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



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

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24           **Abstract**

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

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

## 1. INTRODUCTION

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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.

73 Besides exposure to pollutants, aquatic systems are currently subjected to increased  
74 atmospheric carbon dioxide (CO<sub>2</sub>) concentrations, at present already above ~400 ppm  
75 compared to pre-industrial revolution levels (Pörtner et al., 2014). Such CO<sub>2</sub> increase is  
76 responsible for the rise in global temperature, with a concomitant increase in mean seawater  
77 values of about 0.7°C since pre-industrial times, and a further rise of 3-4 °C is foreseen at the  
78 end of this century (Collins et al., 2013; Pörtner et al., 2014). Additionally, extreme weather  
79 events, including heavy rainy or long drought periods, are expected to increase in frequency  
80 and intensity (Pörtner et al., 2014). Such environmental changes, associated to climate  
81 modification, may contribute to alterations in seawater characteristics, namely in terms of  
82 salinity and temperature. This can further result into changes in organism's sensitivity towards  
83 pollutants but also into modification of pollutants properties and their associated toxicity (Attig et  
84 al., 2014; Byrne, 2012; Coppola et al., 2018a; Izagirre et al., 2014; Manciocco et al., 2014;  
85 Mazzoto et al., 2013). In fact, marine and in particular estuarine organisms are naturally and  
86 simultaneously exposed to multiple stressors, including the ones associated to water  
87 characteristics and pollutants presence, with growing evidences that combined stressors  
88 frequently interact and often amplify effects (Dijkstra et al., 2013). However, interactions  
89 between stressors may be complex and difficult to predict, showing from additive, synergetic to  
90 antagonist effects. According to recent studies, oxidative stress was enhanced in bivalves  
91 exposed to Hg and As under warming conditions (Coppola et al., 2017; Coppola et al., 2018a;  
92 Freitas et al., 2017). On the other way, Nardi et al. (2018) demonstrated that the effects induced  
93 by Cd in *M. galloprovincialis* were not altered by increased temperatures. Recently, Moreira et  
94 al. (2018) demonstrated that changes in salinity and temperature altered the impacts of As in  
95 the embryo-larval development of oysters. The same authors also showed that salinity  
96 influences the biochemical response of *Crassostrea angulata* pyster to As (Moreira et al., 2016).

97 Despite few studies have addressed the combined effects of metal(oid)s and climate  
98 change related factors (see references above), the importance of considering different  
99 environmental variables when evaluating the toxicity of pollutants in aquatic organisms,  
100 including those of emerging concern, has repeatedly been highlighted (see for example De  
101 Marchi et al., 2018; Freitas et al., 2016a). Such information is of utmost relevance in order to  
102 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.  
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## 2. MATERIALS AND METHODS

### 2.1 Experimental conditions

*Mytilus galloprovincialis* (mean total weight  $25.5 \pm 7.5$  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  $17 \pm 1.0$  °C, salinity  $30 \pm 1.0$  and pH  $8.0 \pm 0.1$ , 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 °C). 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 °C; salinity 30 and temperature 17 °C (control condition resembling sampling site characteristics); salinity 35 and temperature 17 °C; salinity 30 and temperature 21 °C; 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).

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

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

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

160

## 161 **2.2 Lead quantifications**

162 Lead concentrations in water samples were directly analysed by inductively coupled  
163 plasma atomization-mass spectrometry (ICP-MS – Thermo X series) after dilution and  
164 acidification with HNO<sub>3</sub> 2 % (v/v), to pH < 2. The limit of quantification (LOQ) of the method was  
165 2 µg/L, with an acceptable relative standard deviation among replicates <10%.

166 Tissue samples were analysed by two techniques, ICP-MS and inductively coupled  
167 plasma optical emission spectrometry (ICP-OES - Jobin Yvon Activa M) for the low and high  
168 concentrations, respectively. LOQ for ICP-MS was of 0.02 µg/g dry weight (DW) and for  
169 ICP-OES was of 1.9 µg/g (DW), and quality control was ensured by analysing all samples in  
170 triplicate and imposing a coefficient of variation of less than 10 %. Prior to analysis, tissue  
171 samples were freeze dried and homogenised for microwave assisted acid digestion sample  
172 preparation method. The digestion was done in closed Teflon vessels, by adding the reagent  
173 mix (1 mL HNO<sub>3</sub> + 2 ml H<sub>2</sub>O<sub>2</sub> + 1 mL H<sub>2</sub>O) to 200 mg of dry tissue and following the heating

174 program: 15 min of temperature increase to 190 °C and then hold at 190 °C for 3 min. After  
175 cooling down, the digests were collected to a final volume of 25 mL with ultrapure water. To  
176 ensure quality control of these results each digestion cycle held a blank sample (< LOQ in both  
177 techniques, n = 4), a sample made in duplicate (coefficient of variation < 10 %; n= 4) and  
178 analysis of certified reference material (Tort-3, lobster hepatopancreas, mean recovery of 116  
179 %; n =5).

