2013; Pörtner et al., 2014). Additionally, extreme weather events, including heavy rainy or long drought periods, are expected to increase in frequency and intensity (Pörtner et al., 2014). Such environmental changes, associated to climate modification, may contribute to alterations in seawater characteristics, namely in terms of salinity and temperature. This can further result into changes in organism's sensitivity towards pollutants but also into modification of pollutants properties and their associated toxicity (Attig et al., 2014; Byrne, 2012; Coppola et al., 2018a; Izagirre et al., 2014; Manciocco et al., 2014; Mazzoto et al., 2013). In fact, marine and in particular estuarine organisms are naturally and simultaneously exposed to multiple stressors, including the ones associated to water characteristics and pollutants presence, with growing evidences that combined stressors frequently interact and often amplify effects (Dijkstra et al., 2013). However, interactions between stressors may be complex and difficult to predict, showing from additive, synergetic to antagonist effects. According to recent studies, oxidative stress was enhanced in bivalves exposed to Hg and As under warming conditions (Coppola et al., 2017; Coppola et al., 2018a; Freitas et al., 2017). On the other way, Nardi et al. (2018) demonstrated that the effects induced by Cd in *M. galloprovincialis* were not altered by increased temperatures. Recently, Moreira et al. (2018) demonstrated that changes in salinity and temperature altered the impacts of As in the embryo-larval development of oysters. The same authors also showed that salinity influences the biochemical response of *Crassostrea angulata* pyster to As (Moreira et al., 2016).

Despite few studies have addressed the combined effects of metal(oid)s and climate change related factors (see references above), the importance of considering different environmental variables when evaluating the toxicity of pollutants in aquatic organisms, including those of emerging concern, has repeatedly been highlighted (see for example De Marchi et al., 2018; Freitas et al., 2016a). Such information is of upmost relevance in order to identify realistic scenarios and protect marine organisms exposed to combined stressors. As

103 extreme weather events will become more frequent, multiple stressor experiments including
104 climate predictions and presence of pollutants should be encouraged. Therefore, considering
105 that Pb is among one of the six regulated substances in the EU Directive and its use in
106 electronic devices is increasing worldwide, the present study aimed to evaluate the toxicity of
107 this metal in the marine species *Mytilus galloprovincialis.* This bivalve was exposed to an
108 environmentally realistic concentration of Pb under different seawater salinity and temperature
109 conditions, resembling actual and predicted climate change scenarios. To this end, the impacts
110 by Pb were assessed in parameters that refer to the mussel's metabolic capacity, antioxidant
111 and biotransformation defences, lipids and protein damage as well as neurotoxicity.
112
113

2. MATERIALS AND METHODS

2.1 Experimental conditions

Mytilus galloprovincialis (mean total weight 25.5 ± 7.5 g) were collected in April 2017 during low tide in a subtidal area located at the Mira Channel (Ria de Aveiro, northwest of Portugal). After sampling, the specimens were placed in aquaria for depuration and acclimation to laboratory conditions for 15 days. During this period the mussels were maintained at 17±1.0 ºC, salinity 30±1.0 and pH 8.0±0.1, resembling conditions at the sampling area, and kept under continuous aeration. Artificial seawater was made using a commercial salt (Tropic Marin®SEA salt) and deionized water. Along the acclimation seawater was renewed 2-3 times per week after which mussels were fed with AlgaMac Protein Plus.

Before starting the experiment water with different salinities (25, 30, 35) was prepared and distributed among different aquaria that were placed in two climatic rooms set at the test temperatures (17 and 21 ºC). The two test water temperatures were reached in each aquarium after 24h being placed in the respective climatic rooms. Afterwards, mussels were distributed among different aquaria, to evaluate the exposure to Pb, under different salinity and temperature values, following 8 conditions: salinity 25 and temperature 17 ºC; salinity 30 and temperature 17 ºC (control condition resembling sampling site characteristics); salinity 35 and temperature 17 ºC; salinity 30 and temperature 21 ºC; all in the presence (50 µg/L) or absence (0 µg/L) of Pb. Lead (Lead nitrate, CAS No: 10099-74-8, EC No: 233-245-9; 1000 mg/L) was purchase from Sigma-Aldrich and the standard solutions was made in miliQ water.

Lead concentration (50 µg/L) was selected considering World Health Organization (WHO) recommendation of Pb in drinking water (WHO, 2013), and concentrations of Pb in highly contaminated coastal ecosystems (among others, Bakary et al., 2015, Vázquez-Sauceda et al., 2012). Furthermore, the concentration of Pb chosen is much lower than that allowed in industrial wastewaters (1.0 mg/L) that can be discharged into aquatic ecosystems (Environmental Protection Agency (EPA 2002); Portuguese Decree-law 236/98). Also, previous studies (data not shown) testing similar water concentration originated mussels Pb concentrations in the range of those chronically present in bivalves from a low contaminated estuary (0.3 to 5 µg/g, Ria de Aveiro, Portugal) (Figueira et al., 2011; Freitas et al., 2012).

Three aquaria were used per condition (3 replicates per condition), with 9 L of capacity and containing 12 mussels each. During the exposure period, water samples from each aquarium were collected immediately after Pb spiking to ensure chemical nominal concentration. At the end of the experimental period (28 days) Pb concentrations were also determined in whole soft tissue of mussels.

During the exposure, mussels were maintained at constant aeration; temperature (17 or 21 ºC) and salinity (25, 30 or 35), parameters that were daily checked and readjusted if necessary. Along the exposure period, mussels were fed with AlgaMac Protein Plus three times per week and seawater was renewed weekly, after which the experimental conditions were re-established, also ensuring seawater parameters and Pb concentration. No mortality was observed during this 28-day experimental period.

After the exposure time, the whole soft tissue of 9 mussels per condition (3 per replicate) was removed from the shells and individually homogenized using a mortar and pestle under liquid nitrogen. The homogenised tissue of each individual was divided into aliquots of 0.5 g fresh weight (FW) of soft tissue, which were used for biomarkers analyses and to determine Pb concentrations.

2.2 Lead quantifications

Lead concentrations in water samples were directly analysed by inductively coupled plasma atomization-mass spectrometry (ICP-MS – Themo X series) after dilution and acidification with HNO_3 2 % (v/v), to pH < 2. The limit of quantification (LOQ) of the method was 2 µg/L, with an acceptable relative standard deviation among replicates <10%.

Tissue samples were analysed by two techniques, ICP-MS and inductively coupled plasma optical emission spectrometry (ICP-OES - Jobin Yvon Activa M) for the low and high concentrations, respectively. LOQ for ICP-MS was of 0.02 µg/g dry weight (DW) and for ICP-OES was of 1.9 µg/g (DW), and quality control was ensured by analysing all samples in triplicate and imposing a coefficient of variation of less than 10 %. Prior to analysis, tissue samples were freeze dried and homogenised for microwave assisted acid digestion sample preparation method. The digestion was done in closed Teflon vessels, by adding the reagent mix (1 mL HNO_3 + 2 ml H_2O_2 + 1 mL H_2O) to 200 mg of dry tissue and following the heating

program: 15 min of temperature increase to 190 ºC and then hold at 190 ºC for 3 min. After cooling down, the digests were collected to a final volume of 25 mL with ultrapure water. To ensure quality control of these results each digestion cycle held a blank sample (< LOQ in both techniques, n = 4), a sample made in duplicate (coefficient of variation < 10 %; n= 4) and analysis of certified reference material (Tort-3, lobster hepatopancreas, mean recovery of 116 %; n =5).

2.3 Biochemical parameters

For each condition, indicators of metabolic capacity (electron transport system activity, ETS), energy reserves (total protein content, PROT; glycogen content, GLY), and oxidative stress status (levels of lipid peroxidation, LPO; and Protein carbonylation, PC; activities of superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx; and glutathione S-transferases, GSTs) were measured. Soft tissue samples were individually sonicated for 15 s and centrifuged for 20 min at 10 000 g (or 3 000 g for ETS) at 4 ºC, using specific buffers for each biomarker at the ratio 1:2 (w/v) (Andrade et al., 2018; 2019; De Marchi et al., 2018). Supernatants were either stored at -80 ºC or immediately analysed. All biochemical parameters were performed in duplicate and using a microplate reader.

2.3.1 Metabolic capacity and energy reserves

ETS activity was measured based on King and Packard (1975) protocol and modifications by De Coen and Janssen (1997). Absorbance was recorded during 10 min at 490 nm with intervals of 25 s. The extinction coefficient (Ɛ) 15,900 $M^{-1}cm^{-1}$ was used to calculate the amount of formazan formed. Results were expressed in nmol per min per g of FW.

GLY quantification was based on the sulphuric acid method (Dubois et al. 1956), using a standard calibration curve of glucose (0–10 mg/mL). Absorbance was read at 492 nm after incubation during 30 min at room temperature. Results were expressed in mg per g of FW.

PROT content was determined according to the spectrophotometric Biuret method (Robinson and Hogden, 1940). Bovine serum albumin (BSA) was used to prepare a standard calibration curve (0–40 mg/mL). Absorbance was read at 540 nm. The results were expressed in mg per g of FW.

204

205 ### 2.3.2 Antioxidant and defences

206 SOD activity was determined by the Beauchamp and Fridovich (1971) method after
207 modifications by Carregosa et al. (2014). The standard calibration curve was obtained using
208 purified SOD (0.25-60 U/mL). Absorbance was read at 560 nm after 20 min incubation at room
209 temperature. Results were expressed in U per g of FW, where one unit (U) represents the
210 quantity of the enzyme that catalyzes the conversion of 1 µmol of substrate per min.

