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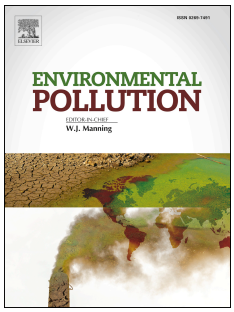

Does salinity modulates the response of *Mytilus galloprovincialis* exposed to triclosan and diclofenac?

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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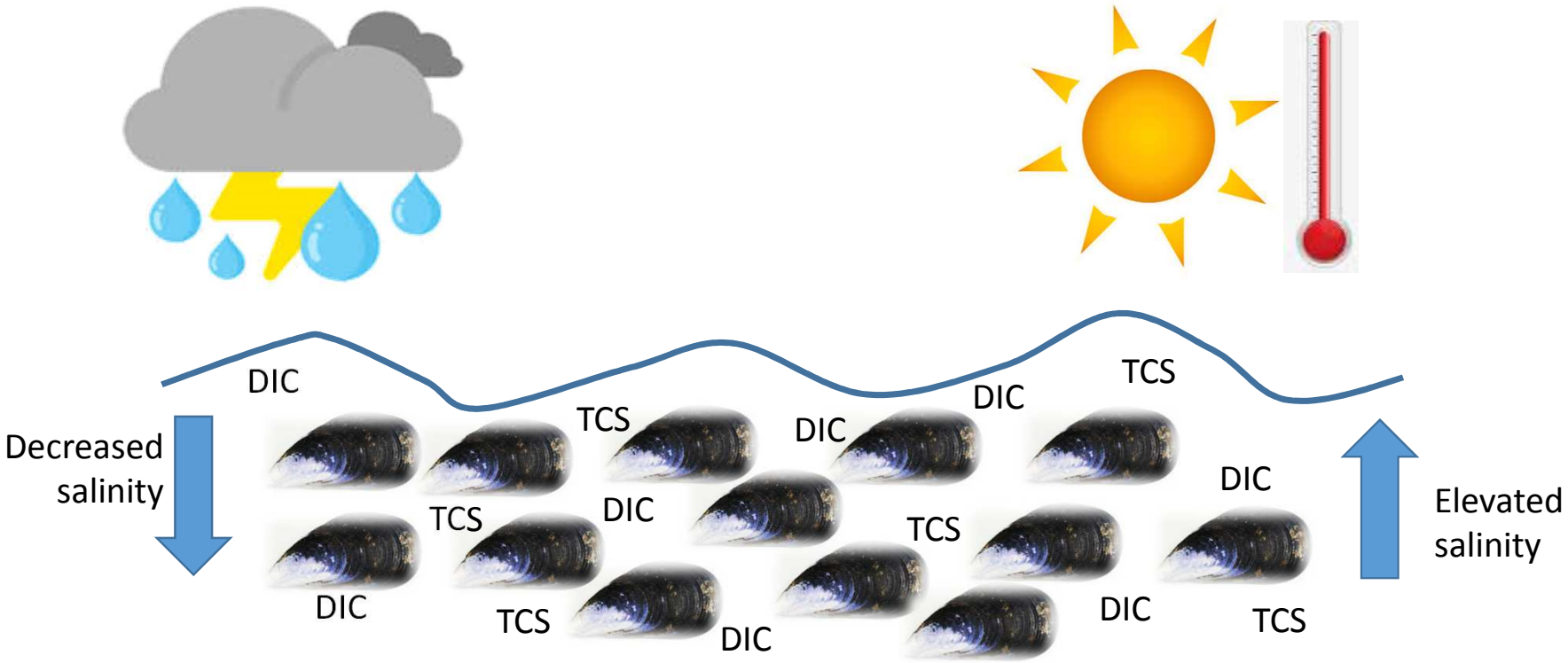

- Mussels lowered their metabolic rate after drug exposures at control salinity
- Mussels increased antioxidant defences when exposed to drugs at all salinities
- GSH/GSSG ratio was consistently reduced when mussels were exposed to TCS and DIC.

