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Does salinity modulates the response of *Mytilus galloprovincialis* exposed to triclosan and diclofenac?

Rosa Freitas, Francesca Coppola, Silvana Costa, Chiara Manzini, Luigi Intorre, Valentina Meucci, Amadeu M.V.M. Soares, Carlo Pretti, Montserrat Solé



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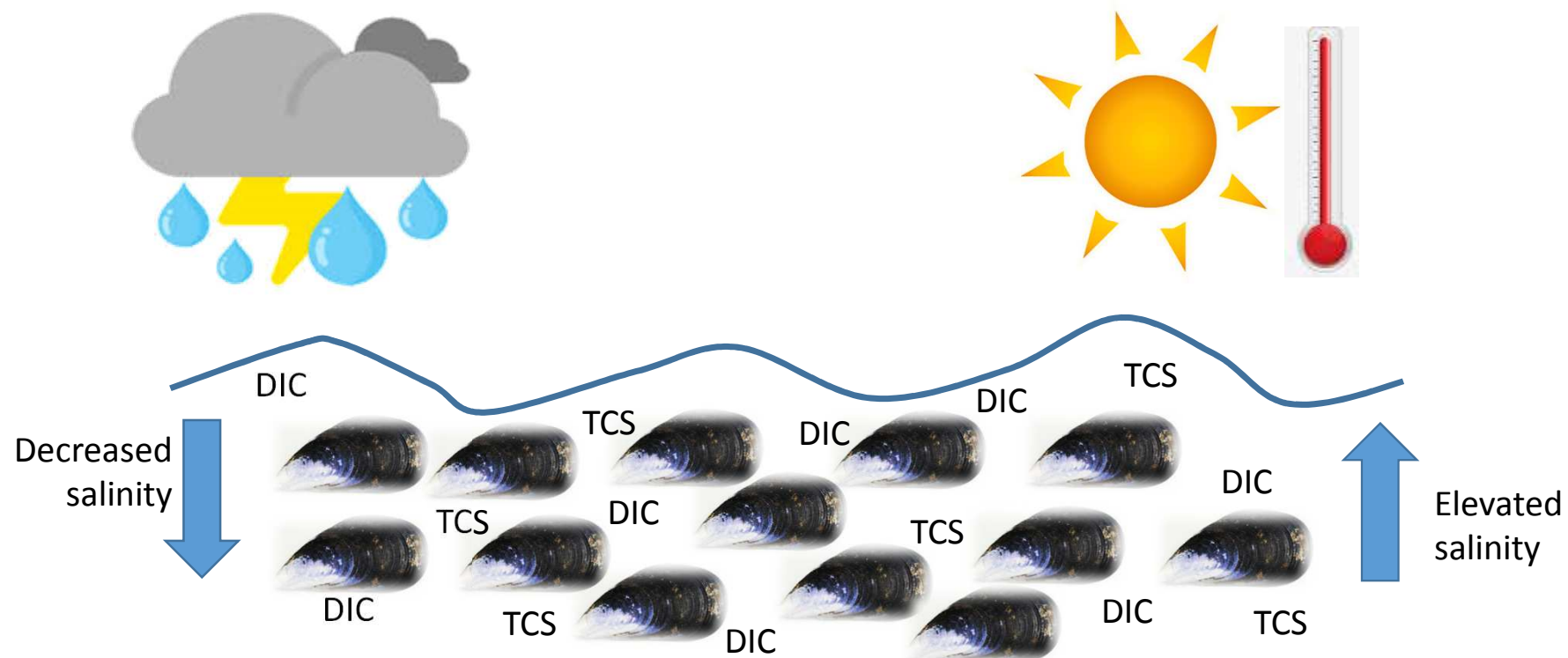
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- Mussels lowered their metabolic rate after drug exposures at control salinity
- Mussels increased antioxidant defences when exposed to drugs at all salinities
- GSH/GSSG ratio was consistently reduced when mussels were exposed to TCS and DIC.

1                   **Does salinity modulates the response of *Mytilus***  
2                   ***galloprovincialis* exposed to Triclosan and Diclofenac?**

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4 Rosa Freitas<sup>a\*</sup>, Francesca Coppola<sup>a</sup>, Silvana Costa<sup>a</sup>, Chiara Manzini<sup>b</sup>, Luigi  
5 Intorre<sup>c</sup>, Valentina Meucci<sup>c</sup>, Amadeu M.V.M. Soares<sup>a</sup>, Carlo Pretti<sup>b,c</sup>, Montserrat  
6 Solé<sup>d</sup>

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8                   <sup>a</sup>Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro,  
9                   Portugal

10                  <sup>b</sup>Consorzio per il Centro Interuniversitario di Biologia Marina ed Ecologia Applicata "G. Bacci"  
11                  (CIBM), Livorno, Italy

12                  <sup>c</sup>Dipartimento di Scienze Veterinarie, Università di Pisa, Italy

13                  <sup>d</sup>Instituto de Ciencias del Mar ICM-CSIC, E-08003 Barcelona, Spain

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

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23

24 **Abstract**

25 In the present study *Mytilus galloprovincialis* mussels were exposed for 28 days to three  
26 salinities: 30 (control), 25 and 35. Simultaneously, organisms at each salinity were exposed to  
27 either the antimicrobial agent Triclosan (TCS) or the pharmaceutical drug Diclofenac (DIC) at 1  
28 µg/L. Salinity alone and exposure to PPCPs exposures changed mussel's metabolic capacity  
29 and oxidative status, but no additive or synergetic effects resulting from the combined exposure  
30 to different stressors were observed. Overall the metabolic capacity of mussels was decreased  
31 when exposed to TCS and DIC under control salinity, which was less pronounced at salinities  
32 out of the control level. TCS had a notorious effect over glutathione peroxidase activity while  
33 DIC exposure enhanced catalase response. Such defence mechanisms were able to prevent  
34 cellular damage but still a clear reduction in GSH/GSSG ratio after PPCPs exposures indicates  
35 oxidative stress which could compromise bivalve's performance to further stressing events.

36

37 **Keywords:** Pharmaceuticals and personal care products; bivalves; oxidative stress;  
38 energy metabolism; extreme weather events.