211 CAT activity was quantified according to the Johansson and Borg (1988) method and
212 modifications by Carregosa et al. (2014). The standard calibration curve was obtained using
213 formaldehyde (0–150 µmol/L). Absorbance was measured at 540 nm. The enzymatic activity
214 was expressed in U per g of FW, where U represents the amount of enzyme that caused the
215 formation of 1.0 nmol formaldehyde per min at 25 °C.

216 GPx activity was quantified following Paglia and Valentine (1967). The absorbance
217 was measured at 340 nm in 10 sec intervals during 5 min and the enzymatic activity was
218 determined using the extinction coefficient (Ɛ) 6.22 $mM^{-1}cm^{-1}$. The results were expressed as U
219 per g of FW, where U represents the amount of enzyme that caused the formation of 1 µmol
220 NADPH oxidized per min.

221 GSTs activity was quantified following Habig et al. (1974) protocol with some
222 adaptations by Carregosa et al. (2014). GSTs activity was measured spectrophotometrically at
223 340 nm, using the extinction coefficient (Ɛ) 9.6 mM^{-1} cm^{-1}. The enzymatic activity was
224 expressed in U per g of FW, where U is defined as the amount of enzyme that catalysis the
225 formation of 1 µmol of dinitrophenyl thioether per min.

226

227 ### 2.3.3 Indicators of cellular damage

228 LPO levels were determined by the quantification of malondialdehyde (MDA), a by-
229 product of lipid peroxidation, according to the method described in Ohkawa et al. (1979).
230 Absorbance was measured at 535 nm and the amount of MDA formed was calculated using the
231 extinction coefficient (Ɛ) 156 mM^{-1} cm^{-1}. The results were expressed in nmol per g of FW.

232 The quantification of carbonyl groups in oxidized proteins (PC) was done following the
233 2,4-dinitrophenylhydrazina (DNPH) alkaline method (Mesquita et al. 2014). Absorbance was

measured at 450 nm and the extinction coefficient (Ɛ) 22,308 M^{-1} cm^{-1} was used to calculated PC levels, expressed in nmol per g of FW.

2.3.4 Neurotoxicity

AChE activity was determined using Acetylthiocholine iodide (ATChI 5 mM) as substrate, according to the methods of Ellman et al. (1961) with modification by Mennillo et al. (2017). The activity was measured at 412 nm during 5 min and expressed in nmol/min per g of FW using the extinction coefficient (Ɛ) 13.6 $mM^{-1}cm^{-1}$.

2.4 Data analysis

To evaluate the bioaccumulation of Pb in mussels' tissues, the bioconcentration factor (BCF) was calculated at each exposure condition. BCF was defined as the ratio of the concentration in the organism in respect to the concentration measured in water. The calculation is based on the equation from Arnot and Gobas (2006):

$$BCF = \frac{concentration\ in\ the\ organism}{concentration\ in\ the\ water}$$

All the biochemical results (ETS, GLY, PROT, SOD, CAT, GPx, GSTs, LPO, PC, AChE) and Pb concentrations, for all conditions, were individually submitted to a non-parametric permutational analysis of variance (PERMANOVA Add-on in Primer v7). A one-way hierarchical design was followed in this analysis. When significant differences were observed in the main test pairwise comparisons were performed. Values lower than 0.05 were considered as significantly different. The null hypotheses tested were: for each biomarker and each Pb concentration (0 or 50 µg/L), no significant differences existed among salinity and temperature levels, represented in figures by letters (lowercase letters for non-contaminated conditions; uppercase letters for contaminated conditions); for each biomarker at each salinity and temperature levels, no significant differences existed between non-contaminated and contaminated mussels, represented in figures with asterisks. For non-contaminated and

262 contaminated organisms no significant differences existed in terms of Pb concentration among
263 different salinity and temperature levels, represented by lower case letters in Tables.

264 The matrix expressing biomarkers and Pb concentrations per condition was
265 normalised and the Euclidean distance calculated and distance among centroids (i.e. the mean
266 position of all the points representing a given sample) was visualized in Principal Coordinates
267 Ordination (PCO) analysis. In the PCO graph, the variables (biomarkers and Pb concentrations)
268 presenting a correlation higher than 75% with conditions spatial distribution were represented
269 as superimposed vectors.

3. RESULTS

3.1 Lead concentrations in water and organisms

Concentrations of Pb measured in water collected immediately after spiking showed neither significant differences among non-contaminated temperature and salinity conditions nor among contaminated ones (Table 1). Trace amounts of Pb were also detected in water of unexposed conditions (1.1-8.2 µg/L), while in those exposed to Pb concentrations ranging between 63.1 and 74.2 µg/L, slightly higher than the targeted nominal concentration (Table 1).

The concentrations of Pb in mussel's soft tissues showed significantly higher (about 3-4 fold) values in organisms exposed to Pb in comparison to non-contaminated ones, with no significant differences among mussels exposed to different conditions (Table 2). The BCF estimated ranged between 0.02-0.03 with no clear influence of salinity and temperature parameters.

3.2 Biochemical parameters

3.2.1 Metabolic capacity and energy reserves

In non-contaminated mussels, significantly lower ETS values were observed in those maintained to salinity 35 in comparison to the remaining conditions. In Pb exposed mussels, significantly lower ETS values were observed at salinities 30 and 35 at control temperature (17 ºC). At salinity 30, ETS increased at 21ºC but decreased at 17ºC in Pb exposed mussels in respect to non-contaminated ones (Figure 1A).

GLY content was only significantly lower at salinity 35 in respect to 30 in non-contaminated mussels. By contrast, in the presence of Pb, GLY was significantly enhanced at the salinity 35. At the control salinity (30), lower GLY content was observed in contaminated mussels maintained at both tested temperatures (Figure 1B).

PROT content in mussels at 17 ºC was significantly higher at salinity 25 both for non-contaminated and Pb contaminated conditions. When considering the temperature influence at salinity 30, PROT reserves were higher in non-contaminated specimens maintained both at 17 and 21 ºC (Figure 1C).

300 *3.2.2 Antioxidant and biotransformation defences*

301 SOD activity in non-contaminated mussels differed at the three tested salinities at 17
302 ºC, with the lowest activity seen at 25. In Pb exposed mussels held at the same temperature,
303 SOD was significantly increased only at salinity 30. At this salinity, the effect of temperature was
304 inverse, while SOD increased in Pb exposed mussels held at 17 ºC, it decreased in
305 contaminated mussels at 21 ºC (Figure 2A).

306 CAT activity was little affected by salinity and it only increased in unexposed mussels
307 at the higher salinity of 35 at 17 ºC. At 21 ºC and salinity 30, CAT activity was significantly
308 higher in non-contaminated mussels than in those exposed to Pb at the same temperature and
309 those held at 17 ºC at the same salinity (Figure 2B).

310 GPx activity was highly salinity dependent, with significantly higher values at salinity
311 25 in non-contaminated mussels; while in all the Pb exposed groups this activity was
312 significantly lower at this salinity condition. In regard to the influence of temperature at salinity
313 30, Pb exposed mussels displayed significantly lower GPx activity than non-contaminated
314 mussels at the two temperatures; with significantly higher GPx values at 17 ºC (Figure 2C).

315 GSTs activity was significantly lower in non-contaminated and contaminated mussels
316 at salinity 25 and temperature 17 ºC. Mussels maintained at salinity 30 and different
317 temperatures (17 and 21 ºC) showed the same response, with significantly higher GSTs values
318 in contaminated mussels (Figure 3).

319

320 *3.2.3 Indicators of cellular damage*

321 LPO values were significantly higher in non-contaminated mussels maintained at
322 salinity 25 but they were significantly lower in the Pb exposed group held at the same condition.
323 At 21 ºC and salinity 30 LPO values significantly increased in Pb exposed mussels, while at 17
324 ºC and salinity 30 an opposite response was observed (Figure 4A).

325 Oxidised proteins measured as PC significantly increased at salinities of 25 and 35
326 even in the non-exposed mussels. Oxidised proteins content was significantly higher in mussels
327 at 21 ºC and salinity 30 in comparison to organisms maintained at the same salinity but 17 ºC
328 (Figure 4B).

329

330 *3.2.4 Neurotoxicity*

331 AChE activity was significantly increased at salinities 25 and 35 in contaminated
332 mussels, while an opposite response was observed in non-contaminated mussels with
333 significantly lower AChE values observed at salinities 25 and 35. No effects were observed due
334 to temperature differences (Figure 5).

335

336 Due to the large number of biomarkers considered (10), physical variables (2
337 temperatures and 3 salinities) and two chemical conditions (presence and absence of Pb) that
338 generate complex responses, an integrative multicomponent analysis was considered. The
339 PCO axis 1 explained 31.7% of the total variation clearly separating Pb contaminated mussels
340 (except organisms at 17 ºC and salinity 25), in the positive side of the axis, from non-
341 contaminated organisms, distributed in the negative side of the axis (Figure 6). The variables
342 that better explained the variation were: Pb concentration, PC levels, CAT and GSTs activities,
343 presenting a high correlation with axis 1 positive side (Pb and GSTs) and negative side (CAT
344 and PC). The PCO vertical dimension (PCO axis 2) explained 27.6% of the total variation
345 separating non-contaminated mussels at control conditions (salinity 30 and temperature 17 ºC)
346 and salinity 25 in the negative side, from the other conditions in the positive side. LPO
347 represents high correlation with PCO axis 2 negative side (Figure 6).