180

## 181 **2.3 Biochemical parameters**

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

191

### 192 **2.3.1 Metabolic capacity and energy reserves**

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

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

200 PROT content was determined according to the spectrophotometric Biuret method  
201 (Robinson and Hogden, 1940). Bovine serum albumin (BSA) was used to prepare a standard  
202 calibration curve (0–40 mg/mL). Absorbance was read at 540 nm. The results were expressed  
203 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  $\mu\text{mol}$  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  $\mu\text{mol/L}$ ). 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 ( $\epsilon$ ) 6.22  $\text{mM}^{-1}\text{cm}^{-1}$ . The results were expressed as U  
219 per g of FW, where U represents the amount of enzyme that caused the formation of 1  $\mu\text{mol}$   
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 ( $\epsilon$ ) 9.6  $\text{mM}^{-1}\text{cm}^{-1}$ . 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  $\mu\text{mol}$  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 ( $\epsilon$ ) 156  $\text{mM}^{-1}\text{cm}^{-1}$ . 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

234 measured at 450 nm and the extinction coefficient ( $\epsilon$ ) 22,308 M<sup>-1</sup> cm<sup>-1</sup> was used to calculated  
235 PC levels, expressed in nmol per g of FW.

236

#### 237 2.3.4 Neurotoxicity

238 AChE activity was determined using Acetylthiocholine iodide (ATChI 5 mM) as  
239 substrate, according to the methods of Ellman et al. (1961) with modification by Mennillo et al.  
240 (2017). The activity was measured at 412 nm during 5 min and expressed in nmol/min per g of  
241 FW using the extinction coefficient ( $\epsilon$ ) 13.6 mM<sup>-1</sup>cm<sup>-1</sup>.

242

243

#### 244 2.4 Data analysis

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

$$249 \quad BCF = \frac{\text{concentration in the organism}}{\text{concentration in the water}}$$

250

251 All the biochemical results (ETS, GLY, PROT, SOD, CAT, GPx, GSTs, LPO, PC,  
252 AChE) and Pb concentrations, for all conditions, were individually submitted to a non-parametric  
253 permutational analysis of variance (PERMANOVA Add-on in Primer v7). A one-way hierarchical  
254 design was followed in this analysis. When significant differences were observed in the main  
255 test pairwise comparisons were performed. Values lower than 0.05 were considered as  
256 significantly different. The null hypotheses tested were: for each biomarker and each Pb  
257 concentration (0 or 50 µg/L), no significant differences existed among salinity and temperature  
258 levels, represented in figures by letters (lowercase letters for non-contaminated conditions;  
259 uppercase letters for contaminated conditions); for each biomarker at each salinity and  
260 temperature levels, no significant differences existed between non-contaminated and  
261 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.

## 271 **3. RESULTS**

### 272 **3.1 Lead concentrations in water and organisms**

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

278 The concentrations of Pb in mussel's soft tissues showed significantly higher (about 3-  
279 4 fold) values in organisms exposed to Pb in comparison to non-contaminated ones, with no  
280 significant differences among mussels exposed to different conditions (Table 2). The BCF  
281 estimated ranged between 0.02-0.03 with no clear influence of salinity and temperature  
282 parameters.

283

### 284 **3.2 Biochemical parameters**

#### 285 *3.2.1 Metabolic capacity and energy reserves*

286 In non-contaminated mussels, significantly lower ETS values were observed in those  
287 maintained to salinity 35 in comparison to the remaining conditions. In Pb exposed mussels,  
288 significantly lower ETS values were observed at salinities 30 and 35 at control temperature (17  
289 °C). At salinity 30, ETS increased at 21°C but decreased at 17°C in Pb exposed mussels in  
290 respect to non-contaminated ones (Figure 1A).

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

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

299

### 3.2.2 Antioxidant and biotransformation defences

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

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

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

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

### 3.2.3 Indicators of cellular damage

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

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

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 °C 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 °C)  
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

350

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

359

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

369

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

380 high temperature (21°C) conditions, which were in turn correlated with increased GLY and  
381 PROT consumption particularly at higher temperature. Previous studies with bivalves already  
382 demonstrated that under control salinity (30) and temperature (17 °C) conditions, exposure to  
383 metals strongly decreased their metabolic capacity (Bielen et al., 2016; Coppola et al., 2017;  
384 Coppola et al., 2018; Izagirre et al., 2014; Nardi et al., 2017), while under combined stressful  
385 conditions (salinity and pollution) their metabolic capacity increased (Moreira et al., 2016). Thus  
386 both strategies can be alternatively adopted in bivalves. The neurotoxicity marker AChE did not  
387 seem to be a mechanism that significantly contributed to the identification of differences among  
388 tested conditions (correlation <75%). Salinities 5 units over and under the control value (salinity  
389 30) either decreased (non-contaminated mussels) or increased (Pb exposed mussels) this  
390 enzymatic activity. The interpretation of this result is difficult in bivalves since a clear  
391 physiological role of this activity, other than the neurotoxicity due to AChE inhibition, is not yet  
392 clear (Sole et al., 2018). Other metals such as Ni (Attig et al., 2010) and Cd (Chalkiadaki et al.,  
393 2014) are reported as AChE inhibitors in bivalves, whereas an increase in AChE in the bivalve  
394 *Perna indica* exposed to As was interpreted as an attempt to reduce the neurotransmitter  
395 excess in the synaptic clefts (Rajkumar, 2013).

