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Salinity-dependent impacts on the effects of antiepileptic and antihistaminic drugs in *Ruditapes philippinarum*

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Abstract

In coastal systems, pollutants as pharmaceutical drugs exert changes from the molecular to the organism level in marine bivalves. Besides pollutants, coastal systems are prone to changes in environmental parameters, as the alteration of salinity values because of Climate Change. Together, these stressors (pharmaceutical drugs and salinity changes) can exert different threats than each stressor acting individually; for example, salinity can change the physical-chemical properties of the drugs and/or the sensitivity of the organisms to them. However, limited information is available on this subject, with variable results, and for this reason, this study aimed to evaluate the impacts of salinity changes (15, 25 and 35) on the effects of the antiepileptic carbamazepine (CBZ, 1 µg/L) and the antihistamine cetirizine (CTZ, 0.6 µg/L), when acting individually and combined (CBZ+CTZ), in the edible clam *Ruditapes philippinarum*. After 28 days of exposure, drugs concentrations, bioconcentration factors and biochemical parameters, related to clam's metabolic capacity and oxidative stress were evaluated. The results showed that clams under low salinity suffered more changes in metabolic, antioxidant and biotransformation activities, in comparison with the remaining salinities under study. However, limited impacts were observed when comparing drug effects at low salinity. Indeed, it seemed that CTZ and CBZ+CTZ, under high salinity (salinity 35) were the worst exposure conditions for the clams, since they caused higher levels of cellular damage. It stands out that salinity changes altered the impact of pharmaceutical drugs on marine bivalves.

Keywords: pharmaceutical drugs, bivalves, salinity, biomarkers, Climate Change

1. INTRODUCTION

Marine bivalves are organisms with a high ecological and socio-economic value that have been frequently used as bioindicator species to assess pollution impacts at coastal systems (Fernández-Tajes et al., 2010; Dallarés et al., 2018; Bendell et al., 2020; Sacchi et al., 2013; Capolupo et al., 2017). In particular, bivalves are known by their wide distribution, sedentary lifestyle, bioaccumulation potential, environmental resistance to different conditions and low procurement costs, making them well suited for environmental monitoring (laboratory and field studies) (Strehse and Maser, 2020).

Pharmaceutical drugs are among the pollutants present in coastal systems that have been monitored using marine bivalves (Almeida et al., 2021b; Álvarez-Muñoz et al., 2015; Caban et al., 2016; Capolupo et al., 2017; McEneff et al., 2014; Moreno-González et al., 2016; Świacka et al., 2019). Both under laboratory and field conditions, impacts of pharmaceutical drugs on marine bivalves have been reported, from molecular changes to the organism level (Fabbri and Franzellitti, 2016; Martínez-Morcillo et al., 2020). Mortality and changes in growth, reproduction, and behavior alterations, at the individual level, as well as changes on physiological, cellular and molecular responses (*e.g.*, immunological, antioxidant, detoxification, neurological and metabolism parameters) at the sub-individual level, were observed in marine bivalves exposed to pharmaceutical drugs under field (*e.g.*, Martínez-Morcillo et al., 2020) but mainly laboratory conditions (Balbi et al., 2021, 2018; Fabbri and Franzellitti, 2016; Freitas et al., 2019; Gonzalez-Rey and Bebianno, 2014; Honkoop et al., 1999; Lacaze et al., 2015; Martin-Diaz et al., 2009; Solé et al., 2010). Pharmaceuticals as carbamazepine (CBZ, antiepileptic drug) and cetirizine (CTZ, antihistaminic drug) have been found in the ng/L range in the marine environment, although higher concentrations (µg/L range) were determined in freshwater ecosystems (*e.g.*, Bahlmann et al., 2014, 2012, 2009; Brumovskỳ et al., 2016; Calisto et al., 2011; Čelić et al., 2019; Ebele et al., 2017; Nödler et al., 2014; Rehrl et al., 2020). Laboratory studies already showed the negative impacts posed by these two pharmaceuticals to marine bivalves, as changes in physiological parameters, metabolic activity and oxidative stress (see for review, Almeida et al., 2020, 2017, 2014; Oliveira et al., 2017; Teixeira et al., 2017).