348

4. DISCUSSION

In the present study, the amount of Pb accumulated in whole mussel tissue demonstrated that fluctuation of salinity (± 5) and temperature (4º C) from the present ones (identified as control salinity 30 and temperature 17ª C), did not influence the concentration of this metal or its BCF values. However, this behaviour can differ from other elements under the similar temperature scenarios. For instance, Coppola et al (2017, 2018) already demonstrated a different bioaccumulation pattern of Hg and As in *M. galloprovincialis* under different temperatures: Hg concentration decreased and As concentrations increased in *M. galloprovincialis* exposed to 21 ºC compared to those held at 17 ºC.

Despite limited variations in Pb bioaccumulation in mussels at different environmental conditions, the biochemical responses varied dependng on the physical water conditions in both Pb-contaminated and non-contaminated mussels. Due to the comprehensive set of biomarkers tested and the complexity of the responses obtained at the different water conditions, the PCO analysis was considered in order to interpretate the extend of the changes as it integrates the various responses measured and reports on which factors may better explain the observed differences. Derived from the PCO analysis, the influence of Pb exposure alone was confirmed while the modulation in the biochemical responses observed by the different temperature and salinity conditions was less obvious.

The parameters related to energy metabolism such as ETS, which corresponds to the overall mitochondrial activity in relation to energy production, was not a mechanism that significantly contributed to the differences observed as it did not show a correlation >75% with all tested conditions, reason why it did not appear as an explanatory vector in the PCO. Neither did the GLY content account for explaining differences among tested conditions. Despite the limited influence of ETS in the overall responses, the highest salinity alone decreased mussel's metabolic capacity regardless of Pb exposure. However, under Pb contamination, mussels significantly increased their metabolism at the salinity 25 and the highest temperature (21 ºC). Thus, two strategies were seen adopted by mussels: one, by decreasing their metabolism at higher salinity regardless of Pb presence but also under Pb exposure at actual salinity (control salinity 30), and this way avoiding the accumulation of Pb as a protective measure; and a second strategy by increasing their metabolic rate under Pb exposure at lower salinity (25) and

high temperature (21ºC) conditions, which were in turn correlated with increased GLY and PROT consumption particularly at higher temperature. Previous studies with bivalves already demonstrated that under control salinity (30) and temperature (17 ºC) conditions, exposure to metals strongly decreased their metabolic capacity (Bielen et al., 2016; Coppola et al., 2017; Coppola et al., 2018; Izagirre et al., 2014; Nardi et al., 2017), while under combined stressful conditions (salinity and pollution) their metabolic capacity increased (Moreira et al., 2016). Thus both strategies can be alternatively adopted in bivalves. The neurotoxicity marker AChE did not seem to be a mechanism that significantly contributed to the identification of differences among tested conditions (correlation <75%). Salinities 5 units over and under the control value (salinity 30) either decreased (non-contaminated mussels) or increased (Pb exposed mussels) this enzymatic activity. The interpretation of this result is difficult in bivalves since a clear physiological role of this activity, other than the neurotoxicity due to AChE inhibition, is not yet clear (Sole et al., 2018). Other metals such as Ni (Attig et al., 2010) and Cd (Chalkiadaki et al., 2014) are reported as AChE inhibitors in bivalves, whereas an increase in AChE in the bivalve *Perna indica* exposed to As was interpreted as an attempt to reduce the neurotransmitter excess in the synaptic clefts (Rajkumar, 2013).

From the PCO analysis (Figure 6) three clear groups can be outlined. One encompassing 3 out of 4 Pb exposed conditions, those being highly correlated with GSTs activity. These results clearly demonstrated that biotransformation enzymes (GSTs) were activated in the presence of Pb, independently on the salinity and temperature levels. The capacity to increase the activity of these enzymes to detoxify their cells from Pb was formerly demonstrated in bivalves exposed to metals (Attig et al., 2010; Oliveira et al., 2018; Monteiro et al., 2019). A second group included control salinity (30) and high temperature unexposed mussels with high correlation with PROT reserves, the antioxidant CAT activity and the ocurrence of oxidised proteins (PC). This second group with increased PROT reserves and antioxidant CAT defences seemed to be sufficient to prevent LPO occurrence but not protein oxidation. An increase in PROT content was already showed by Freitas et al. (2016b) in *Ruditapes philippinarum* exposed to increased As concentrations (0, 4 and 17 mg/L) and a range of salinities (14, 21, 28, 35 and 42) as a measure to face stress. A third group included low salinity and low temperature unexposed mussels with a close relationship with GPx activity

and LPO levels. This third group, although included the mussels exhibiting the highest antioxidant GPx activity, it lacked the action of other efficient antioxidant defences such as CAT and SOD. In fact, SOD activity was the lowest under temperature 17 ºC and salinity 25, and consequently LPO occurrence was not prevented in this particular group. Such antioxidant response patterns indicate that even non-contaminated mussels tried to avoid cellular damage when facing unfavourable water conditions (i.e., out of salinity 30 and temperature 17 ºC) by increasing particular antioxidant defences. The response of antioxidant enzymes to unfavourable physical water parameters was already described in mussels and other bivalves, with i) inhibition of SOD under highly stressful conditions of salinity (Gonçalves et al., 2017), or ii) increase of antioxidant defenses in bivalves exposed to salinity decreases (Freitas et al., 2017; Velez et al., 2016) or salinity increases (Rahman et al., 2019); also to temperature elevation (Coppola et al., 2018; Rahman et al., 2019; Verlecar et al., 2007) or decreased pH (Matozzo et al., 2013). The present results highlight that the presence of Pb generated complex antioxidant responses under unfavourable salinity and temperature conditions. That is it under 9 stressing situations 5 decreases and 1 increase were recorded for the three antioxidant enzymes measured. Three out of the 5 decreases were due to increased temperature evidencing that antioxidant defenses may be compromised under warming conditions and Pb contamination in mussels. A more limited capacity of these enzymes to act when under combined stressful conditions was already demonstrated by other authors (Maria and Bebianno, 2011; Freitas et al., 2017).

As a consequence of mussel's efficent activation of their defence mechanisms, in general, no LPO or PC ocurrence was observed in Pb contaminated mussels. Only one exception being LPO elevation in Pb exposed mussels reared at higher temperature and highlights this as the worst case situation. Efficent defence response were also observed in *M. galloprovincialis* exposed to Cd and Hg (Coppola et al., 2017; Rocha et al., 2015), demonstrating the capacity of bivalves to avoid cellular damage by increasing their antioxidant defences.

Conclusion

This study demonstrates the usefullness of aplying multicomponent tools when assessing the effects of several physico-chemical conditions in a comprehensive set of variables embracing aspects that relate to energy metabolism, antioxidant defenses, oxidative stress damage and neurotoxicity. Exposure to Pb induced mostly the conjugation detoxification reactions by GSTs regardless of salinity or temperature conditions. Nonetheless, salinities of 25 and 35 when compared to the control one (30) were also revealed as stressful situations that did not prevent the ocurrence of oxidised lipids (measured as LPO levels at salinity 25) or oxidised proteins (measured as PC at salinity 35) even in uncontaminated mussels. Temperature alone had more influence in modulating the responses in non-contaminated mussels (separated in the PCO) than those exposed to Pb since the presence of the contaminant seem to mask the effect of the temperature and they appear highly related in the PCO axis.

Acknowledgments

Francesca Coppola, and Rui Monteiro benefited from PhD grants (SFRH/BD/118582/2016 and SFRH/BD/108535/2015, respectively), given by the National Funds through the Portuguese Science Foundation (FCT), supported by FSE and Programa Operacional Capital Humano (POCH) e da União Europeia. Rosa Freitas was funded by national funds (OE), through FCT – Fundação para a Ciência e a Tecnologia, I.P., in the scope of the framework contract foreseen in the numbers 4, 5 and 6 of the article 23, of the Decree-Law 57/2016, of August 29, changed by Law 57/2017, of July 19. This work was also financially supported by the project BISPECIAl: BIvalveS under Polluted Environment and CIImate chAnge PTDC/CTA-AMB/28425/2017 (POCI-01-0145-FEDER-028425) funded by FEDER, through COMPETE2020 - Programa Operacional Competitividade e Internacionalização (POCI), and by national funds (OE), through FCT/MCTES. Thanks are due for the financial support to CESAM (UID/AMB/50017/2019), to FCT/MEC through national funds, and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020. Rosa Freitas and Montserrat Solé are membersof the RED Iberoamericana RIESCOS (419RT0578) funded by CYTED.

REFERENCES:

Al Naggar, Y., Khalil, M. S., Ghorab, M.A. (2018). Environmental pollution by heavy metals in the aquatic ecosystems of Egypt. Open Acc J of Toxicol. 3, 1.

Anderson M.J., Gorley R.N, Clarke K.R. 2008. PERMANOVAþ for PRIMER: Guide to software and statistical methods. University of Auckland and PRIMER-E, Plymouth, UK.

Andrade, M., De Marchi, L., Pretti, C., Chiellini, F., Morelli, A., Soares, A.M.V.M., Rocha, R.J.M., Figueira, E., Freitas, R. (2018). Are the impacts of carbon nanotubes enhanced in *Mytilus galloprovincialis* submitted to air exposure? Aquat. Toxicol. 202, 163-172.