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

410 and LPO levels. This third group, although included the mussels exhibiting the highest  
411 antioxidant GPx activity, it lacked the action of other efficient antioxidant defences such as CAT  
412 and SOD. In fact, SOD activity was the lowest under temperature 17 °C and salinity 25, and  
413 consequently LPO occurrence was not prevented in this particular group. Such antioxidant  
414 response patterns indicate that even non-contaminated mussels tried to avoid cellular damage  
415 when facing unfavourable water conditions (i.e., out of salinity 30 and temperature 17 °C) by  
416 increasing particular antioxidant defences. The response of antioxidant enzymes to  
417 unfavourable physical water parameters was already described in mussels and other bivalves,  
418 with i) inhibition of SOD under highly stressful conditions of salinity (Gonçalves et al., 2017), or  
419 ii) increase of antioxidant defenses in bivalves exposed to salinity decreases (Freitas et al.,  
420 2017; Velez et al., 2016) or salinity increases (Rahman et al., 2019); also to temperature  
421 elevation (Coppola et al., 2018; Rahman et al., 2019; Verlecar et al., 2007) or decreased pH  
422 (Matozzo et al., 2013). The present results highlight that the presence of Pb generated complex  
423 antioxidant responses under unfavourable salinity and temperature conditions. That is it under 9  
424 stressing situations 5 decreases and 1 increase were recorded for the three antioxidant  
425 enzymes measured. Three out of the 5 decreases were due to increased temperature  
426 evidencing that antioxidant defenses may be compromised under warming conditions and Pb  
427 contamination in mussels. A more limited capacity of these enzymes to act when under  
428 combined stressful conditions was already demonstrated by other authors (Maria and Bebianno,  
429 2011; Freitas et al., 2017).

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

437

438

## Conclusion

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

451

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670 [df](https://www.who.int/gho/publications/world_health_statistics/WHS2013_IndicatorCompendium.pdf)

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## 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 °C, Pb-contaminated and non-contaminated mussels at salinity 30 and at temperature 21 °C. 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.

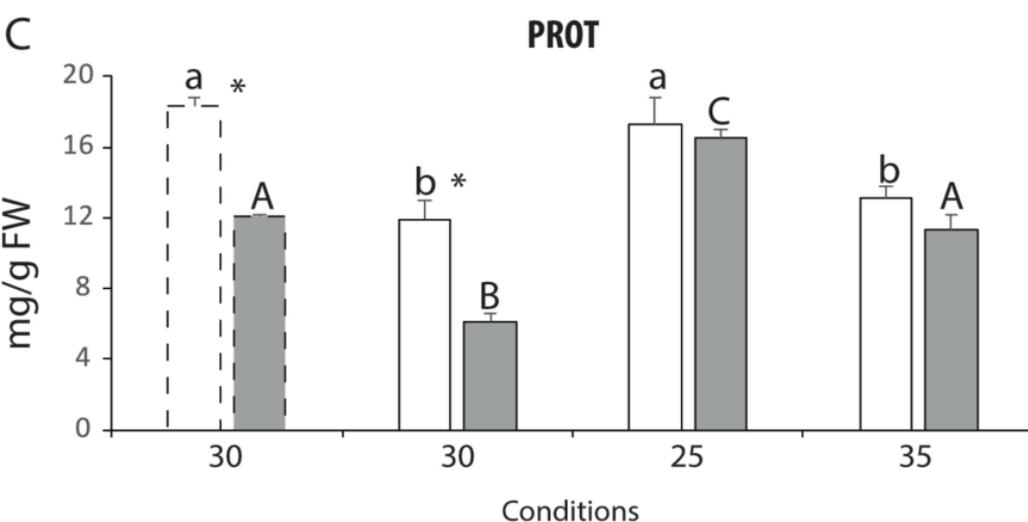
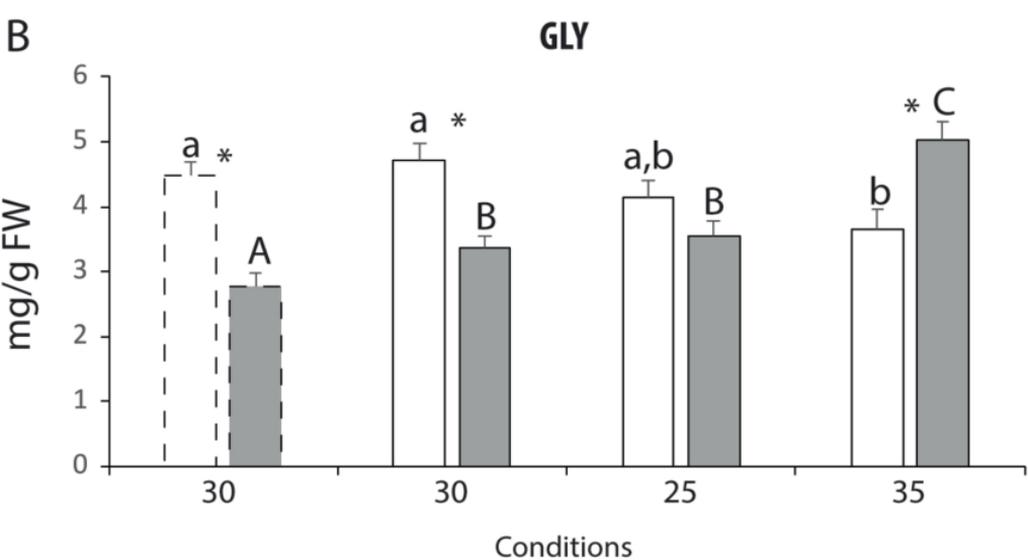
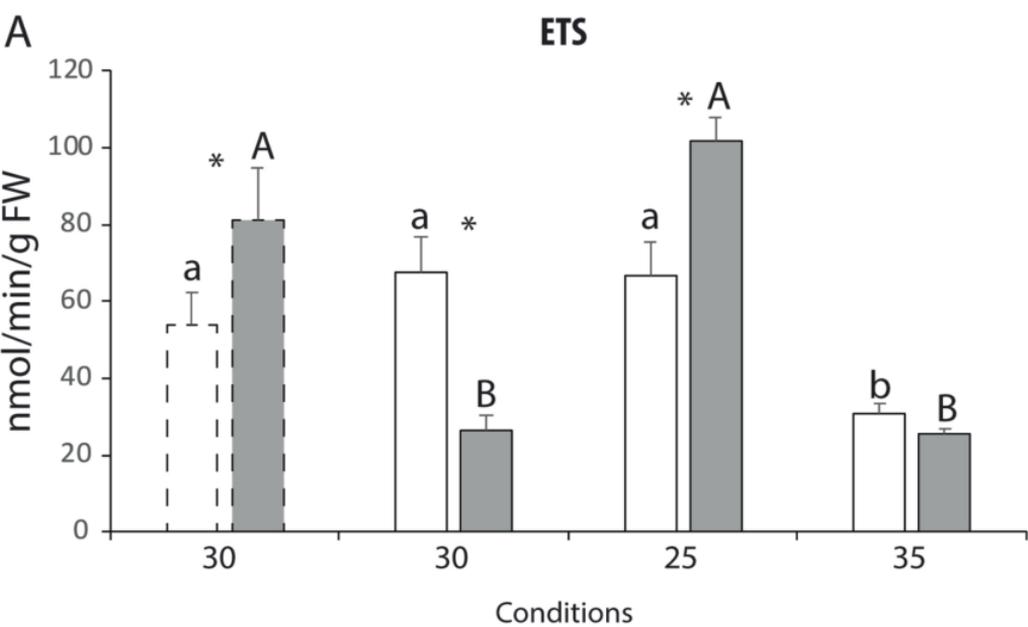
Figure 6. Centroids ordination diagram (PCO) based on Pb concentrations and biochemical markers measured in *Mytilus galloprovincialis* under different conditions (see legend Figure 1). Black letters represented contaminated mussels while grey letters represent non-contaminated mussels. Pearson correlation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