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

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Abstract

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

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

1. INTRODUCTION

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

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

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

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

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

2. MATERIALS AND METHODS

2.1 Experimental conditions

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

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

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

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

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

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

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

2.2 Triclosan and Diclofenac concentrations

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

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

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

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

A Series 200 PerkinElmer gradient pump coupled to a Series 200 PerkinElmer variable UV detector, which was set at 280 nm, was used as HPLC system. The mobile phase consisted of acetonitrile and 0.2% formic acid in water, at a ratio of 60:40 (v:v). The reversed-phase column was a Haisil, LC column (5 μm, 150x4.60 mm, Higgins). The column was kept at room temperature. Turbochrome software was used for data processing. The DIC recovery was >80% for water samples and >77% for soft tissues. The detection limit, calculated as a signal-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 1).

2.3 Biochemical parameters

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

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

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

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

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

3.2.3 Indicators of cellular damage

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

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

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

4. DISCUSSION

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

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

Salinity influences sorption and therefore bioavailability of hydrophobic chemicals, including TCS (Wu et al., 2016; Xie et al., 2008). In this case, the lower salinity could have determined the higher bioavailability of TCS that explains its higher bioaccumulation in mussels exposed to the lowest salinity. Moreover, bioaccumulation and toxicity in bivalves do not always parallel each other since metabolism of parental compounds take place and in some cases despite the absence of the chemical, the toxic responses have already taken place (Kock et al., 2010). In fact, TCS is a compound easily metabolised to methyl-TCS in the presence of mussels in the medium (Kookana et al., 2013). The explanation for the discrepancy in bioaccumulation at the lowest salinity in respect to the others tested could partly been justified by the salting-out effect although the toxicological consequences of the exposures were similar at all salinities. Since photodegradation appeared to be one of the main degradation route in aquatic environments (Fang et al., 2010), it could be also hypothesized that observed toxicity might be due to the formation of by-products such as 2,8-dichlorodibenzo-*p*-dioxin, 2,4-dichlorophenol and possibly dichlorohydroxydibenzofuran. Also Mezcua et al. (2004) detected photodegradation of TCS; in particular, under natural sunlight the disappearance of TCS 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_2O_2
359 produced by SOD into H_2O and O_2 while GPx reduces other peroxides in addition to H_2O_2 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

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

5. CONCLUSION

The present results clearly revealed metabolic and oxidative stress impacts of both TCS and DIC in *M. galloprovincialis*, regardless the salinity tested. In fact, salinity changes alone were responsible for more metabolic and oxidative parameter responses in mussels than the PPCPs themselves. DIC showed preferentially enhanced CAT activity while TCS strongly increased GPx activity and both PPCPs caused enhanced GSTs activities. Damage measured as increased LPO levels was evident only at the lowest salinity while the GSH/GSSG balance was the parameter more consistently affected by salinity changes and PPCPs exposures.

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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).

TRICLOSAN

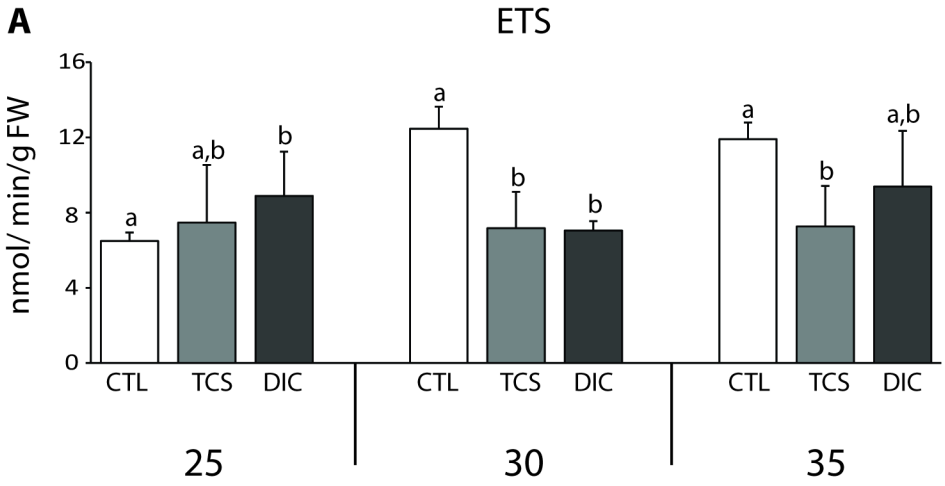
Condition	**Control**	**Water**	**Tissue**
T 17ºC Salinity		LOD water: 0.008 µg/L LOQ water: 0.025 µg/L	LOD tissue: 0.13 ng/g d.w LOQ tissue: 0.4 ng/g d.w
25	<LOQ	$0.74 \pm 0.10^{a,b}$	10.5 ± 1.78^{a}
30	<LOQ	0.85 ± 0.07^{b}	0.55 ± 0.11^{b}
35	<LOQ	0.71 ± 0.07^{a}	0.44 ± 0.05^{b}