Andrade, M., De Marchi, L., Soares, A.M.V.M., Rocha, R.J.M., Figueira, E., Freitas, R. (2019). Are the effects induced by increased temperature enhanced in *Mytilus galloprovincialis* submitted to air exposure? Sci Total Environ. 647, 431–440.

Ansari, T.M., Marr, I.L.M., Tariq, N.T. (2004). Heavy metals in marine pollution perspective–A Mini Review. J. Appl. Sci. Res. 4, 1–20.

Arnot, J. A., and Gobas, F. A. (2006). A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. Environ. Rev. 14(4), 257–297.

Attig, H., Dagnino, A., Negri, A., Jebali, J., Boussetta, H., Viarengo, A., Banni, M. (2010). Uptake and biochemical responses of mussels *Mytilus galloprovincialis* exposed to sublethal nickel concentrations. Ecotoxicol. Environ. Saf. 73, 1712–1719.

Attig, H., Kamel, N., Sforzini, S., Dagnino, A., Jamel, J., Boussetta, H., Viarengo, A., Banni, M. (2014). Effects of thermal stress and nickel exposure on biomarkers responses in *Mytilus galloprovincialis* (Lam). Mar. Environ. Res. 94, 65-71.

Beauchamp, C., Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276–287.

Bielen, A., Bošnjak, I., Sepčić, K., Jaklič, M., Cvitanić, M., Lušić, J., Hudina, S. (2016). Differences in tolerance to anthropogenic stress between invasive and native bivalves. Sci. Total Environ. 543, 449–459.

Bielen, A., Bošnjak, I., Sepčić, K., Jaklič, M., Cvitanić, M., Lušić, J., Hudina, S. (2015). Differences in tolerance to anthropogenic stress between invasive and native bivalves. Sci. Total Environ. 543, 449–459.

Byrne, M. (2012). Global change ecotoxicology: Identification of early life history bottlenecks in marine invertebrates, variable species responses and variable experimental approaches. Mar. Environ. Res. 76, 3-15.

Carregosa, V., Figueira, E., Gil, A. M., Pereira, S., Pinto, J., Soares, A. M. V. M., Freitas, R. (2014). Tolerance of *Venerupis philippinarum* to salinity: Osmotic and metabolic aspects. Comp Biochem Physiol C Toxicol Pharmacol. 171, 36–43.

Chakraborty, P., Raghunadh Babu, P. V, Sarma, V.V. (2012). A study of lead and cadmium speciation in some estuarine and coastal sediments. Chem. Geol. 294-295, 217-225.

Chalkiadaki, O., Dassenakis, M., Paraskevopoulou, V., Lydakis-Simantiris, N. (2014). Experimental study of cadmium bioaccumulation in three Mediterranean marine bivalve species: Correlation with selected biomarkers. Pure Appl. Chem. 86, 1189– 1204.

Company, R., Serafim, A., Bebianno, M.J., Cosson, R., Shillito, B., Fiala-Médioni, A. (2004). Effect of cadmium, copper and mercury on antioxidant enzyme activities and lipid peroxidation in the gills of the hydrothermal vent mussel *Bathymodiolus azoricus*. Mar. Environ. Res. 58, 377–381.

Coppola F., Henriques B., Soares A.M.V.M., Figueira E., Pereira E., Freitas R. (2018a). Influence of temperature rise on the recovery capacity of *Mytilus galloprovincialis* exposed to mercury pollution. Ecol Indic. 93, 1060-1069.

Coppola, F., Almeida, Â., Henriques, B., Soares, A. M. V. M., Figueira, E., Pereira, E., and Freitas, R. (2018b). Biochemical responses and accumulation patterns of *Mytilus galloprovincialis* exposed to thermal stress and Arsenic contamination. Ecotoxicol. Environ. Saf. 147, 954–962.

Coppola, F., Almeida, Â., Henriques, B., Soares, A. M. V. M., Figueira, E., Pereira, E., Freitas, R. (2017). Biochemical impacts of Hg in *Mytilus galloprovincialis* under present and predicted warming scenarios. Sci. Total Environ. 601, 1129–1138.

De Coen, W.M.D., Janssen, C.R. (1997). The use of biomarkers in Daphnia magna toxicity testing. IV. Cellular Energy Allocation: a new methodology to assess the energy budget of toxicant-stressed Daphnia populations. J. Aquat. Ecosyst. Stress Recovery 6, 43–55.

De Marchi L., Neto V., Pretti C., Figueira E., Chiellini F., Morelli A., Soares A.M.V.M., Freitas R. (2018). Does the exposure to salinity variations and water dispersible carbon nanotubes induce oxidative stress in *Hediste diversicolor*? Mar. Environ. Res. 141: 186-195.

De Marchi L., Neto V., Pretti C., Figueira E., Chiellini F., Morelli A., Soares A.M.V.M., Freitas R. (2018). The influence of salinity on the effects of Multi-walled carbon nanotubes on polychaetes. Sci. Rep. 8, 8571.

de Souza Machado, A. A., Spencer, K., Kloas, W., Toffolon, M., Zarfl, C. (2016). Metal fate and effects in estuaries: A review and conceptual model for better understanding of toxicity. Sci. Total Environ., 541, 268–281.

Dijkstra, J. A., Buckman, K. L., Ward, D., Evans, D. W., Dionne, M., Chen, C. Y. (2013). Experimental and natural warming elevates mercury concentrations in estuarine fish. PLoS ONE. 8,3.

Dubois, M.K., Gilles, A., Hamilton, J.K., Rebers, P.A., Sith, F. (1956). Calorimetric method for determination of sugars and related substances. Anal. Chem. 28, 350-356.

Ellman, G., Courtney, K., Andres Jr., V., Featherstone, R. (1961). A new rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88-95.

Freitas, R., Coppola, F., De Marchi, L., Codella, V., Pretti, C., Chiellini, F., Morelli, A., Polese, G., Soares, A.M.V.M., Figueira, E. (2018). The influence of Arsenic on the toxicity of carbon nanoparticles in bivalves. J. Hazard. Mater. 358, 484-493.

Freitas, R., Coppola, F., Henriques, B., Wrona, F., Figueira, E., Pereira, E., Soares, A.M.V.M. (2017a). Does pre-exposure to warming conditions increase *Mytilus galloprovincialis* tolerance to Hg contamination? Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 203, 1-11.

548 Freitas, R., De Marchi, L., Bastos, M., Moreira, A., Velez, C., Chiesa, S., Wrona, F.J.,
549 Figueira, E., Soares, A.M.V.M. (2017b). Effects of seawater acidification and salinity alterations
550 on metabolic, osmoregulation and oxidative stress markers in *Mytilus galloprovincialis*. Ecol.
551 Indic. 79, 54-62.
552 Freitas R., Almeida A., Calisto V., Velez C., Moreira A., Schneider R.J., Esteves V.I.,
553 Wrona F.J., Figueira E., Soares A.M.V.M. (2016a) The impacts of pharmaceutical drugs under
554 ocean acidification: New data on single and combined long-term effects of carbamazepine on
555 Scrobicularia plana. Sci. Total Environ. 541, 977-985.
556 Freitas, R., Salamanca, L., Velez, C., Wrona, F. J., Soares, A. M. V. M., and Figueira,
557 E. (2016b). Multiple stressors in estuarine waters: Effects of arsenic and salinity on *Ruditapes*
558 *philippinarum*. Sci. Total Environ. 541, 1106–1114.
559 Freitas, R., Martins, R., Antunes, S., Velez, C., Moreira, A., Cardoso, P., Pires A,
560 Soares AM, Figueira, E. (2014). *Venerupis decussata* under environmentally relevant lead
561 concentrations: Bioconcentration, tolerance, and biochemical alterations. Environ. Toxicol.
562 Chem. 33(12), 2786–2794.
563 Gagnaire, B., Thomas-Guyon, H., & Renault, T. (2004). In vitro effects of cadmium
564 and mercury on Pacific oyster, Crassostrea gigas (Thunberg), haemocytes. Fish Shellfish
565 Immunol. 16, 501–512.
566 Gonçalves, A.M.M., Barroso, D.V., Serafim, T.L., Verdelhos, T., Marques, J.C.,
567 Gonçalves, F. (2017). The biochemical response of two commercial bivalve species to exposure
568 to strong salinity changes illustrated by selected biomarkers. Ecol. Indic. 77, 59-66.
569 Habig, W.H., Pabst, M.J., Jakoby, W.B. (1974). Glutathione S-Transferases. The fist
570 enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.
571 IPCC, 2014: Climate Change 2014: Synthesis Report. Contribution of Working Groups
572 I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change
573 [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151 pp.
574 Izagirre, U., Errasti, A., Bilbao, E., Múgica, M., Marigómez, I. (2014). Combined effects
575 of thermal stress and Cd on lysosomal biomarkers and transcription of genes encoding
576 lysosomal enzymes and HSP70 in mussels, *Mytilus galloprovincialis*. Aquat .Toxicol. 149, 145–
577 156.
578 Johansson, L.H., Borg, L.A. (1988). A spectrophotometric method for determination of
579 catalase activity in small tissue samples. Anal. Biochem. 174(1), 331–336.
580 King, F. D., and Packard, T. T. (1975). Respiration and the activity of the respiratory
581 electron transport system in marine zooplankton. Limnol. Oceanogr. 20, 849–854.
582 Manciocco, A., Calamandrei, G., Alleva, E. (2014). Global warming and environmental
583 contaminants in aquatic organisms: The need of the etho-toxicology approach. Chemosphere
584 100, 1–7.
585 Maria, V. L., and M. J. Bebianno. (2011). Antioxidant and lipid peroxidation responses in
586 Mytilus galloprovincialis exposed to mixtures of benzo(a)pyrene and copper. Comp. Biochem.
587 Physiol. C Toxicol. Pharmacol. 154, 56–63.