Besides pollutants, coastal systems are characterized by high environmental variability in terms of abiotic factors such as temperature, pH, salinity, nutrients, among others (Harley et al.,

2006). In addition to the natural environmental variability, abiotic factors are expected to change due to Climate Change. In particular, salinity fluctuations are expected due to the increasing frequency and intensity of extreme weather events (*e.g.*, long drought; heavy rainfall periods) and changes in ice-melting regimes (Harley et al., 2006). Bivalves' life-stages and adults' survival, growth and reproduction were shown to be affected by salinity changes. Indeed, salinity variations led to negative impacts on osmoregulation, feeding activity and oxygen uptake, causing metabolic shifts and oxidative stress at the biochemical level (*e.g.*, Carregosa et al., 2014; Cole et al., 2016; Dickinson et al., 2013; Gamain et al., 2016; Grenier et al., 2020; Ivanina et al., 2020; Pourmozaffar et al., 2020). Nevertheless, in aquatic environments, organisms are exposed to a combination of different stressors which creates a range of associated environmental and ecotoxicological risks. Threats posed by the combination of stressors can differ from the effects caused by each stressor acting individually. Still, limited information is available on the influence of salinity on the behavior of pharmaceutical drugs and their effects (bioconcentration and toxicity) towards marine species. Studies regarding the effects posed to marine organisms due to pharmaceutical drugs under different salinity levels were performed on fish (Blewett et al., 2013a, 2013b; Meina et al., 2013; Scott et al., 2019), bivalves (Campos et al., 2016; Chang et al., 2012; Correia et al., 2016; Freitas et al., 2020, 2019) and crustaceans (González-Ortegón et al., 2013). Salinity changes can alter pharmaceutical drugs' physical-chemical properties, as the "salting-out" effect of sulfamethoxazole in the freshwater species *Danio rerio*, observed by Chen et al. (2017), as well as the marine species sensitivity, possibly affecting the uptake, retention and detoxification of drugs resulting in toxicity. Also, salinity fluctuations can reduce disease resistance in bivalves, making them more vulnerable to various pathogens (as reviewed by Pourmozaffar et al. (2020)) and possibly to the presence of other contaminants as pharmaceutical drugs.

As previously referred, salinity fluctuations are expected due to Climate Change consequences, especially associated with extreme weather events. In the present study the hypothesis tested was that predicted salinity fluctuations would increase the harmful effects of pharmaceuticals, namely to marine bivalves. For that, bioconcentration and biochemical effects exerted on the edible clam *Ruditapes philippinarum*, after chronic exposure (28 days), by two

pharmaceutical drugs (CBZ, 1 µg/L; CTZ, 0.6 µg/L), were evaluated at different salinity levels (15, 25 (salinity control) and 35).

2. MATERIALS AND METHODS

2.1 Experimental conditions

The clam *Ruditapes philippinarum* (Adams & Reeve, 1850) (mean length: 4.1±0.3 cm; mean width: 3.2±0.2 cm) was collected in the Mira channel (Ria de Aveiro, Portugal) to be used as model species in this study.

After collection, clams were submitted to a period of depuration and acclimation to laboratory conditions, following the procedure described by Almeida et al. (2018). *R. philippinarum* occurs in the mud-sandy beaches of the intertidal zone, where the salinity level ranges from 16 to 36 (Kim et al., 2001). Salinities 15 and 35 (tolerance limits) were chosen to be tested in this study for comparison with salinity control 25, applied in previous studies. Although salinity in the natural habitat conditions can vary beyond the used control condition, the authors selected salinity 25 to minimize interferences on the adopted drugs quantification procedure (Almeida et al., 2014). In the laboratory, one-third of the organisms was maintained at the control salinity (25) and the remaining two-thirds of the organisms were submitted to a slow decrease (for salinity 15) or increase (for salinity 35) of salinity (2/3 units per day) until the desired salinity level was obtained.

After acclimation, clams were placed in different aquaria (three per treatment) to test the chronic (28 days) effects of carbamazepine (CBZ) and cetirizine (CTZ), acting alone, or combined (drug *vs* drug), under different salinities (15-low, 25 = control and 35-high). Thus, the following treatments were tested for each studied salinity: CTL (0 µg/L CTZ, 0 µg/L CBZ), CBZ (1 µg/L), CTZ (0.6 µg/L), CBZ+CTZ (CBZ, 1 µg/L + CTZ, 0.6 µg/L). The concentrations tested in this study were selected based on drug concentrations found at the Ria de Aveiro as well as in other aquatic systems (*e.g.*, Bahlmann et al., 2012; Calisto et al., 2011) and on concentrations already used in laboratory experiments in toxicological studies with bivalves (*e.g.*, Aguirre-Martínez et al., 2013, 2016; Almeida et al., 2018; Tsiaka et al., 2013). Each aquarium used in the chronic exposure was filled with 15 L of artificial seawater (salinities 15, 25 and 35 ± 1 g/L) with six clams. During the exposure period, water was renewed every week and the conditions of aeration, room temperature, photoperiod, food and drugs concentrations were reestablished. Mortality was checked daily.