39

## 40 1. INTRODUCTION

41 A vast variety of substances arrives currently to the aquatic environment, including  
42 newly developed chemicals and products. Among them are pharmaceuticals and personal care  
43 products (PPCPs) worldwide produced and used (Daughton and Ternes, 1999; Fabbri and  
44 Franzellitti, 2016; Fent et al., 2006), being actually identified as emerging environmental  
45 contaminants as most of them are ubiquitous, persistent and biologically active (Wang and  
46 Wang, 2016). PPCPs may enter direct or indirectly in the environment as a result of human or  
47 animal waste, after incomplete absorption and excretion from the body, or emissions of medical,  
48 industrial, agricultural, or household discharges. Among the most widely used PPCPs are the  
49 antimicrobial agent Triclosan (TCS) and the nonsteroidal anti-inflammatory drug (NSAID) drug  
50 Diclofenac (DIC). The occurrence in the environment has been addressed (Bonnefille et al.,  
51 2018; Dann and Hontela, 2011; Fabbri and Franzellitti, 2016), revealing removal rates in waste  
52 water treatment plants (WWTPs) up to 99 % for Triclosan and 80% for Diclofenac (Wang et al.,  
53 2018; Zhang et al., 2008) and concentrations ranging from ng/L to µg/L in sewage treatment  
54 plants influents and effluents, rivers, groundwater, and coastal areas (Al Aukidy et al., 2012;  
55 Gaw et al., 2014; Lolic et al., 2015; Mezzelani et al., 2016; Olaniyan et al., 2016; Pal et al.,  
56 2010). In what regards to the effects of PPCPs in aquatic organisms a limited number of studies  
57 have evaluated water exposures at realistic concentrations of TCS (Gatidou et al., 2010;  
58 Matozzo et al., 2012) and DIC (Fontes et al., 2018; Gonzalez-Rey and Bebianno, 2014;  
59 Goodchild et al., 2016; Mezzelani et al., 2016; Mezzelani et al., 2018; Munari et al., 2018;  
60 Schmidt et al., 2014).

61 In the aquatic environment pollutants are not acting alone, with environmental changes  
62 playing an important role on their fate and impacts, with changes on species sensitivity and  
63 pollutants toxicity. In particular, recent studies already demonstrated that increased sensitivity of  
64 invertebrates to pharmaceuticals, nanoparticles or metals may result from exposure to climate  
65 change related factors, such as seawater acidification and warming (Coppola et al., 2018;  
66 Freitas et al., 2017a; Moreira et al., 2018; Munari et al., 2018; Nardi et al., 2018; Velez et al.,  
67 2016a) but fewer studies have focussed on salinity variations (De Marchi et al., 2018a; b; Velez  
68 et al., 2016b). Also, the increasing frequency and intensity of extreme weather events, namely  
69 long drought and heavy rainfall periods, may change pollutants characteristics and toxicity. For

70 example, the transformation behaviour of ibuprofen differed between freshwater and seawater  
71 (Weigel et al., 2004) and prochlorperazine was more stable in seawater than freshwater  
72 (Spongberg et al., 2011). Still, little is known on the impact of climate change events, in  
73 particular salinity changes, on the toxicity of PPCPs within the marine environment.

74 Biomarkers related to metabolic capacity, available energy reserves, oxidative stress  
75 defences and damage are very informative as they reveal the capacity of organisms to face  
76 challenging situations derived from chemical exposures and/or physical unfavourable conditions  
77 (Monserrat et al., 2007; Regoli and Giuliani, 2014). Marine bivalves and mussels in particular  
78 that reside in close proximity to coastal sites are under the influences to strong variations of  
79 water properties and constantly subjected to the action of anthropogenic chemicals. Despite  
80 their natural adaptation to a changing environment, the strength and duration of the insults  
81 could compromise their physiological and biochemical performance, with several studies  
82 demonstrating the impacts of pollutants and climate change factors on mussels (Andrade et al.,  
83 2019; Coppola et al., 2017; Freitas et al., 2017a; Munari et al., 2018).

84 Thus the aim of the present study was to assess the toxicity caused by chronic  
85 exposure (after 28 days) to TCS and DIC independently and at environmentally realistic  
86 concentrations (1 µg/L) each in the mussel *Mytilus galloprovincialis* under 3 salinity regimes:  
87 salinity at the study area (30) and a  $\pm 5$  units change (25 and 35). The parameters evaluated  
88 refer to energy balance, antioxidant defences, glutathione balance and oxidative stress  
89 damage.

90

## 91 2. MATERIALS AND METHODS

### 92 2.1 Experimental conditions

93 *Mytilus galloprovincialis* were collected at the Mira Channel (Ria de Aveiro, a coastal  
94 lagoon, northwest of Portugal), in September 2018. After sampling, the specimens (mean length  
95  $6.3\pm 0.4$  cm, mean width  $3.7\pm 0.3$  cm) were placed in aquaria for depuration and acclimation to  
96 laboratory conditions for 7 days. Artificial seawater (salinity  $30 \pm 1$ ), made with artificial salt  
97 (Tropic Marin®SEA SALT from Tropic Marine Center) and deionized water, was used. During  
98 this period the organisms were maintained at  $18^{\circ}\text{C} \pm 1.0$  °C, salinity  $30 \pm 1.0$  and pH  $8.0 \pm 0.1$ ,  
99 resembling conditions at the sampling area during their collection, and kept under continuous  
100 aeration with a 12 h light: 12 h dark photoperiod. During this period seawater was renewed two-  
101 three times per week and organisms were fed with AlgaMac Protein Plus, Aquafauna Bio-  
102 Marine, Inc (150000 cells/animal).

103 After acclimation, organisms were distributed in different aquaria, to test the impacts  
104 induced by the exposure to Triclosan (TCS) and Diclofenac (DIC) under different salinities,  
105 testing the following 9 conditions: **CTL**, 0 µg/L PPCPs at salinities 25, 30 and 35; **TCS**, 1 µg/L of  
106 TCS and 0 µg/L of DIC at salinities 25, 30 and 35; **DIC**, 1 µg/L DIC of and 0 µg/L of TCS at  
107 salinities 25, 30 and 35.

108 TCS (CAS: 3380-34-5. REF: PHR1338) and DIC (CAS: 15307-86-5. REF: D6899) were  
109 purchase from Sigma-Aldrich and used to prepare the stock solutions.

110 Per condition four glass containers were used, with three organisms in each container  
111 filled with 3 L of seawater. During the experiment, immediately after water renewal and spiking,  
112 water samples from the control and contaminated tanks were collected to ensure nominal  
113 chemical concentration. At the end of the exposure period (28 days) TCS and DIC  
114 concentrations were also determined in whole soft tissue of mussels.

115 During the 28-day experimental period mussels were maintained at constant aeration.  
116 Temperature and salinity were checked daily and readjusted if necessary. Along the exposure  
117 period mussels were also fed with the same commercial preparation as described before at a  
118 periodicity of three times per week, and seawater was renewed weekly, after which the  
119 experimental conditions were re-established, including seawater parameters and TCS and DIC

120 concentrations to ensure the same exposure conditions during the experiment. No mortality was  
121 observed throughout the experiment.

122 After exposure, organisms were individually frozen and manually homogenized with a  
123 mortar and a pestle under liquid nitrogen. Each homogenized organism was divided into  
124 aliquots of 0.5 g, which were used for biomarker analyses and quantification of TCS and DIC  
125 concentrations. The whole soft tissue of each organism was used with the aim to assess the  
126 general status of each specimen and not the response of a given organ or tissue.