588 Marques, A., Piló, D., Carvalho, S., Araújo, O., Guilherme, S., Santos, M. A., Vale, C.,
589 Pereia, F., Pacheco, M., Pereira, P. (2018). Metal bioaccumulation and oxidative stress profiles
590 in *Ruditapes philippinarum* – insights towards its suitability as bioindicator of estuarine metal
591 contamination. Ecol Indic. 95, 1087–1099.
592 Matozzo, V., Chinellato, A., Munari, M., Bressan, M., Marin, M.G. (2013). Can the
593 combination of decreased pH and increased temperature values induce oxidative stress in the
594 clam *Chamelea gallina* and the mussel *Mytilus galloprovincialis*? Mar Pollut Bull. 72, 34-40.
595 Mennillo, E., Casu, V., Tardelli, F., De Marchi, L., Freitas, R., and Pretti, C. (2017).
596 Suitability of cholinesterase of polychaete Diopatra neapolitana as biomarker of exposure to
597 pesticides: In vitro characterization. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 191, 152–
598 159.
599 Mesquita, C. S., Oliveira, R., Bento, F., Geraldo, D., Rodrigues, J.V., Marcos, J.C.
600 (2014). Simplified 2,4-dinitrophenylhydrazine spectrophotometric assay for quantification of
601 carbonyls in oxidized proteins. Anal. Biochem. 458, 69–71.
602 Monteiro, R., Costa, S., Coppola, F., Freitas, R., Vale, C., Pereira, E. (2019).
603 Evidences of metabolic alterations and cellular damage in mussels after short pulses of Ti
604 contamination. Sci. Total Environ. 650, 987-995.
605 Moreira A., Freitas R., Figueira E., Ghirardini A.V., Soares A.M.V.M., Radaelli M.,
606 Guida M., Libralato G. (2018) Combined effects of arsenic, salinity and temperature on
607 Crassostrea gigas embryotoxicity. Ecotox. Environ. Saf. 147, 251-259.
608 Moreira, A., Figueira, E., Soares, A. M. V. M., Freitas, R. (2016). Salinity influences
609 the biochemical response of *Crassostrea angulata* to Arsenic. Environ Pollut. 214, 756–766.
610 Nardi, A., Benedetti, M., Fattorini, D., and Regoli, F. (2018). Oxidative and interactive
611 challenge of cadmium and ocean acidification on the smooth scallop *Flexopecten glaber*. Aquat.
612 Toxicol. 196, 53–60.
613 Nardi, A., Mincarelli, L. F., Benedetti, M., Fattorini, D., d'Errico, G., Regoli, F. (2017).
614 Indirect effects of climate changes on cadmium bioavailability and biological effects in the
615 Mediterranean mussel *Mytilus galloprovincialis*. Chemosphere 169, 493–502.
616 Oliveira, P., Lírio, A.V., Canhoto, S., Guilhermino, L. (2018). Toxicity of mercury and
617 post-exposure recovery in *Corbicula fluminea*: Neurotoxicity, oxidative stress and oxygen
618 consumption. Ecol Indic. 91, 503-51.
619 Ohkawa, H., Ohishi, N., Yagi, K. (1979). Assay for lipid peroxides in animal tissues by
620 thiobarbituric acid reaction. Anal. Biochem. 95(2), 351–358.
621 Paglia, D. E., and Valentine, W. N. (1967). Studies on the quantitative and qualitative
622 characterization of erythrocyte glutathione peroxidase. TJ. Lab. Clin. Med. 70(1), 158–169.
623 Pörtner, H.-O., Karl, M.D., Boyd, P.W., Cheung, W.W.L., Lluch-Cota, S.E., Nojiri, Y.,
624 Schmidt D.N., Zavialov P.O. (2014). Impacts, adaptation, and vulnerability. Part A : Global and
625 Sectoral Aspects. Contribution of Working Group II to the Fifth Assessment Report of the
626 Intergovernmental Panel on Climate Change, Cambridge University Press.

Rahman, M.A., Henderson, S., Miller-Ezzy, P., Li, X.X., Qin, J.G. (2019). Immune response to temperature stress in three bivalve species: Pacific oyster *Crassostrea gigas*, Mediterranean mussel *Mytilus galloprovincialis* and mud cockle *Katelysia rhytiphora*. Fish Shellfish Immunol. 86, 868-874.

Rajkumar, J. S. I. (2013). Reduced glutathione and acetylcholinesterase expressions in *Perna indica* exposed to trivalent arsenic. Int. J. Biol. Res. Pregnancy. 1, 1–4.

Regoli, F., and Giuliani, M. E. (2014). Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Mar. Environ. Res. 93, 106–117.

Robinson, H., and Hodgen, C. (1940). The biuret reaction in the determination of serum proteins: A study of the conditions necessary for the production of a stable color which bears a quantitative relationship to the protein concentration. J. Biol. Chem. 135, 707.

Rocha, T. L., Gomes, T., Mestre, N. C., Cardoso, C., Bebianno, M. J. (2015). Tissue specific responses to cadmium-based quantum dots in the marine mussel *Mytilus galloprovincialis*. Aquat Toxicol. 169, 10–18.

Samuel, S., Kathirvel, R., Jayavelu, T., Chinnakkannu, P. (2005). Protein oxidative damage in arsenic induced rat brain: influence of Dl-α-lipoic acid. Toxicol. Lett. 155, 27–34.

Singh, R., Gautam, N., Mishra, A., Gupta, R. (2011). Heavy metals and living systems: An overview. Indian J Pharmacol. 43, 246–253.

Solé, M., Bonsignore, M., Rivera-Ingraham, G., Freitas, R. (2018). Exploring alternative biomarkers of pesticide pollution in clams. Mar. Pollut. Bull. 136, 61–67.

Srichandan, S., Panigrahy, R. C., Baliarsingh, S. K., Rao B., S., Pati, P., Sahu, B. K., and Sahu, K. C. (2016). Distribution of trace metals in surface seawater and zooplankton of the Bay of Bengal, off Rushikulya estuary, East Coast of India. Mar. Pollut Bull. 111(1–2), 468–475.

Vázquez-Sauceda, M. de la L., Pérez-Castañeda, R., Sánchez-Martínez, J. G., and Aguirre-Guzmán, G. (2012). Cadmium and lead levels along the estuarine ecosystem of Tigre River-San Andres Lagoon, Tamaulipas, Mexico. Bull. Environ. Contam. Toxicol. 89(4), 782–785.

Velez, C., Figueira, E., Soares, A.M.V.M., Freitas, R. (2016). Combined effects of seawater acidification and salinity changes in *Ruditapes philippinarum*. Aquat. Toxicol. 176, 141-150,

Velez, C., Galvão, P., Longo, R., Malm, O., Soares, A.M.V.M., Figueira, E., Freitas, R. (2015). *Ruditapes philippinarum* and *Ruditapes decussatus* under Hg environmental contamination. Environ. Sci. Pollut. Res. 22, 11890–11904.

Verlecar, X.N., Jena, K.B., Chainy, G.B.N. (2007). Biochemical markers of oxidative stress in Perna viridis exposed to mercury and temperature. Chem. Biol. Interact. 167, 219-226.

Wadige, M.C. P. M., Taylor, A. M., Maher, W. A., Krikowa, F. (2014). Bioavailability and toxicity of zinc from contaminated freshwater sediments: Linking exposure-dose–response relationships of the freshwater bivalve *Hyridella australis* to zinc-spiked sediments. Aquat. Toxicol. 156, 179–190.

Zhang, K., Dwivedi, V., Chi, C., Wu, J. (2010). Graphene oxide/ferric hydroxide composites for efficient arsenate removal from drinking water. J Hazard Mater. 182, 162–168.

WHO 2013.Trace Elements in Human Nutrition and Health. Retrieved from https://www.who.int/gho/publications/world_health_statistics/WHS2013_IndicatorCompendium.pdf

Figure captions

Figure 1. A: Electron transport system activity (ETS), B: Glycogen content (GLY) and C: Protein content (PROT), in *Mytilus galloprovincialis* under different conditions: Pb-contaminated and non-contaminated mussels at salinities 30, 25 and 35 and at temperature 17 ºC, Pb-contaminated and non-contaminated mussels at salinity 30 and at temperature 21 ºC. Values are presented as mean + standard deviation. Significant differences ($p \leq 0.05$) among salinity and temperature conditions are represented with different letters: lowercase letters for non-contaminated mussels and uppercase letters for contaminated mussels. Significant differences ($p \leq 0.05$) between non-contaminated and contaminated mussels for each salinity and temperature condition are represented with an asterisks. White bars represent non-contaminated mussels. Grey bars represent contaminated mussels.

Figure 2. Activities of A: Superoxide dismutase (SOD); B: Catalase (CAT); and C: Glutathione peroxidase (GPx), in *Mytilus galloprovincialis* under different conditions (see legend Figure 1). Values are presented as mean + standard deviation. Significant differences ($p \leq 0.05$) among salinity and temperature conditions are represented with different letters: lowercase letters for non-contaminated mussels and uppercase letters for contaminated mussels. Significant differences ($p \leq 0.05$) between non-contaminated and contaminated mussels for each salinity and temperature condition are represented with an asterisks. White bars represent non-contaminated mussels. Grey bars represent contaminated mussels.