DICLOFENAC

Condition	**Control**	**Water**	**Tissue**
T 17ºC Salinity		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
25	<LOQ	0.96 ± 0.11^{a}	47.0 ± 1.41^{a}
30	<LOQ	0.96 ± 0.05^{a}	28.5 ± 14.6^{b}
35	<LOQ	0.96 ± 0.01^{a}	$40.0 \pm 11.31^{a,b}$

Table 2. Pairwise comparisons (*p*-values) between salinity levels for each tested condition (0 µg/L-CTL, 1 µg/L TCS or 1 µg/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≤0.05) are highlighted in bold.

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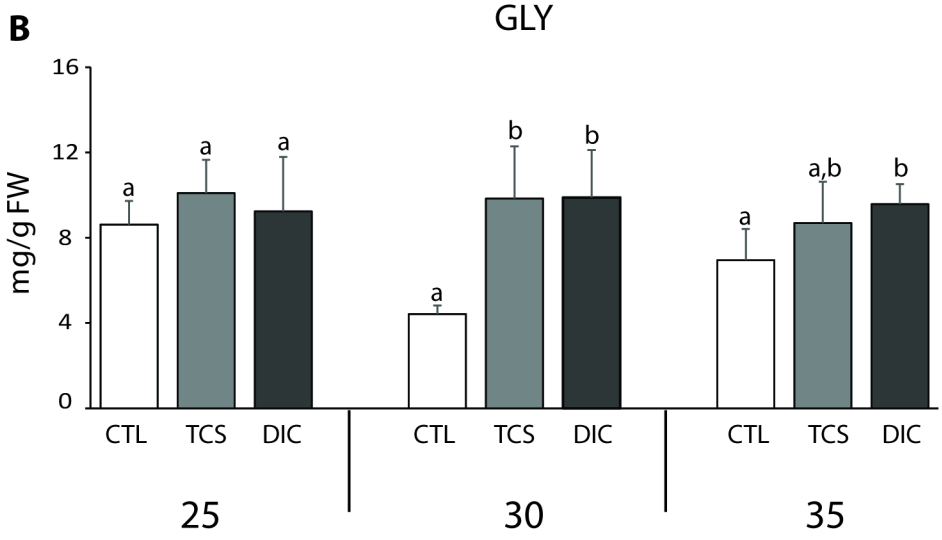
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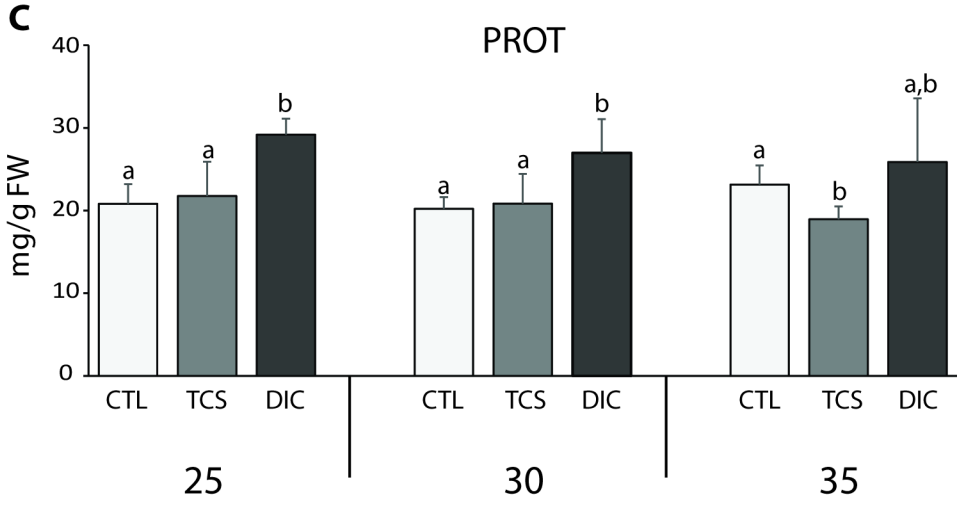
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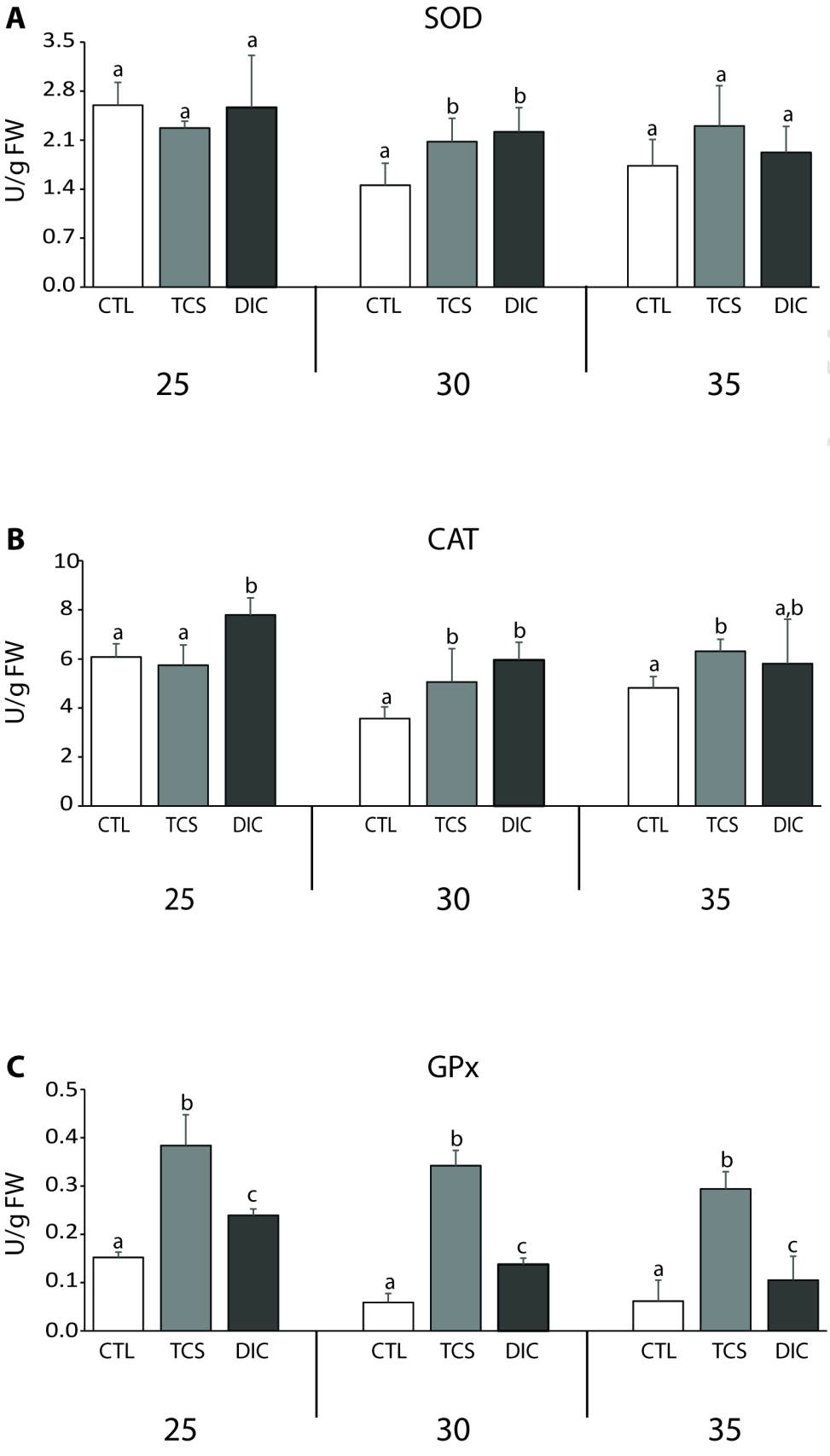
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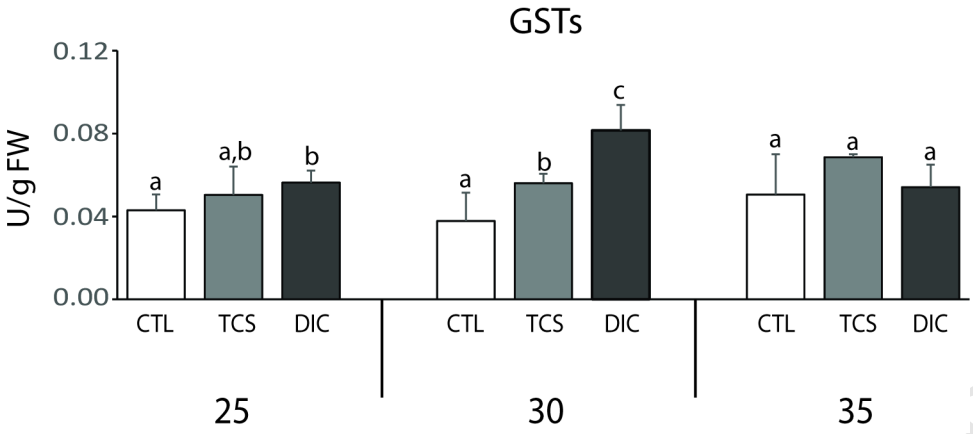