To follow drug concentrations during the experiments, aliquots of water were collected from each aquarium containing the exposure medium immediately before the water renewal (end of the exposure week) and immediately after spiking (beginning of the exposure week).

At the end of the exposure period, organisms were immediately frozen with liquid nitrogen. Afterward, the soft tissues of each clam were removed and mechanically pulverized with liquid nitrogen according to Almeida et al. (2018). Aliquots of 0.3 g fresh weight (FW) were prepared from each homogenized clam (six organisms per treatment), to be used both for biochemical analyses and drugs quantification.

2.2. Determination of drugs concentrations

The concentrations of CBZ and CTZ in water samples from the exposure medium and in clams' tissues were determined by a directive competitive ELISA (Enzyme-Linked Immunosorbent Assay). Drugs in clams' tissues were extracted with deionized water (1:2 *w/v*), disrupted in TissueLyser II, for 25 s at 4 ºC and centrifuged for 20 min at 10000 g at 4 ºC. Supernatants were collected and stored at −20 °C or directly used to determine drug concentrations. Water samples were analysed directly without no sample pre-treatment, only a dilution when necessary. This assay was validated by Bahlmann et al. (2014, 2012, 2009) and Calisto et al. (2011) and tested for drug determination in bivalves' tissues by Almeida et al. (2014). The same assay was used to quantify both pharmaceuticals due to the cross-reactivity of CTZ against the monoclonal antibody for detecting CBZ. The affinity of the monoclonal antibody to CTZ is highly dependent on the pH during the interaction step, which was controlled by the sample buffer. A sample buffer at pH 7.6 was applied in samples contaminated only with one drug (CBZ-single and CTZ-single). When both drugs were acting together (CBZ-combined and CTZ-combined), sample buffers at pH 4.5 and 10.5 were used. Then, a system of equations was applied according to Bahlmann et al. (2012) and Calisto et al. (2011) to calculate CBZ and CTZ concentrations. To perform the analysis, 96-well high-binding microtiter plates were coated with a polyclonal anti-mouse antibody overnight. After, the plates were washed, and the monoclonal antibody incubated for 1 h. After this period, another washing step was performed and the tracer solution, the standards and sample buffer were incubated for 30 min and washed again. The substrate solution was added to each well and the plate incubated for

30 min. The enzyme reaction was stopped by the addition of 1 M sulphuric acid. The absorbance was measured at 450 nm and referenced to 650 nm. All samples and standards (0–100 µg/L) were determined in triplicate on each plate. A four-parametric logistic equation (Findlay and Dillard, 2007) was fitted to the mean values of the standards to obtain a calibration curve. To analyze exposure medium water samples, standard solutions were prepared at salinities 15, 25 and 35, according to the salinity of the sample under analysis. For clams' tissues, standard solutions were prepared in ultrapure water.

2.3 Biochemical analyses

Biochemical analyses were determined in the whole soft tissues of *R. philippinarum*, using protocols previously described (Almeida et al., 2018a, 2017). For each biochemical analysis, 0.3 g FW of soft tissue per organism was extracted with a specific buffer (1:2 *w/v*) (Almeida et al., 2018a) to assess energy metabolism (electron transport system activity, ETS; glycogen content, GLY; total protein content, PROT); the antioxidant and biotransformation capacity (activities of the enzymes: superoxide dismutase, SOD; catalase, CAT; glutathione S-transferases, GSTs) and oxidative damage (lipid peroxidation levels, LPO; protein carbonylation levels, PC), the redox balance (ratio between reduced (GSH) and oxidized (GSSG) glutathione; GSH/GSSG)). For the extraction, the samples were simultaneously disrupted using a TissueLyser II, for 25 s at 4 ºC. After this procedure, samples were centrifuged for 20 min at 10 000 g (3 000 g for ETS activity) at 4 °C. Supernatants were stored at −20 °C or directly used to measure the above-mentioned

biomarkers. All the measurements of biochemical parameters were performed in duplicate.