Figure 3. Activity of Glutathione S-transferases (GSTs), in *Mytilus galloprovincialis* under different conditions (see legend Figure 1). Values are presented as mean + standard deviation. Significant differences ($p \leq 0.05$) among salinity and temperature conditions are represented with different letters: lowercase letters for non-contaminated

mussels and uppercase letters for contaminated mussels. Significant differences ($p \leq 0.05$) between non-contaminated and contaminated mussels for each salinity and temperature condition are represented with an asterisks. White bars represent non-contaminated mussels. Grey bars represent contaminated mussels.

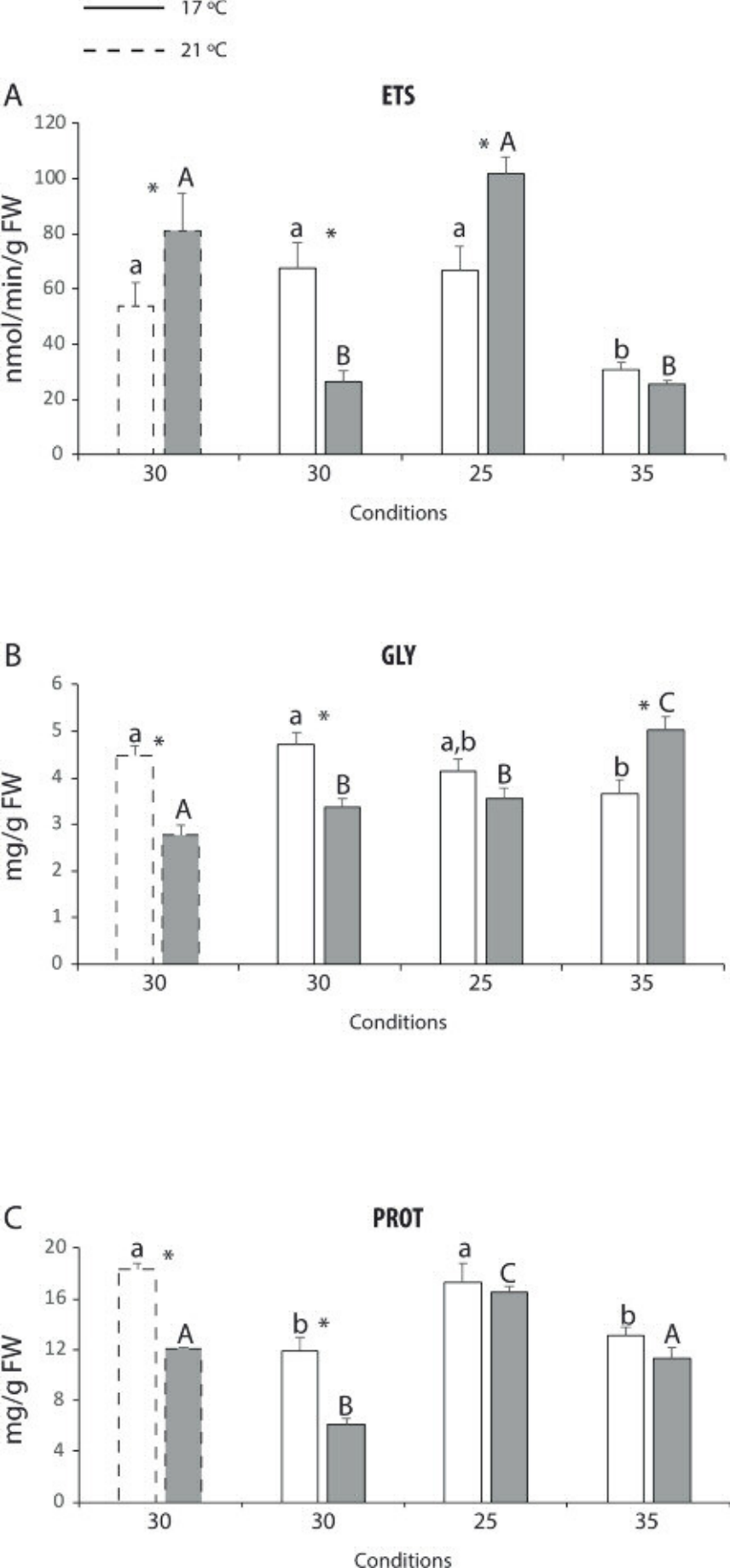
Figure 4. Levels of A: Lipid peroxidation (LPO); B: Protein carbonylation (PC); and C: reduced/oxidised glutathione ratio (GSH/GSSG), in *Mytilus galloprovincialis* under different conditions (see legend Figure 1). Values are presented as mean + standard deviation. Significant differences ($p \leq 0.05$) among salinity and temperature conditions are represented with different letters: lowercase letters for non-contaminated mussels and uppercase letters for contaminated mussels. Significant differences ($p \leq 0.05$) between non-contaminated and contaminated mussels for each salinity and temperature condition are represented with an asterisks. White bars represent non-contaminated mussels. Grey bars represent contaminated mussels.

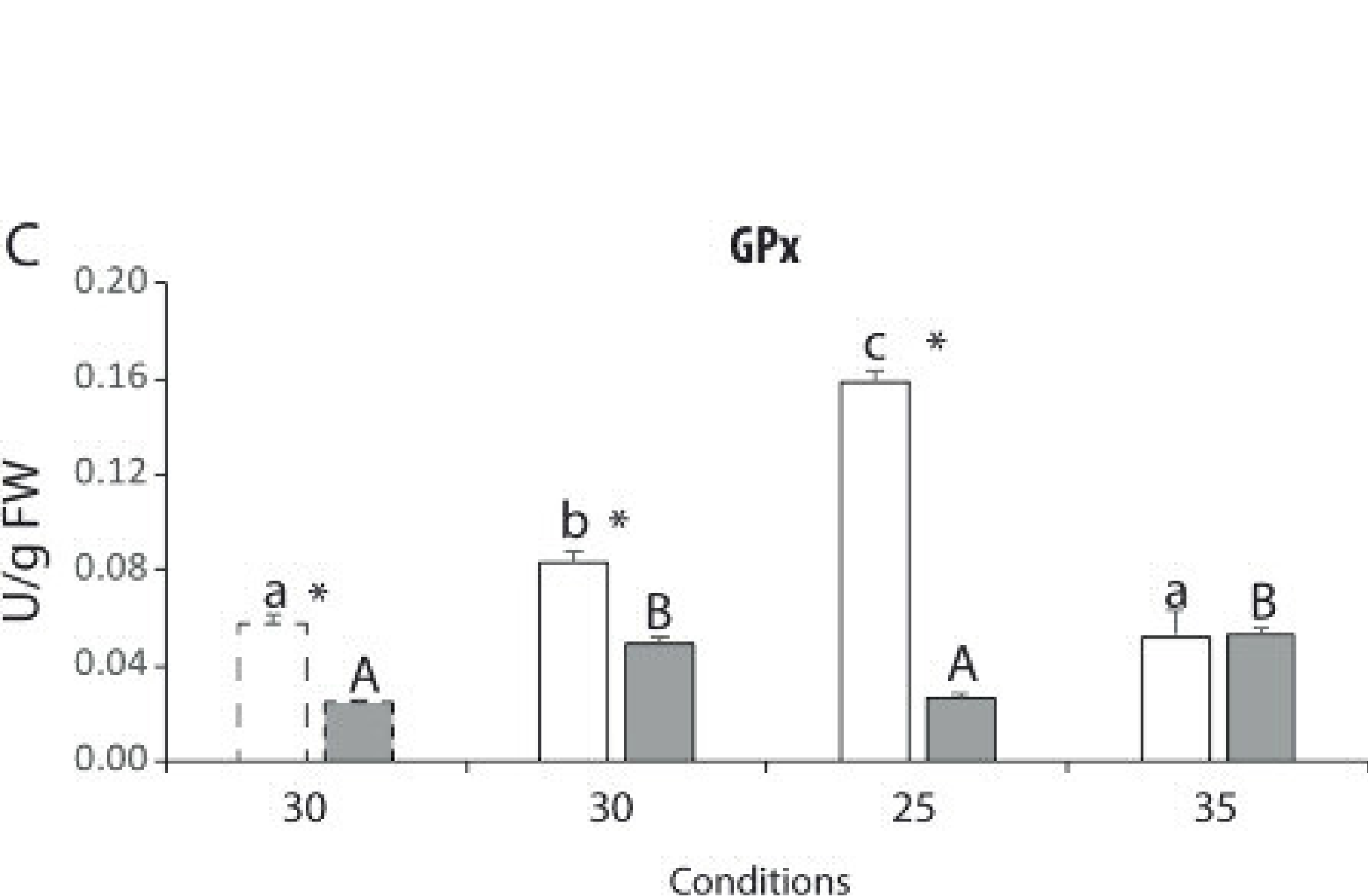
Figure 5. Activity of Acetylcholinesterase (AChE), in *Mytilus galloprovincialis* under different conditions (see legend Figure 1). Values are presented as mean + standard deviation. Significant differences ($p \leq 0.05$) among salinity and temperature conditions are represented with different letters: lowercase letters for non-contaminated mussels and uppercase letters for contaminated mussels. Significant differences ($p \leq 0.05$) between non-contaminated and contaminated mussels for each salinity and temperature condition are represented with an asterisks. White bars represent non-contaminated mussels. Grey bars represent contaminated mussels.