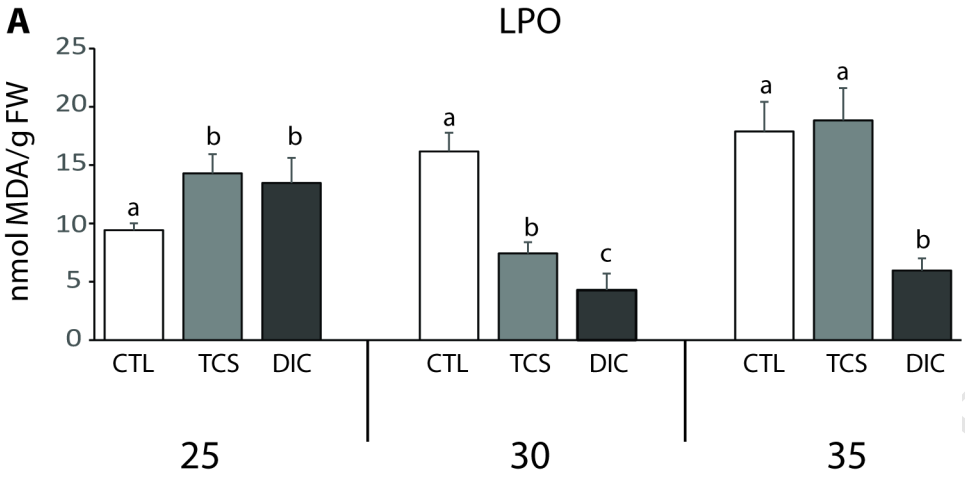

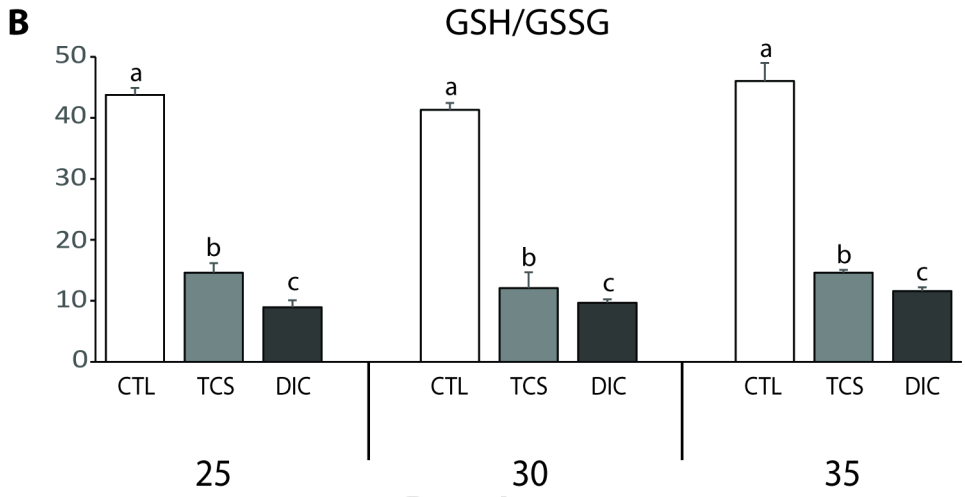

- Mussels lowered their metabolic rate after drug exposures at control salinity
- Mussels increased antioxidant defences when exposed to drugs at all salinities
- GSH/GSSG ratio was consistently reduced when mussels were exposed to TCS and DIC.
- Impacts by TCS and DIC affected particularly GPx and CAT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