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

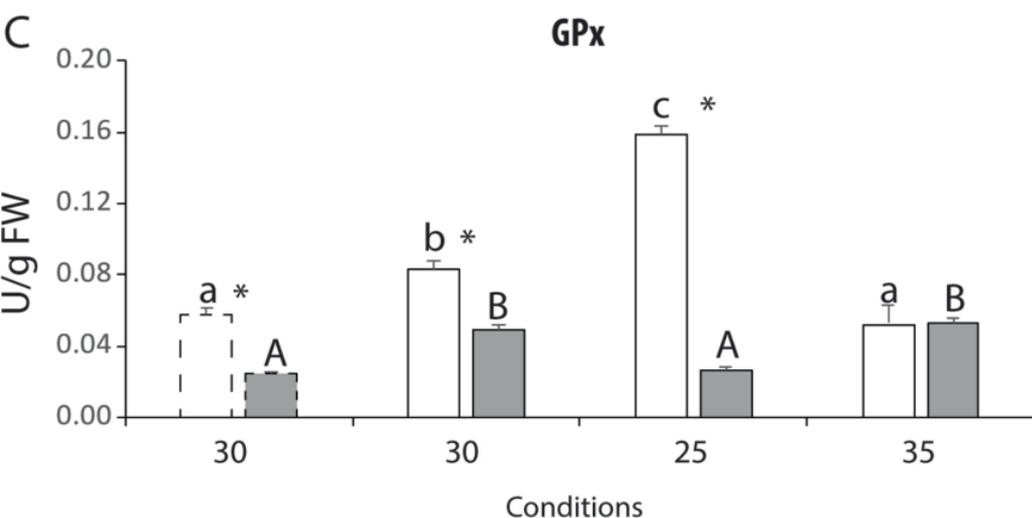
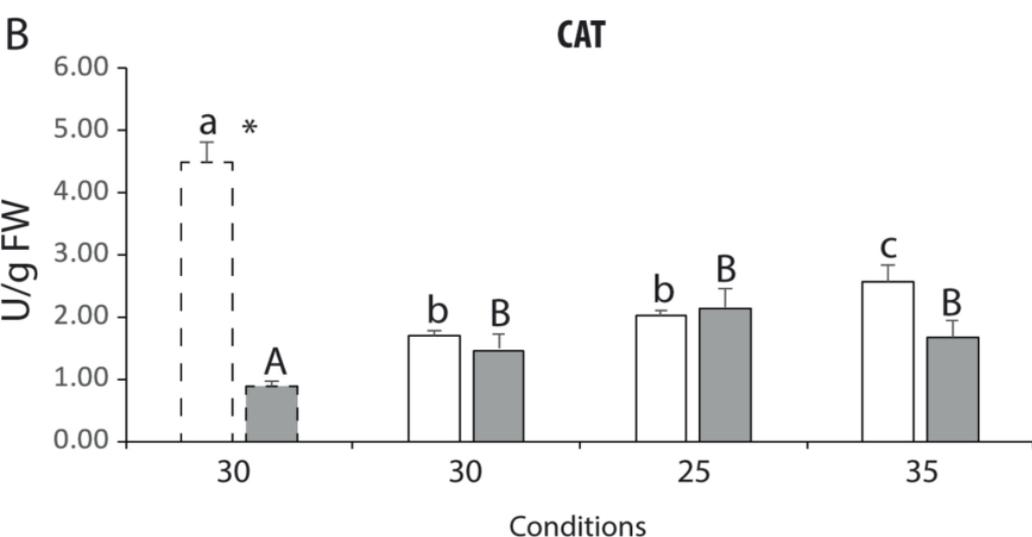
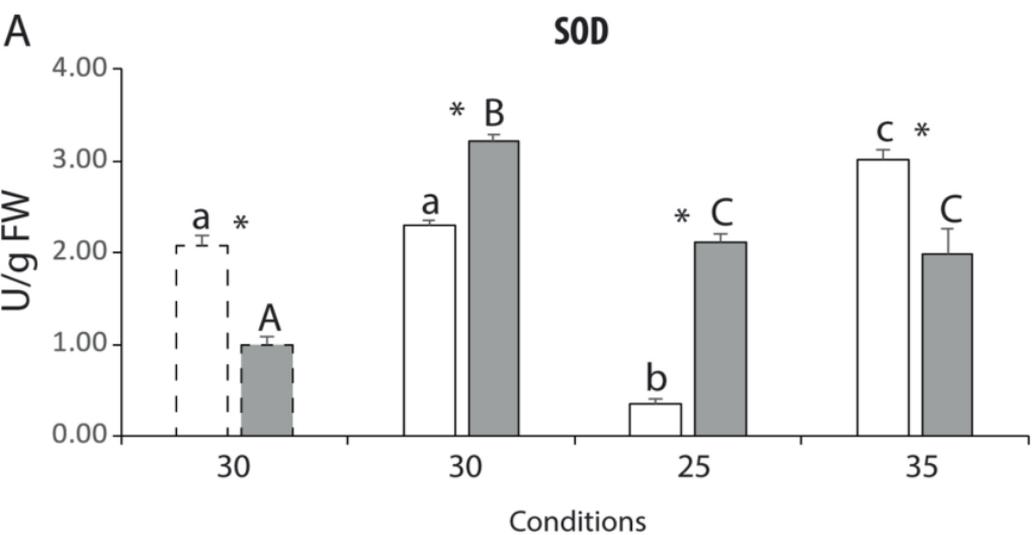
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- Metabolism increased at lower salinity and higher temperature in Pb exposed mussels.
- Overall, exposure to Pb increased detoxification activity measured as GSTs.
- Antioxidant defences failed to prevent LPO at the lowest salinity in controls.
-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