127

128

## 129 **2.2 Triclosan and Diclofenac concentrations**

130 TCS in water samples was extracted with C-18 SPE cartridges (HYPERSEP, 6 mL, 1 g,  
131 Thermo Scientific) in a SPE manifold (Thermo Scientific) under vacuum after acidification (pH 3)  
132 of samples (Cheng et al., 2011). Dichloromethane eluates were then dried and dissolved in  
133 toluene for GC analysis. The QuEChERS (Agilent Technologies) method (Schmidt and Snow,  
134 2016) was used for extracting TCS in soft tissues.

135 The extracts were then analyzed with a GC-MS method (Tohidi and Cai, 2015).  
136 Calibration curve was performed with TCS standard (Sigma-Aldrich) in dichloromethane. All  
137 samples were analyzed by the use of a GC Trace 1300 (Thermo Scientific) coupled to a TriPlus  
138 RSH autosampler and a triple quadrupole mass spectrometer TSQ Duo with an electron impact  
139 ionization source (EI) (Thermo Scientific); the column was an Agilent DB-5MS. The detection  
140 limit (LOD), calculated as a signal-to-noise ratio of 3:1, was 0.008 µg/L for water samples and  
141 0.13 ng/g d.w. for soft tissues. The percent of recovery was >91 for water samples and >89 for  
142 soft tissues (see Table 1).

143 High performance liquid chromatography-ultraviolet (HPLC-UV) detection was used for  
144 the determination of DIC concentrations both in water and soft tissues. The method  
145 of Madikizela and Chimuka (2017) was used for the analysis of water samples that were  
146 extracted by solid phase extraction (Oasis MAX 6cc 150 mg solid-phase extraction cartridges,  
147 Waters). The pH of water samples was adjusted to 2.5, then 100 mL of each sample was  
148 loaded onto a pre-conditioned cartridge. The SPE cartridge was rinsed with methanol: water  
149 (10:90%, v:v) prior to sequential elution of DIC with 2 mL methanol, followed by 2 mL methanol



150 and acetic acid (90:10, v:v) and 2 mL of 2% (v:v) formic acid diluted using a mixture of methanol  
151 and acetic acid (40:60, v:v). The analytes from the SPE cartridge were quantified using HPLC.  
152 DIC in soft tissues samples were analyzed by using the method of (Gatidou et al., 2007). Whole  
153 tissue samples of 3 individuals (1.5 g) were dehydrated and sonicated at 50 °C for 30 min using  
154 16 ml of mixture of methanol (10 ml) and Milli-Q water (6 ml) as the extraction solvent. The  
155 supernatant was collected after centrifugation and diluted to a final volume of 100 mL using  
156 Milli-Q grade water and then purified with solid phase extraction as reported for water samples.

157 A Series 200 PerkinElmer gradient pump coupled to a Series 200 PerkinElmer variable  
158 UV detector, which was set at 280 nm, was used as HPLC system. The mobile phase consisted  
159 of acetonitrile and 0.2% formic acid in water, at a ratio of 60:40 (v:v). The reversed-phase  
160 column was a Haisil, LC column (5 µm, 150x4.60 mm, Higgins). The column was kept at room  
161 temperature. Turbochrome software was used for data processing. The DIC recovery was  
162 >80% for water samples and >77% for soft tissues. The detection limit, calculated as a signal-  
163 to-noise ratio of 3:1, was 0.10 µg/L for water samples and 5 ng/g d.w. for soft tissues (see Table  
164 1).

165

### 166 **2.3 Biochemical parameters**

167 After 28 days exposure, mussels used for biomarker analysis (2 per replicate, 8 per  
168 condition) were treated with specific buffers according each biomarker determination at the  
169 proportion 1:2 w/v (Almeida et al., 2014; Andrade et al., 2018). For each biochemical  
170 determination, 0.5 g fresh weight (FW) of soft tissue per organism was used. For each condition  
171 indicators of metabolic capacity (electron transport system activity, ETS), energy reserves (total  
172 protein content, PROT; glycogen content, GLY), oxidative stress (lipid peroxidation levels, LPO;  
173 ratio reduced (GSH) / oxidized (GSSG) glutathione; superoxide dismutase activity, SOD;  
174 catalase activity, CAT; glutathione peroxidase activity, GPx; glutathione S-transferases activity,  
175 GSTs) were determined. The samples were homogenized for 15 s at 4 °C using a small bead  
176 mill (TissueLyser II, QIAGEN) and centrifuged for 20 min at 10 000 g (or 3000 g for ETS) at 4 °C  
177 (Thermo Scientific Heraeus® Multifuge® 3SR). Supernatants were either stored at -80 °C or  
178 immediately used. All biochemical parameters were performed in duplicate as described in

179 detailed elsewhere (Almeida et al., 2014; Andrade et al., 2018). All measurements were done  
180 using a microplate reader (Biotek).

181

## 182 **2.4 Data analysis**

183 Concentrations of TCS and DIC in the water and mussels soft tissues as well as the  
184 biochemical parameters (ETS, GLY, PROT, SOD, CAT, GPx, GSTs, LPO, GSH/GSSG) were  
185 separately submitted to a non-parametric permutational analysis of variance (PERMANOVA  
186 Add-on in Primer v7). A one-way hierarchical design was followed in this analysis. The pseudo-  
187 F p-values in the PERMANOVA main tests were evaluated in terms of significance. When  
188 significant differences were observed in the main test pairwise comparisons were performed.  
189 Values lower than 0.05 were considered as significantly different. The null hypotheses tested  
190 were: for each PPCP, no significant differences exist in terms of concentration among salinity  
191 levels, both for water and tissue samples; for each biomarker, no significant differences existed  
192 among the 9 exposure conditions (CTL sal 25, TCS sal 25, DIC sal 25, CTL sal 30, TCS sal 30,  
193 DIC sal 30, CTL sal 35, TCS sal 35, DIC sal 35). For each PPCP and sample type (water or  
194 tissue), differences among salinities were represented with different letters in the Table. For  
195 each salinity level (25, 30 and 35), significant differences among exposure concentrations (0  
196 µg/L-CTL, 1 µg/L TCS or 1 µg/L DIC) were represented with different letters in the graphs. For  
197 each exposure concentration (0 µg/L-CTL, 1 µg/L TCS or 1 µg/L DIC), differences among  
198 salinity levels (25, 30 and 35) were represented with *p*-values in a table format and the  
199 significant ones indicated in bold.

200

201

## 3. RESULTS

### 3.1 Triclosan and Diclofenac concentrations

In Table 1 the water and tissue concentrations for both PPCPs are presented. Water concentration of TCS immediately after spiking ranged from 0.64 to 0.92 regardless of the salinity condition. Nonetheless, the tissue bioaccumulation of TCS significantly differed between the lower salinity (25) and the other conditions (30 and 35). TCS was about 20-fold higher at the hypo saline condition. DIC concentration in water was more stable 0.85-0.95 at the 3 salinity conditions while bioaccumulated DIC was significantly higher at the extreme salinities (45.6-51.3 ng/g d.w) in relation to the control one ( $28.5 \pm 14.6$  ng/g d.w).