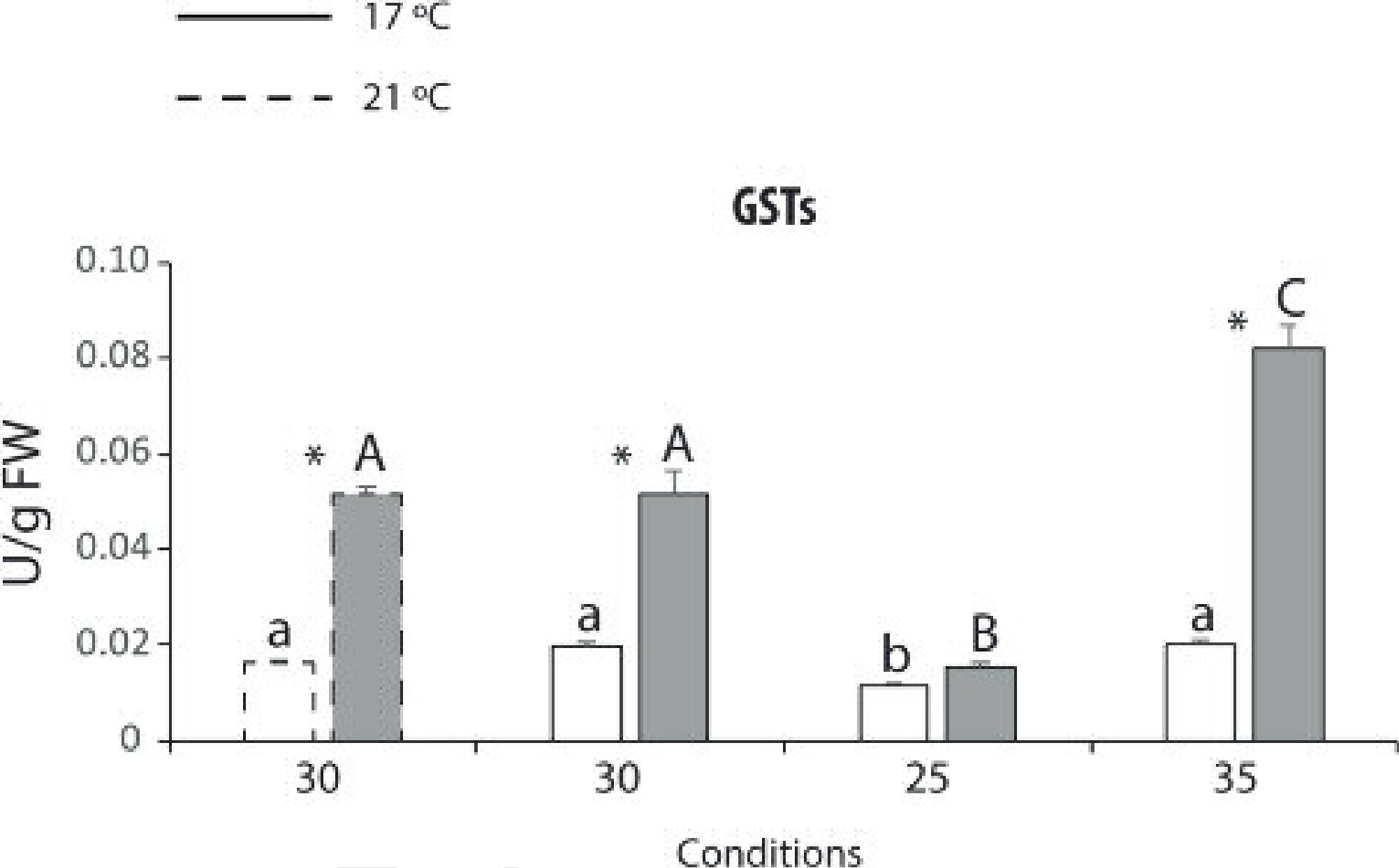
Figure 6. Centroids ordination diagram (PCO) based on Pb concentrations and biochemical markers measured in *Mytilus galloprovincialis* under different conditions (see legend Figure 1). Black letters represented contaminated mussels while grey letters represent non-contaminated mussels. Pearson correlation vectors are

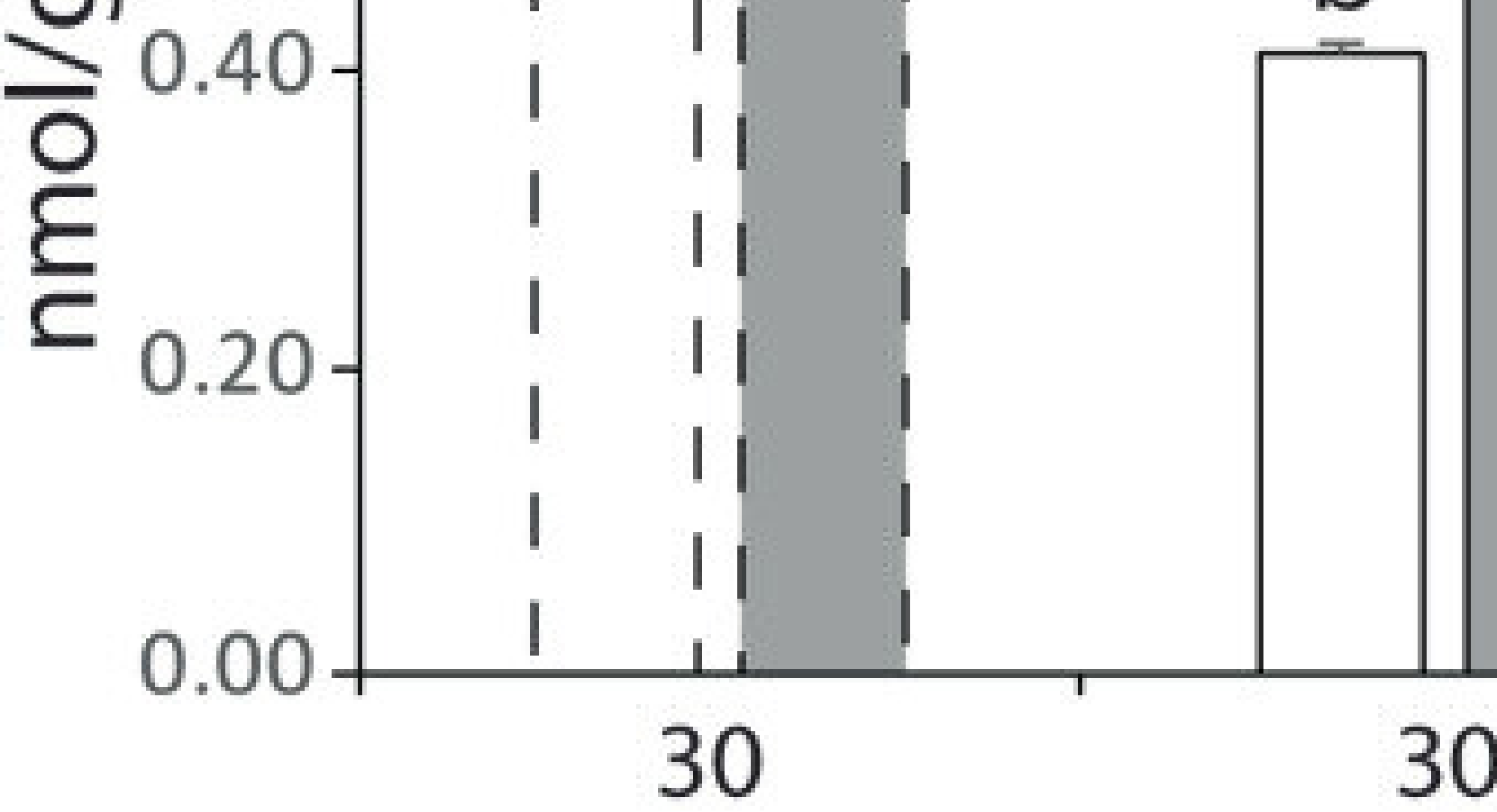
superimposed as supplementary variables, namely biochemical data ($r > 0.75$): PC, CAT, PROT, GPx, Pb, GSTs, LPO.