— 17 °C  
- - - 21 °C

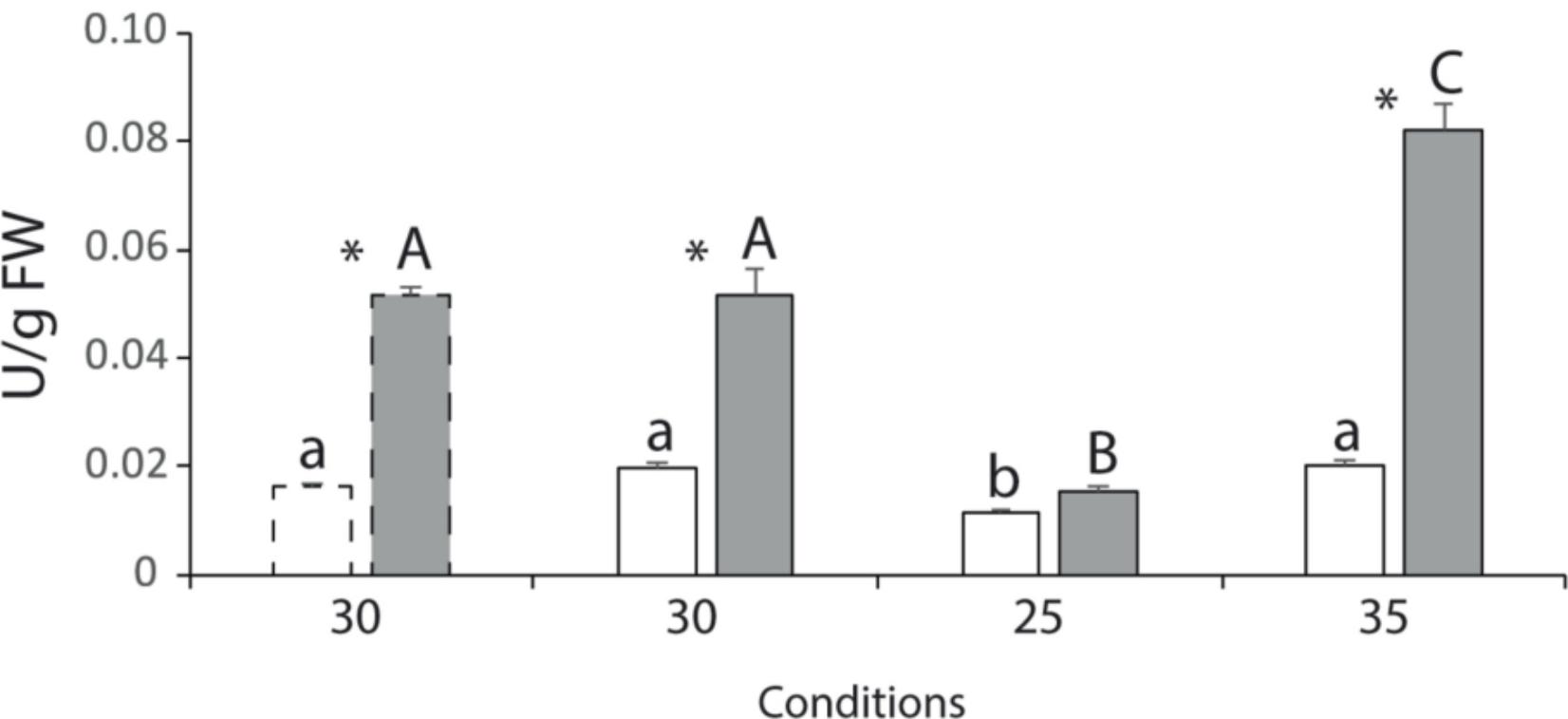


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