### 3.2 Biochemical responses

#### 3.2.1 Metabolic capacity and energy reserves

ETS activity in control and exposed mussels is illustrated in Figure 1A. At salinity 25 mussels exposed to DIC significantly increased ETS activity in respect to control organisms. By contrast, at salinities 30 and 35 contaminated mussels tended to decrease ETS activity in comparison to unexposed specimens, with significantly lower values in mussels exposed to TCS and DIC at salinity 30 and mussels exposed to TCS at salinity 35. ETS values observed in unexposed mussels were significantly lower at salinity 25 compared to salinities 30 and 35 (Table 2).

GLY content (Figure 1B) increased in mussels contaminated with TCS and DIC at salinity 30 and mussels exposed to DIC at salinity 35 in respect to their controls. Except between CTL25 and CTL35, GLY values varied significantly among salinities for non-contaminated mussels, with significantly lower values at salinity 30 (Table 2).

At salinities 25 and 30 mussels exposed to DIC showed significantly higher PROT content in comparison to mussels exposed to TCS or unexposed (Figure 1C). At salinity 35 mussels exposed to TCS showed significantly lower PROT content in comparison to CTL organisms. PROT values were significantly different between control mussels maintained at salinities 25 and 35 as well as between those at salinities 30 and 35 (Table 2).

#### 3.2.2 Antioxidant and biotransformation defences

232 SOD activity (Figure 2A) between non-contaminated (CTL) and contaminated mussels  
233 (TCS and DIC) was only enhanced at the control salinity (30). SOD activity also varied  
234 significantly among salinities for unexposed mussels, with the lowest values attained in mussels  
235 held at salinity 30 (Table 2).

236 CAT activity (Figure 2B) was enhanced regardless of the salinity tested, in mussels  
237 exposed to TCS (at salinity 30 and 35) and DIC (at 25 and 30) in comparison to their controls.  
238 CAT activity in unexposed organisms also varied significantly as a function of salinity, with the  
239 lowest value also at control salinity (30). Organisms exposed to DIC showed significantly higher  
240 values at salinity 25 compared to salinities 30 and 35 (Table 2).

241 GPx activity (Figure 2C) was strongly enhanced at all salinities in mussels exposed to  
242 TCS but also DIC in respect to their controls, with higher values in contaminated mussels  
243 maintained at salinity 25. Unexposed mussels at salinity 25 showed significantly lower GPx  
244 values than non-contaminated organisms at salinities 30 and 35. Mussels exposed to TCS and  
245 salinity 35 showed significantly lower GPx values than organisms at salinities 25 and 30 (Table  
246 2).

247 GSTs activity (Figure 3), contaminated mussels tended to increase their activity in  
248 comparison to CTL organisms, with significantly higher values in organisms exposed to DIC (at  
249 salinity 25) and to TCS and DIC (at salinity 30). Unexposed mussels at salinity 35 showed  
250 significantly higher GSTs values than non-contaminated organisms at salinities 25 and 30. A  
251 similar trend was observed in organisms exposed to TCS while exposure to DIC was  
252 responsible for higher GSTs values at salinity 30 in respect to the others (Table 2).

253

### 254 *3.2.3 Indicators of cellular damage*

255 LPO levels (Figure 4A) in mussels at salinity 25 and exposed to TCS and DIC  
256 significantly increased in comparison to non-contaminated organisms. By contrast at salinities  
257 30 and 35 contaminated mussels tended to decrease their LPO levels in comparison to non-  
258 contaminated mussels, with significantly lower values in mussels exposed to TCS and DIC at  
259 salinity 30 and mussels exposed to DIC at salinity 35. Salinity levels and PPCP exposures  
260 affected LPO levels in mussels, with the exception for organisms exposed to DIC at salinities 30

261 and 35 where no significant differences were observed between mussels exposed to these  
262 conditions (DIC 30 vs DIC 35) (Table 2).

263           Regardless of salinity, exposure to PPCPs caused significantly lower GSH/GSSG  
264 values in comparison to their respective controls, with the highest reduction attained after  
265 exposure to DIC (Figure 4B). Unexposed mussels showed significantly higher GSH/GSSG ratio  
266 at salinity 35 in comparison to salinity 30. Less evident although still significant, mussels  
267 exposed to TCS and salinity 30 showed significantly lower ratio values than those at salinities  
268 25 and 35. Exposure to DIC caused significant lower GSH/GSSG values in mussels at salinity  
269 25 in respect to those held at salinity of 35 (Table 2).

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#### 4. DISCUSSION

273

274 Analysed water concentrations matched the nominal ones (1 µg/L) soon after the spiking  
275 for Triclosan (TCS) as well as Diclofenac (DIC) regardless of water salinity. However, TCS  
276 levels were more variable than those corresponding to DIC (Table 1). So far, we are not aware  
277 of any study confirming expected nominal concentrations of TCS with real ones, although 1 µg/L  
278 was already adopted in bivalve studies (Binelli et al., 2011; Matozzo et al., 2012). Matching real  
279 and nominal water concentrations were also confirmed in other studies with bivalves after DIC  
280 exposures (Fontes et al., 2018; Schmidt et al., 2014).

281

282 Bioaccumulated TCS in whole mussels tissue was highly dependent on the salinity of the  
283 medium. However, even when more abundant, TCS concentration was lower than in other  
284 studies using comparable water exposures and times (Gatidou et al., 2010; Riva et al., 2012).  
285 By contrast tissue concentration of DIC was more similar at all salinities and in line or higher  
286 than levels reported in bivalves in other lab experiments using comparable nominal water  
287 concentrations (Ericson et al., 2010; Mezzelani et al., 2016; Mezzelani et al., 2018).