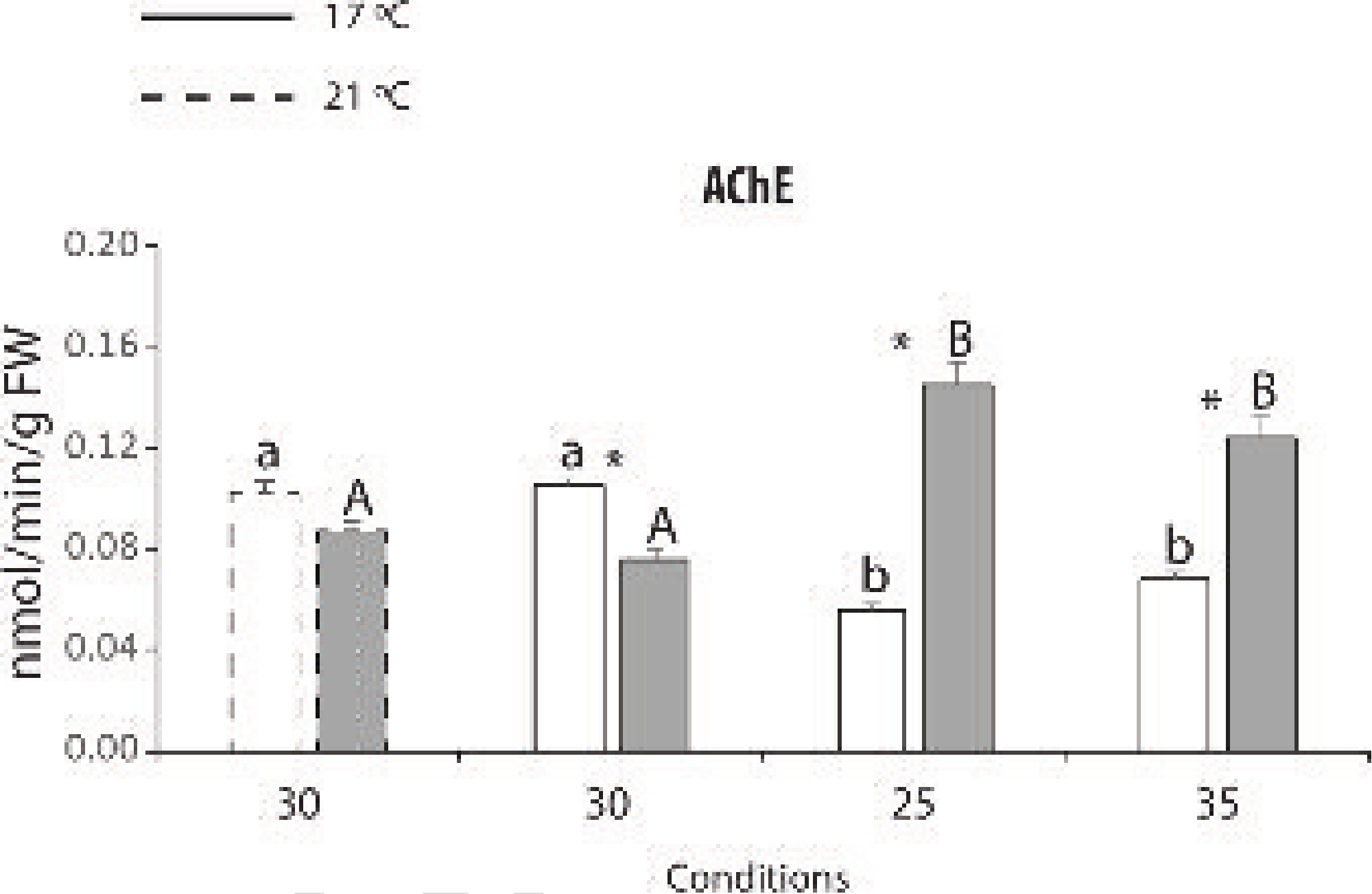
- Metabolism increased at lower salinity and higher temperature in Pb exposed mussels.
- Overall, exposure to Pb increased detoxification activity measured as GSTs.
- Antioxidant defences failed to prevent LPO at the lowest salinity in controls.
- Damaged proteins occurred at the highest salinity in unexposed mussels.


17 °C
21 °C
A
ETS
nmol/min/g FW
120
100
80
60
40
20
0
a
A
a
B
a
A
b
B
30
30
25
35
Conditions
B
GLY
mg/g FW
6
5
4
3
2
1
0
a
A
a
B
a,b
B
b
C
30
30
25
35
Conditions
C
PROT
mg/g FW
20
16
12
8
4
0
a
A
b
B
a
C
b
A
30
30
25
35
Conditions


C
GPx
U/g FW
0.20
0.16
0.12
0.08
0.04
0.00
a
*
A
b
*
B
c
*
A
a
B
30
30
25
35
Conditions


17 °C
21 °C
GSTs
0.10
0.08
0.06
0.04
0.02
0
U/g FW
*
A
*
A
*
C
a
a
b
B
a
30
30
25
35
Conditions


nmol/
0.40
0.20
0.00
30
30


17 °C
21 °C
AChE
nmol/min/g FW
0.20
0.16
0.12
0.08
0.04
0.00
a
A
a
*
A
*
B
b
*
B
b
30
30
25
35
Conditions

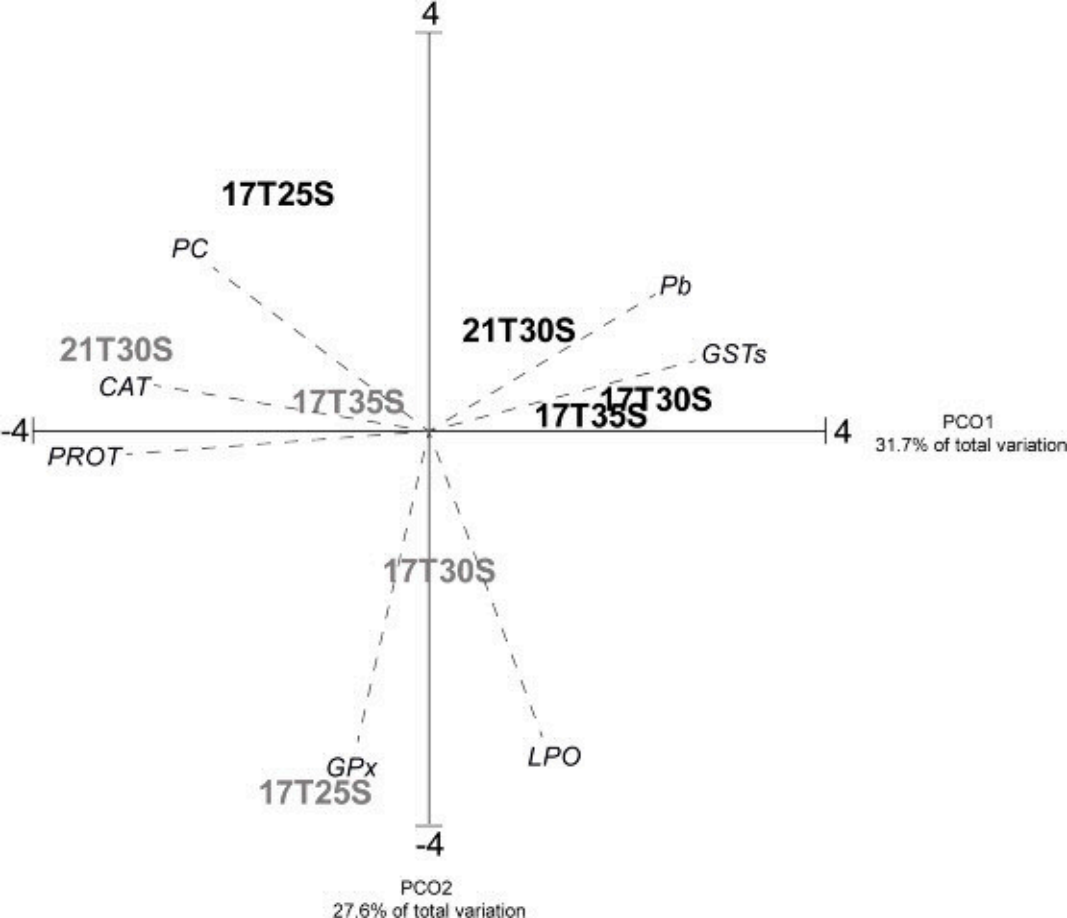

4
17T25S
PC
Pb
21T30S
21T30S
GSTs
CAT
17T35S
17T30S
17T35S
-4
4
PROT
PCO1
31.7% of total variation
17T30S
GPx
LPO
17T25S
-4
PCO2
27.6% of total variation

Table 1. Mean Lead concentrations (µg/L) in water samples weekly and immediately sampled after spiking during the experimental period (28 days), at each condition. Non-contaminated (mussels exposed to 0 µg/L of Lead) and contaminated (mussels exposed to 50 µg/L of Lead) conditions. For non-contaminated and contaminated mussels, significant differences ($p \leq 0.05$) among different salinity and temperature conditions are represented with different lower case letters.

Conditions	**Salinity**	**Temperature (ºC)**	**Water Pb concentrations (µg/L)**
Non-contaminated	25	17	5.3±2.9 [a]
	30	17	4.6±2.5 [a]
	35	17	3.6±2.5 [a]
	30	21	6.3±0.6 [a]
Contaminated	25	17	66.6±7.5 [a]
	30	17	69.9±4.3 [a]
	35	17	67.7±2.1 [a]
	30	21	65.2±2.1 [a]

Table 2. Mean Lead concentrations (µg/g) in mussel's soft tissues collected at the end of the experimental period (28 days), at each water condition. Non-contaminated (mussels exposed to 0 µg/L of Lead) and contaminated (mussels exposed to 50 µg/L of Lead) conditions. For non-contaminated and contaminated mussels, significant differences ($p \leq 0.05$) among different salinity and temperature conditions are represented with different lower case letters. BCF- Bioconcentration factor.

Conditions	**Salinity**	**Temperature (ºC)**	**Tissue Pb concentrations (µg/g)**	**BCF**
Non-contaminated	25	17	0.63 ± 0.10^{a}	-
	30	17	0.44 ± 0.03^{b}	-
	35	17	$0.43\pm0.07^{b,c}$	-
	30	21	0.38 ± 0.01^{c}	-
Contaminated	25	17	1.8 ± 0.2^{a}	0.027
	30	17	1.6 ± 0.2^{a}	0.023
	35	17	1.3 ± 0.2^{a}	0.019
	30	21	1.4 ± 0.2^{a}	0.021