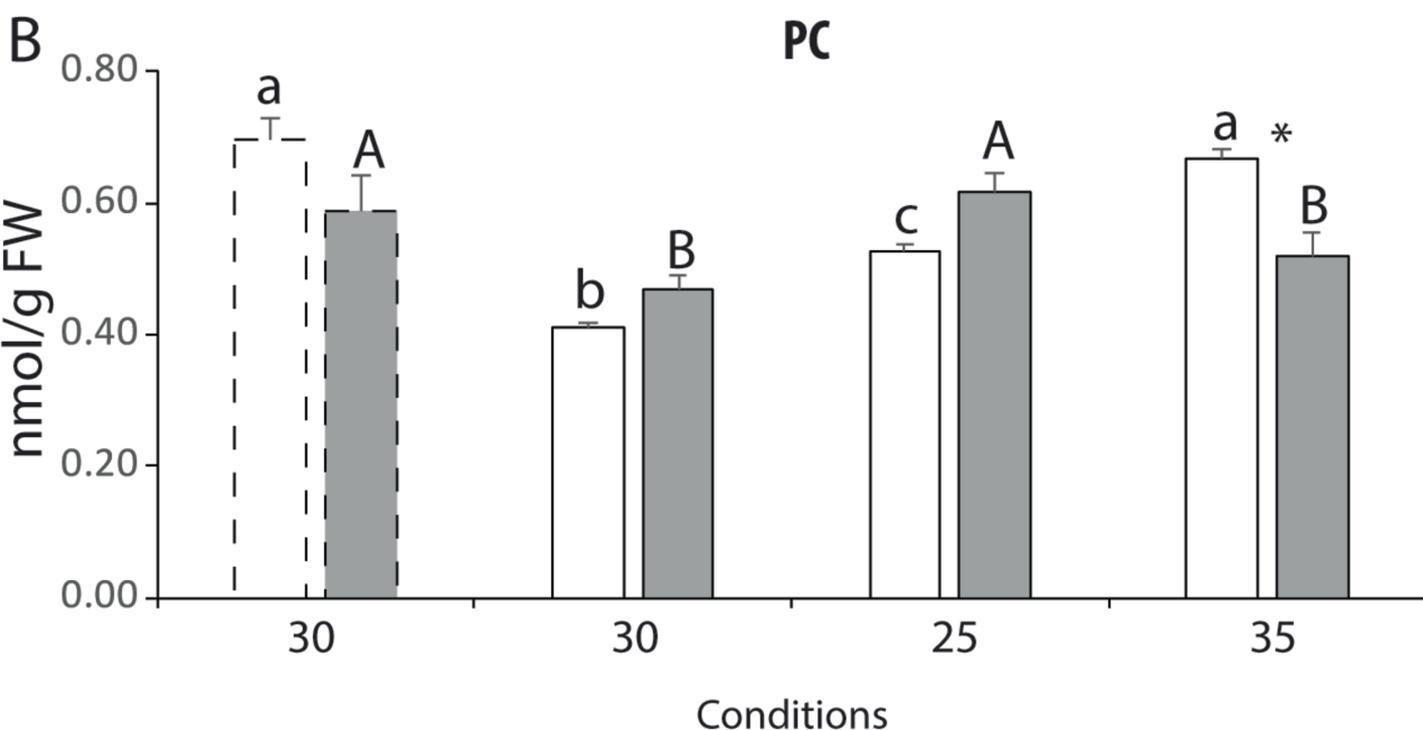
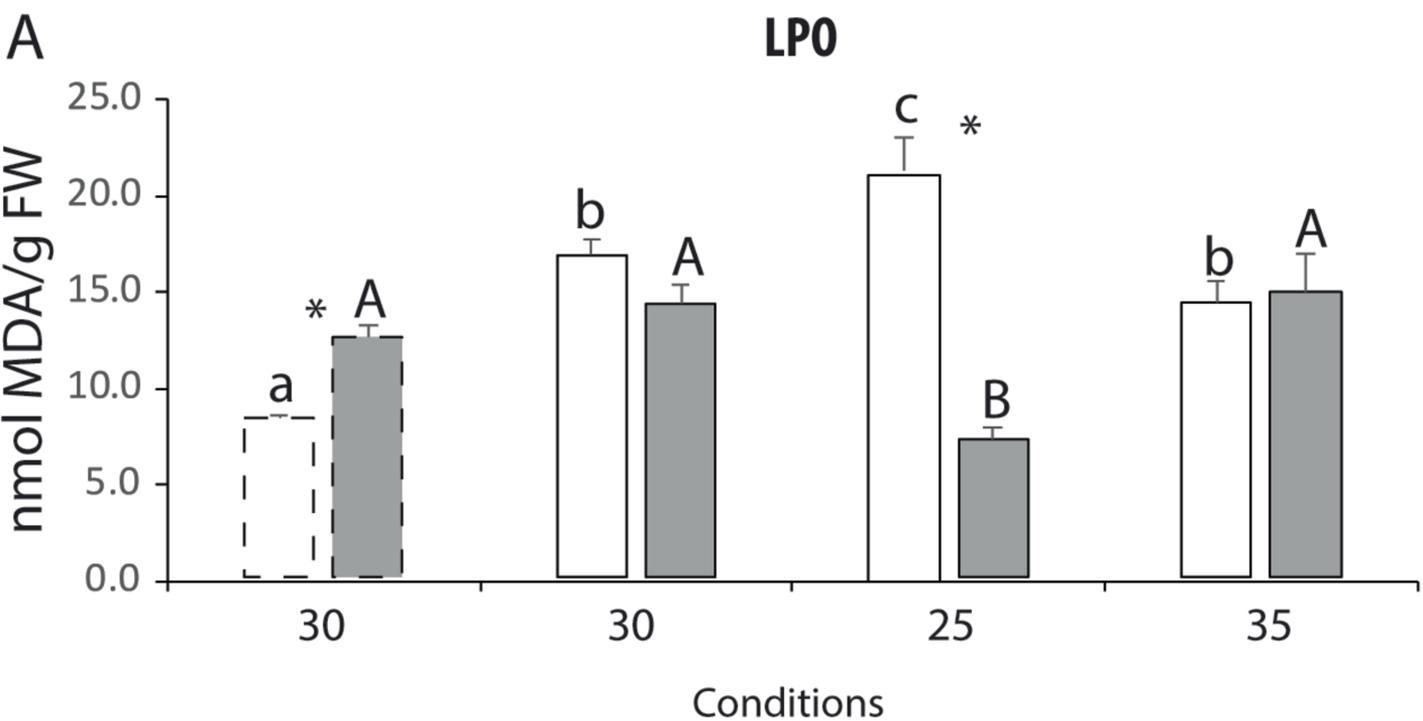