288

289 Salinity influences sorption and therefore bioavailability of hydrophobic chemicals,  
290 including TCS (Wu et al., 2016; Xie et al., 2008). In this case, the lower salinity could have  
291 determined the higher bioavailability of TCS that explains its higher bioaccumulation in mussels  
292 exposed to the lowest salinity. Moreover, bioaccumulation and toxicity in bivalves do not always  
293 parallel each other since metabolism of parental compounds take place and in some cases  
294 despite the absence of the chemical, the toxic responses have already taken place (Kock et al.,  
295 2010). In fact, TCS is a compound easily metabolised to methyl-TCS in the presence of mussels  
296 in the medium (Kookana et al., 2013). The explanation for the discrepancy in bioaccumulation at  
297 the lowest salinity in respect to the others tested could partly been justified by the salting-out  
298 effect although the toxicological consequences of the exposures were similar at all salinities.  
299 Since photodegradation appeared to be one of the main degradation route in aquatic  
300 environments (Fang et al., 2010), it could be also hypothesized that observed toxicity might be  
301 due to the formation of by-products such as 2,8-dichlorodibenzo-*p*-dioxin, 2,4-dichlorophenol  
302 and possibly dichlorohydroxydibenzofuran. Also Mezcua et al. (2004) detected  
303 photodegradation of TCS; in particular, under natural sunlight the disappearance of TCS  
304 together with the appearance of 2,7/2,8-dibenzodichloro-*p*-dioxin (as the major product of

303 photolysis) was observed. As described by Sanchez-Prado et al. (2006) together with Kanetoshi  
304 et al. (1988), the photodegradation of TCS and formation of chlorinated compounds (particularly  
305 2,8-dichlorodibenzo-*p*-dioxin) occurs over a wide range of pH levels (3.0–9.0), with the rate of  
306 formation being faster at basic pH. In what concerns to DIC, lower concentrations observed in  
307 mussels exposed to salinity 30 may result from higher GSTs detoxification activity of these  
308 enzymes at this condition in comparison to salinities 25 and 30. Similarly, Quinn et al. (2011)  
309 showed an increase in GSTs activity along the increase concentration of DIC in the mussel  
310 *Dreissena polymorpha*.

311 In order to assess if PPCPs levels in mussels tissues were able to modify their responses  
312 in a salinity-dependent manner, biomarkers related to energy balance, oxidative defences and  
313 stress damage were evaluated both in non-contaminated and contaminated organisms  
314 maintained at each different salinity.

315 At control (unexposed) conditions, salinity alone was responsible for the modulation of  
316 several biochemical responses, especially at the lowest salinity tested (25) with decreased  
317 energetic metabolism and increased energy reserves, as well as an increase in antioxidant  
318 defences that were effective in preventing cellular damages. Similarly, higher energy reserves  
319 content was also observed in *M. galloprovincialis* exposed for 28 days to salinity 14 (Freitas et  
320 al., 2017b). A previous study with two euryhaline bivalves *Corbicula fluminea* and *Scrobicularia*  
321 *plana* acclimatized to their respective extreme salinity tolerances for 7 days had also an impact  
322 on their metabolic reserves and GSTs activity although no impact on ETS (Bertrand et al.,  
323 2017). Also, the increased antioxidant defence observed at the lowest salinity observed in *M.*  
324 *galloprovincialis* in the present study is well in agreement with observations in other bivalve  
325 species (Bertrand et al., 2017; Carregosa et al., 2014).

326 Our findings further revealed that under control salinity the impacts of PPCPs were  
327 noticed, especially with the reduction of mussel's metabolism and increased energy reserves  
328 content, increased antioxidant and biotransformation enzymes activities and lower LPO.  
329 Although the magnitude of ROS production was not measured in the present study, all the  
330 antioxidant defences considered (SOD, CAT, GPx and GSTs) were enhanced at the natural  
331 salinity condition (30) which clearly suggests ROS production. Nevertheless, the negative  
332 consequences of ROS, measured as LPO levels, did not prove the existence of any damage,

333 which probably results from the combined reduced metabolism (lower ETS) and the efficient  
334 action of the antioxidant responses in comparison to control organisms. Moreover, a strong  
335 decrease in the ratio GSH/GSSG was observed in mussels exposed to TCS and DIC in  
336 comparison to unexposed ones, revealing a general increase of the oxidative status in *M.*  
337 *galloprovincialis* exposed to those PPCPs. This ratio is considered as a reliable biomarker for  
338 monitoring the effects of xenobiotics (van der Oost et al., 2003). In the present study the ratio  
339 GSH/GSSG was also consistently lower under PPCPs exposures. In fact, the involvement of  
340 GSH was demonstrated in a field study under the influence of a salinity gradient (from 35 to 43),  
341 in which the marine limpet *Patella rustica* enhanced all GSH dependent enzymatic responses  
342 (GSTs, GPx and GR) (Benaissa et al., 2017). The key role of GSH alerts for the consequences  
343 that the low GSH/GSSG balance revealed under PPCPs exposures may pose to the bivalves  
344 when facing stronger salinity gradients or other challenging conditions. Other studies with  
345 bivalves also support the use of the GSH/GSSG ratio in the assessment of impacts caused by  
346 pollutants (Almeida et al., 2015; Coppola et al., 2017; De Marchi et al., 2018b; Grintzalis et al.,  
347 2012; Xia et al., 2016).

348

349 In comparison to salinity control (30), at salinities 25 and 35 similar biochemical  
350 responses were observed, namely in terms of energy reserves and defence mechanisms. The  
351 present results further highlight that mussels responses to PPCPs under the different salinities  
352 were less significant than the responses due solely to salinity, especially in terms of energy  
353 metabolism. Similarly, Munari et al. (2018) demonstrated that the biochemical parameters  
354 measured in *Ruditapes philippinarum* and *M. galloprovincialis* were more influenced by the  
355 reduced pH than by DIC or the pH\*contaminant interaction. Although under both extreme  
356 salinities (25 and 35) our findings evidenced a general increase on antioxidant defences, the  
357 results obtained showed that the GPx activity varied more specifically to TCS while CAT  
358 responded more particularly to DIC. Since CAT is only involved in the transformation of H<sub>2</sub>O<sub>2</sub>  
359 produced by SOD into H<sub>2</sub>O and O<sub>2</sub> while GPx reduces other peroxides in addition to H<sub>2</sub>O<sub>2</sub> and it  
360 also involved in the transformation of 2 GSH molecules to 1 GSSG, we may hypothesise that  
361 the use of GPx is more energy-consuming to the organisms which can led to higher impacts  
362 when in the presence of DIC. Nevertheless, at salinities 25 and 35 the defence mechanisms



363 activated were not sufficient to prevent the occurrence of cellular damage (measured by LPO).  
364 Although no studies are known on the effects of TCS and DIC under stressful salinity  
365 conditions, similar impacts were observed by other authors that exposed bivalves, under control  
366 salinity conditions, to the chemicals TCS and/or DIC. In particular, oxidative stress, evidenced  
367 by increased antioxidant and biotransformation enzymes activity as well as cellular damage,  
368 was also observed in bivalves exposed to TCS and DIC, namely in *R. philippinarum* and *M.*  
369 *galloprovincialis* exposed to DIC (Gonzalez-Rey and Bebianno, 2014; Gonzalez-Rey and  
370 Bebianno, 2014; Mezzelani et al., 2016; 2018; Munari et al., 2018); *Mytilus* spp. exposed to DIC  
371 (Schmidt et al., 2011); and *Dreissena polymorpha* exposed to DIC (Quinn et al., 2011). Similar  
372 results were also observed for the species mussels *Elliptio complanata* exposed to TCS  
373 (Goodchild et al., 2016) and *Unio tumidus* exposed to TCS (Falfushinskaya et al., 2015),  
374 indicating that the effects of both PPCPs were also observed at freshwater conditions.

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## 5. CONCLUSION

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## Figure captions

Figure 1. A: Electron transport system (ETS) activity, B: Glycogen (GLY) content and C: Protein (PROT) content, in *Mytilus galloprovincialis* under three salinities (25, 30-control and 35) and exposed to Triclosan (TCS) and Diclofenac (DIC) at 1 µg/L each. Values are presented as mean + standard deviation. For each salinity level, significant differences ( $p \leq 0.05$ ) among treatments are represented with different letters.

Figure 2. A: Superoxide dismutase (SOD) activity; B: Catalase (CAT) activity; and C: Glutathione peroxidase (GPx) activity, in *Mytilus galloprovincialis* under three salinities (25, 30-control and 35) and exposed to Triclosan (TCS) and Diclofenac (DIC) at 1 µg/L each. Values are presented as mean + standard deviation. For each salinity level, significant differences ( $p \leq 0.05$ ) among treatments are represented with different letters.

Figure 3. Glutathione S-transferases (GSTs) activity, in *Mytilus galloprovincialis* under three salinities (25, 30-control and 35) and exposed to Triclosan (TCS) and Diclofenac (DIC) at 1 µg/L each. Values are presented as mean + standard deviation. For each salinity level, significant differences ( $p \leq 0.05$ ) among treatments are represented with different letters.

Figure 4. A: Lipid peroxidation (LPO) levels; and B: reduced/oxidised glutathione (GSH/GSSG) ratio, in *Mytilus galloprovincialis* under three salinities (25, 30-control and 35) and exposed to Triclosan (TCS) and Diclofenac (DIC) at 1 µg/L each. Values are presented as mean + standard deviation. For each salinity level, significant differences ( $p \leq 0.05$ ) among treatments are represented with different letters.

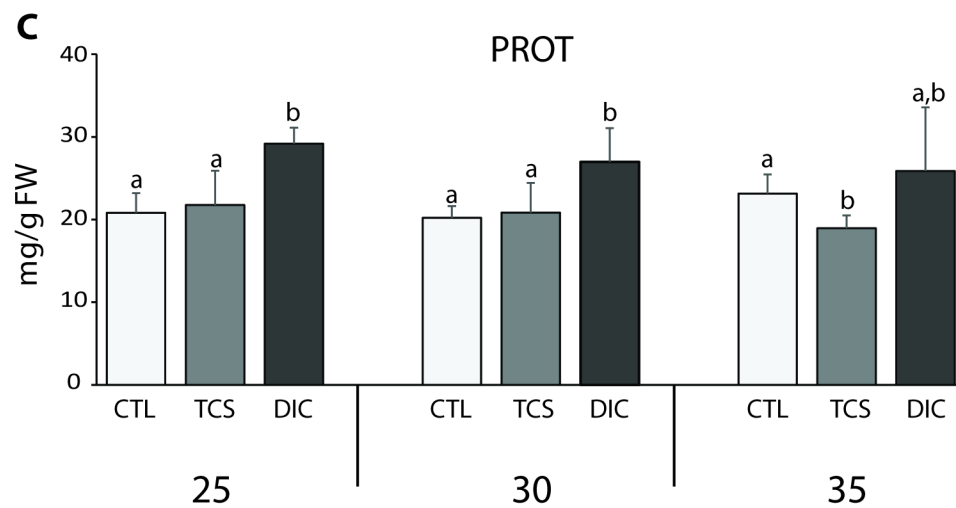
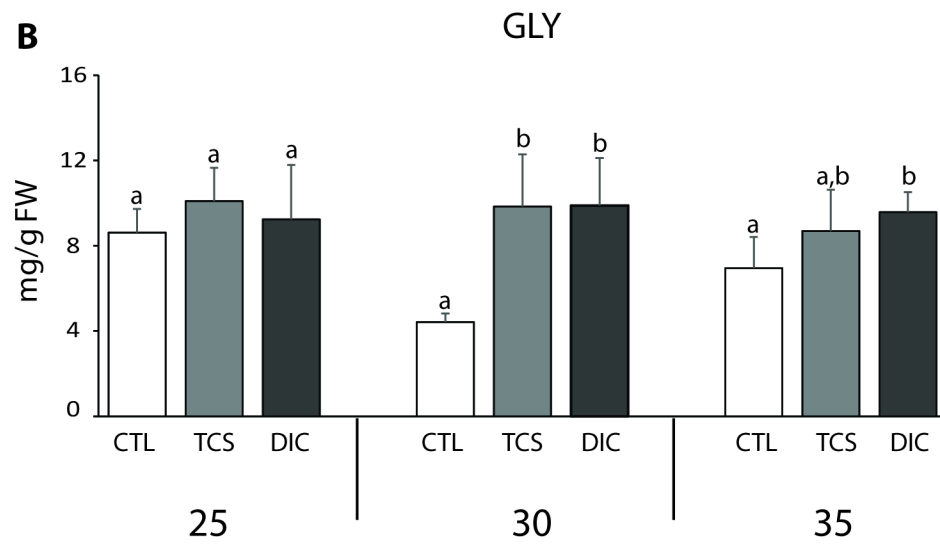
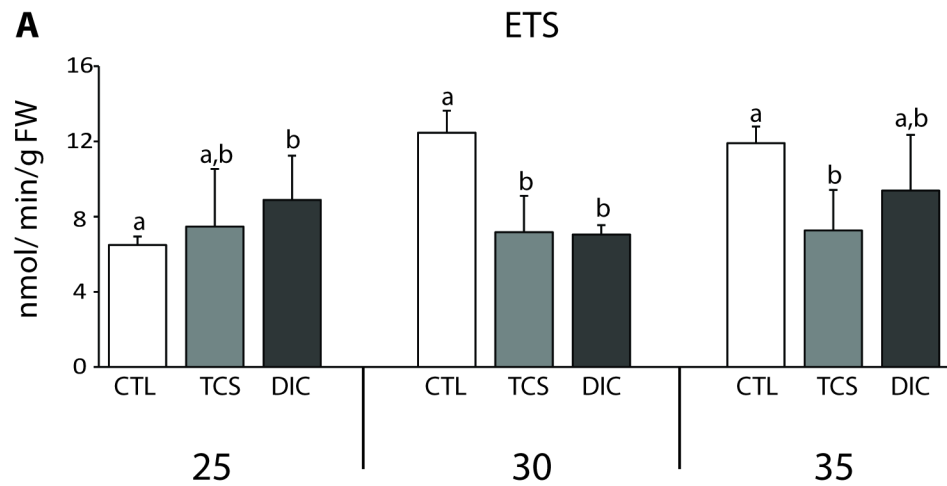