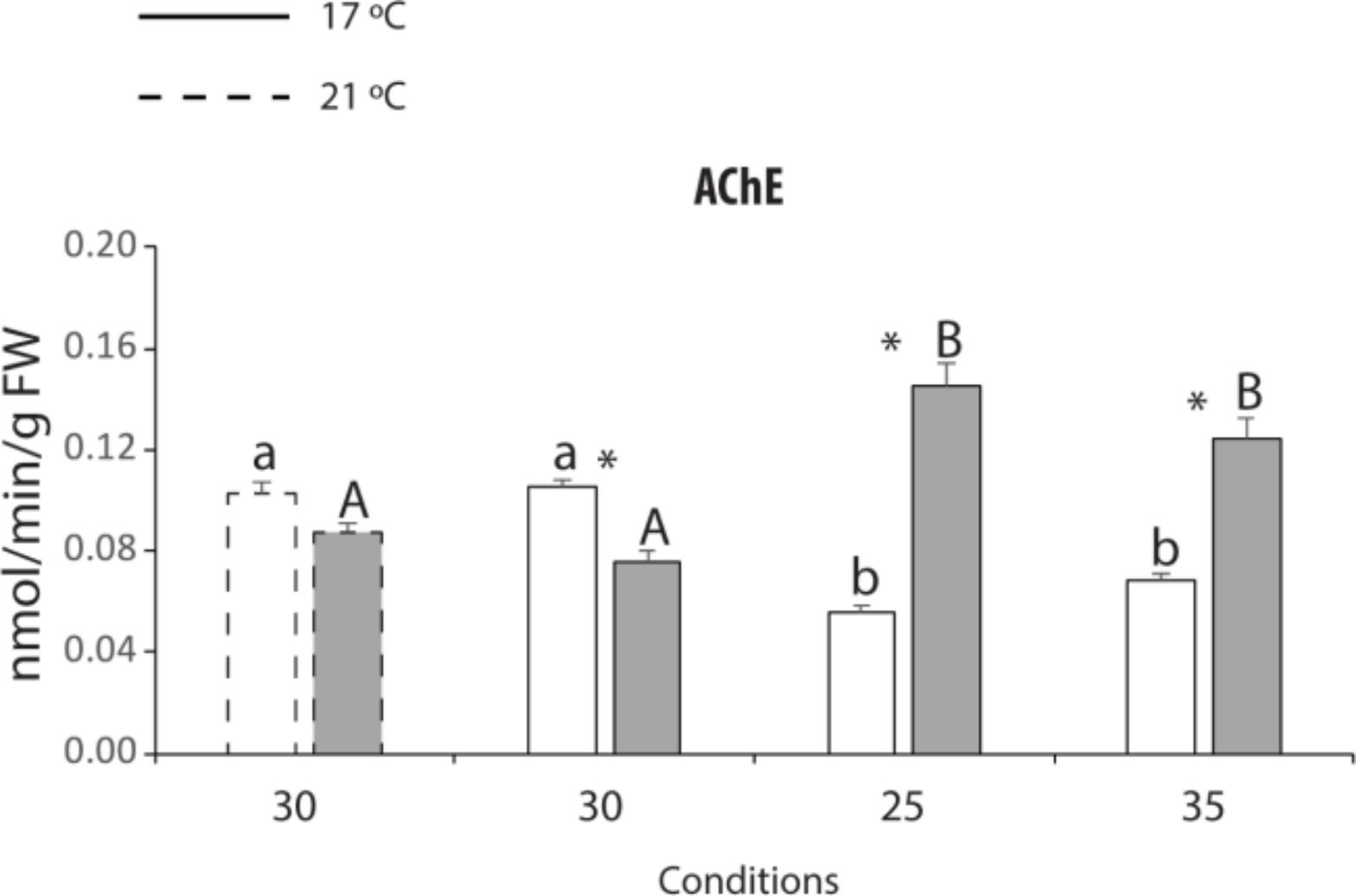
— 17 °C  
- - - 21 °C

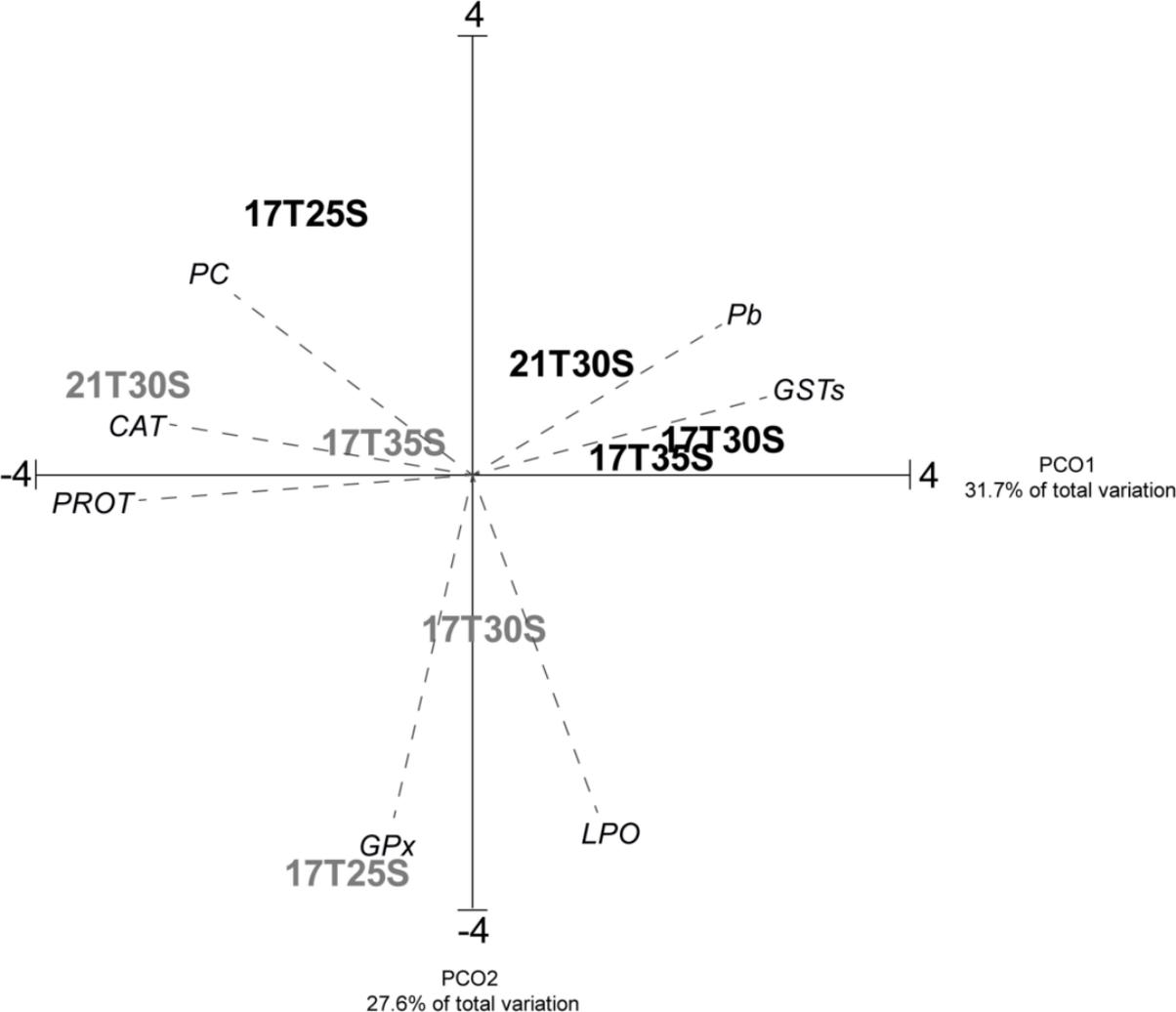
### GSTs



— 17 °C  
- - - 21 °C







**Table 1.** Mean Lead concentrations ( $\mu\text{g/L}$ ) in water samples weekly and immediately sampled after spiking during the experimental period (28 days), at each condition. Non-contaminated (mussels exposed to  $0 \mu\text{g/L}$  of Lead) and contaminated (mussels exposed to  $50 \mu\text{g/L}$  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 ( $^{\circ}\text{C}$ )	Water Pb concentrations ( $\mu\text{g/L}$ )
Non-contaminated	25	17	$5.3 \pm 2.9^a$
	30	17	$4.6 \pm 2.5^a$
	35	17	$3.6 \pm 2.5^a$
	30	21	$6.3 \pm 0.6^a$
Contaminated	25	17	$66.6 \pm 7.5^a$
	30	17	$69.9 \pm 4.3^a$
	35	17	$67.7 \pm 2.1^a$
	30	21	$65.2 \pm 2.1^a$

**Table 2.** Mean Lead concentrations ( $\mu\text{g/g}$ ) 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  $\mu\text{g/L}$  of Lead) and contaminated (mussels exposed to 50  $\mu\text{g/L}$  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 (°C)	Tissue Pb concentrations ( $\mu\text{g/g}$ )	BCF
Non-contaminated	25	17	0.63±0.10 <sup>a</sup>	-
	30	17	0.44±0.03 <sup>b</sup>	-
	35	17	0.43±0.07 <sup>b,c</sup>	-
	30	21	0.38±0.01 <sup>c</sup>	-
Contaminated	25	17	1.8±0.2 <sup>a</sup>	0.027
	30	17	1.6±0.2 <sup>a</sup>	0.023
	35	17	1.3±0.2 <sup>a</sup>	0.019
	30	21	1.4±0.2 <sup>a</sup>	0.021