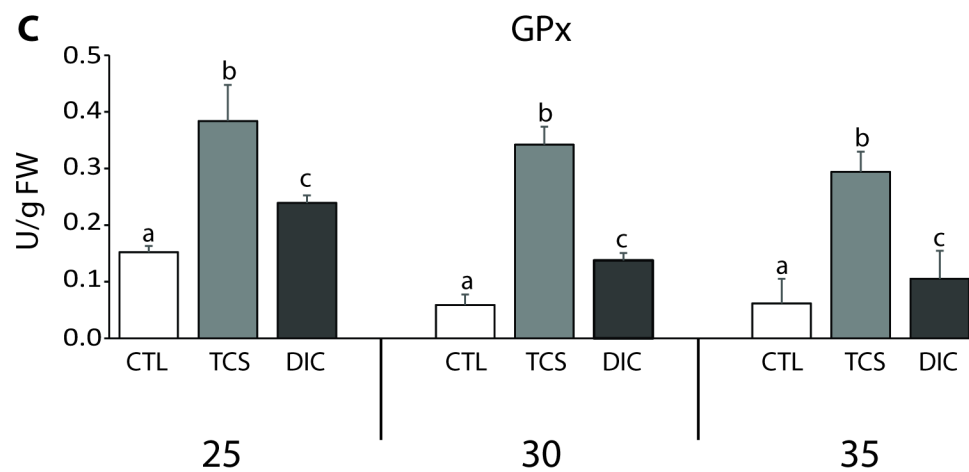
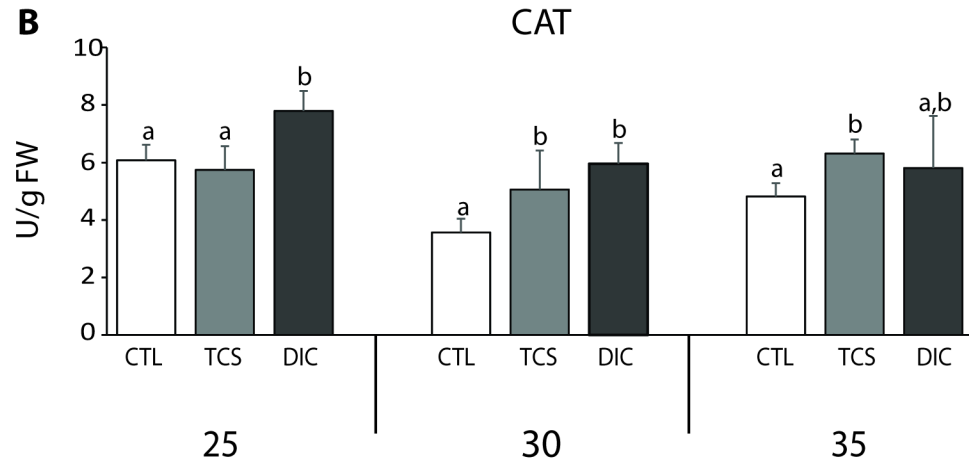
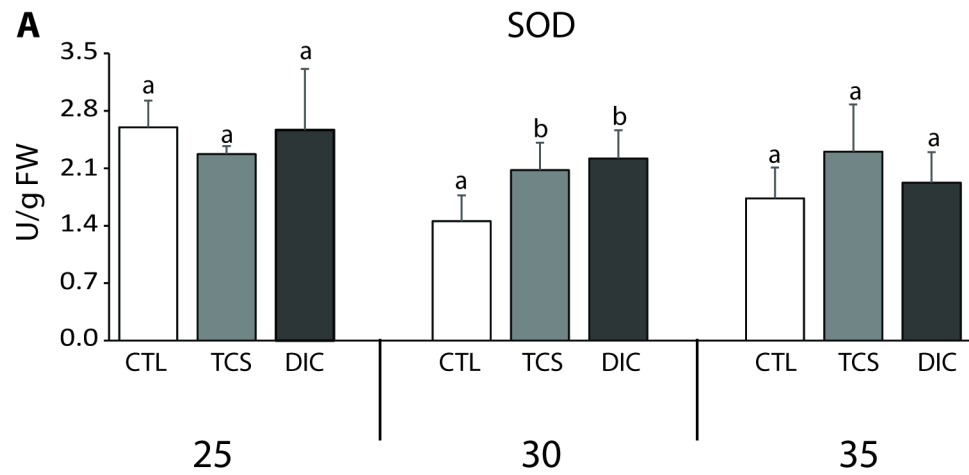
Table 1. Water and tissue concentrations of Triclosan and Diclofenac. Water samples were analysed soon after spiking while tissue samples were analysed after 28-days exposure period. Water and tissue samples at control conditions presented PPCPs lower than the LOQ. LOD: limit of detection; LOQ: limit of quantification. Different letters represent significant differences among salinity levels, for each PPCP (Triclosan or Diclofenac) and sample type (water or tissue).

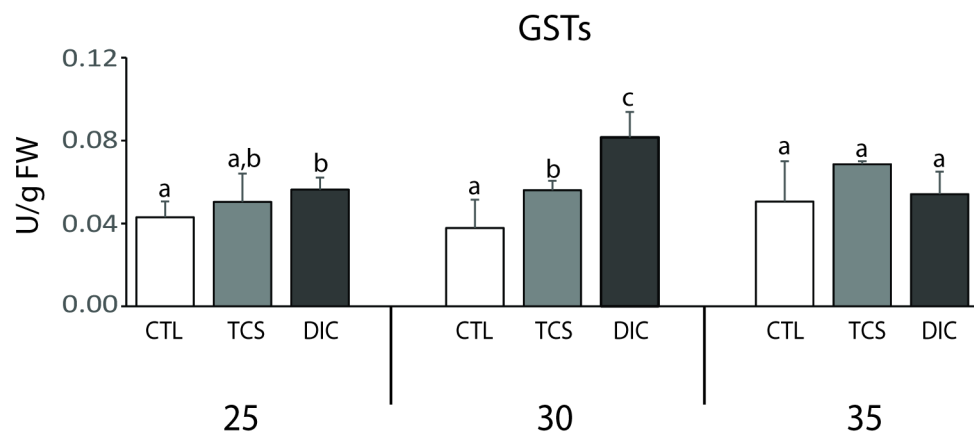
<b>TRICLOSAN</b>			
Condition	Control	Water	Tissue
T 17°C		LOD water: 0.008 µg/L LOQ water: 0.025 µg/L	LOD tissue: 0.13 ng/g d.w LOQ tissue: 0.4 ng/g d.w
Salinity			
25	<LOQ	0.74 ± 0.10 <sup>a,b</sup>	10.5 ± 1.78 <sup>a</sup>
30	<LOQ	0.85 ± 0.07 <sup>b</sup>	0.55 ± 0.11 <sup>b</sup>
35	<LOQ	0.71 ± 0.07 <sup>a</sup>	0.44 ± 0.05 <sup>b</sup>
<b>DICLOFENAC</b>			
Condition	Control	Water	Tissue
T 17°C		LOD water: 0.10 µg/L LOQ water: 0.30 µg/L	LOD tissue: 5 ng/g d.w LOQ tissue: 15 ng/g d.w
Salinity			
25	<LOQ	0.96 ± 0.11 <sup>a</sup>	47.0 ± 1.41 <sup>a</sup>
30	<LOQ	0.96 ± 0.05 <sup>a</sup>	28.5 ± 14.6 <sup>b</sup>
35	<LOQ	0.96 ± 0.01 <sup>a</sup>	40.0 ± 11.31 <sup>a,b</sup>

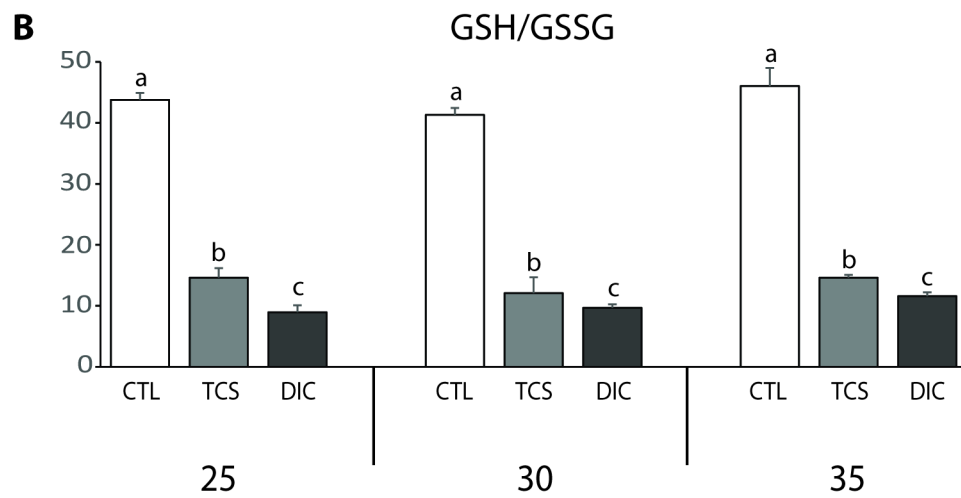
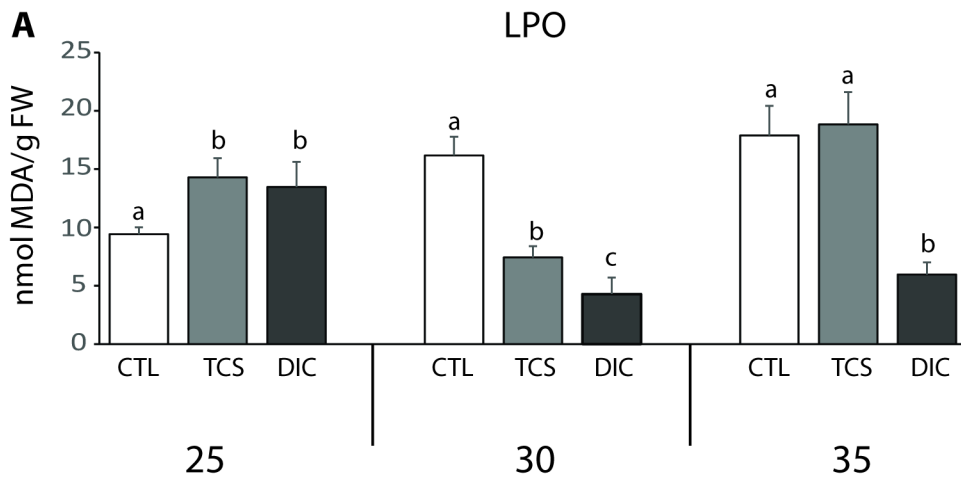
Table 2. Pairwise comparisons ( $p$ -values) between salinity levels for each tested condition (0  $\mu\text{g/L}$ -CTL, 1  $\mu\text{g/L}$  TCS or 1  $\mu\text{g/L}$  DIC) and biochemical parameter (electron transport system activity, ETS; glycogen content, GLY; protein content, PROT; superoxide dismutase activity, SOD; catalase activity, CAT; glutathione peroxidase activity, GPx; glutathione-S-transferases activity, GSTs; lipid peroxidation levels, LPO; ratio between reduced (GSH) and oxidized (GSSG) glutathione). Significant differences ( $p \leq 0.05$ ) are highlighted in bold.

	ETS	GLY	PROT	SOD	CAT	GPx	GSTs	LPO	GSH/GSSG
CTL 25 vs CTL 30	<b>0.0001</b>	<b>0.0001</b>	0.6129	<b>0.0003</b>	<b>0.0001</b>	<b>0.0001</b>	0.4323	<b>0.0001</b>	0.4493
CTL 25 vs CTL 35	<b>0.0001</b>	0.0573	<b>0.0220</b>	<b>0.0059</b>	<b>0.0013</b>	<b>0.0079</b>	<b>0.0045</b>	<b>0.0001</b>	0.5271
CTL 30 vs CTL 35	0.4477	<b>0.0002</b>	<b>0.0035</b>	<b>0.0408</b>	<b>0.0008</b>	0.4468	<b>0.0103</b>	<b>0.0496</b>	<b>0.0001</b>
TCS 25 vs TCS 30	0.8353	0.8390	0.6843	0.1988	0.3227	0.1808	0.3600	<b>0.0001</b>	<b>0.0424</b>
TCS 25 vs TCS 35	0.8957	0.1992	0.1570	0.9011	0.1736	<b>0.0119</b>	<b>0.0089</b>	<b>0.0057</b>	0.8952
TCS 30 vs TCS 35	0.9399	0.3898	0.2617	0.4170	0.0570	<b>0.0352</b>	<b>0.0002</b>	<b>0.0001</b>	<b>0.0192</b>
DIC 25 vs DIC 30	0.0949	0.6527	0.2504	0.3223	<b>0.0017</b>	0.1271	<b>0.0009</b>	<b>0.0001</b>	0.2602
DIC 25 vs DIC 35	0.8179	0.7666	0.3312	0.0893	<b>0.0330</b>	0.0642	0.6795	<b>0.0003</b>	<b>0.0109</b>
DIC 30 vs DIC 35	0.2366	0.7590	<b>0.7696</b>	0.1778	0.8497	0.1477	<b>0.0020</b>	0.1464	0.0761









- Mussels lowered their metabolic rate after drug exposures at control salinity
- Mussels increased antioxidant defences when exposed to drugs at all salinities
- GSH/GSSG ratio was consistently reduced when mussels were exposed to TCS and DIC.
- Impacts by TCS and DIC affected particularly GPx and CAT activities, respectively.
- Salinity alone induced greater metabolic and oxidative stress impacts than PPCPs

ACCEPTED MANUSCRIPT