Indicators of energy metabolism

ETS activity was determined based on King and Packard (1975) methodology with the modifications presented by De Coen and Janssen (1997). The molar extinction coefficient (Ɛ) 15,900 M^{-1} cm^{-1} was used to determine the amount of formazan formed and the results expressed in nmol per min per g FW. GLY content was quantified based on the sulphuric acid method of Dubois et al. (1956), using glucose standards (0-5 mg/mL). Concentrations of GLY were expressed in mg per g FW. The Biuret method of Robinson and Hogden (1940) was

applied to determine PROT content, using bovine serum albumin (BSA) as standards (0–40 mg/mL). Concentrations of PROT were expressed in mg per g FW.

Oxidative stress-related enzymatic activity

SOD activity was determined based on Beauchamp and Fridovich (1971) work, using SOD standards (0.25–60 U/mL). Results were expressed in U/g FW (U = µmol/min). CAT activity was quantified according to Johansson and Borg (1988), using formaldehyde standards (0–150 µM). The results of CAT activity were expressed in U/g FW (U = nmol/min). The activity of GSTs was quantified following Habig et al. (1974), using Ɛ = 9.6 mM^{-1} cm^{-1} and the activity expressed in U per g FW (U = µmol/min).

Indicators of cellular damage

LPO was measured by the quantification of TBARS (ThioBarbituric Acid Reactive Substances) according to Ohkawa et al. (1979). The molar extinction coefficient, Ɛ = 156 mM^{-1} cm^{-1}, was used to determine LPO content expressed in nmol of malondialdehyde formed per g FW. PC levels were determined following the methodology described by Mesquita et al. (2014) and Levine et al. (2000), using Ɛ = 22.308 mM^{-1} cm^{-1}. PC content was expressed in µmol per g FW. The quantification of GSH and GSSG was performed based on Rahman et al. (2006) work, using reduced and oxidized glutathione standards (0–60 µmol/L). Results were expressed in nmol per g FW. GSH/GSSG was calculated dividing the GSH values by 2x the amount of GSSG.

2.4 Data and statistical analyses

For each condition, the bioconcentration factor (BCF) (Arnot and Gobas, 2006) was calculated according to Almeida et al. (2018), except, for this case, it was used an average of the concentrations in the exposure medium, obtained immediately before the water renewal (end). The average concentrations in the clams' tissues were divided by the average concentrations in the exposure medium (e.g., for salinity 25 at CBZ treatment, dividing the concentration in tissues = 1.02 ng/g FW per the concentration in water = 1.023 ng/mL gives a BCF of 0.99 mL/g = L/kg).

Although it was not possible to use the concentrations obtained for CBZ-combined and CTZ-combined at salinity 35 (not available, in Table 1) due to salinity interferences on the quantification procedure applied when both drugs were combined, BCF was calculated for these treatments using nominal concentrations (CBZ-combined = 1 µg/L, CTZ-combined = 0.6 µg/L).

Drug concentrations from the exposure medium and in clam's soft tissues, BCF and biochemical parameters obtained from each treatment, were submitted to hypothesis testing using permutational analysis of variance with PERMANOVA+ for PRIMER v6 statistical software (Anderson et al., 2008).

The null hypotheses tested were: a) for each salinity level (15, 25, 35), no significant differences exist among treatments (CTL, CBZ, CTZ, CBZ+CTZ) for drug concentrations in water from the exposure medium, drug concentrations in clams' tissues, BCF and biochemical responses; b) for each treatment (CTL, CBZ, CTZ, CBZ+CTZ), no significant differences exist among salinity levels (15, 25, 35) for drug concentration in water from the exposure medium, drug concentrations in clams' tissues, BCF and biochemical responses. For hypothesis a), significant differences ($p < 0.05$) were represented in the Figures and Table 1 with different letters. For hypothesis b), significant differences ($p < 0.05$) were represented in Tables 2 (for results of drug quantification and BCF) and 3 (for biochemical responses).

The matrix containing the biomarkers per treatment for each salinity value was normalised and the Euclidean distance calculated among centroids was used to construct a Principal Coordination Ordination (PCO) analysis (Figure 5). In the PCO graph, the variables presenting a correlation higher than 0.85 were represented as superimposed vectors.

3. RESULTS

3.1 Mortality

Mortality was observed in clams exposed to salinity 15 at each drug treatment (CBZ, CTZ, CBZ+CTZ, 11% for each) and salinity 35 at CTZ treatment (6%). No mortality was observed at salinity 25, regardless the drug treatment.

3.2 Drugs concentrations (water and tissues) and bioconcentration factor

In general, regardless the treatment, drug concentrations in water collected from the exposure medium at the end of the exposure week (Table 1) were in a range of +/- 10% of the nominal concentrations. For each drug and at a given salinity, no significant differences were observed between single and combined treatments, except for CTZ at salinity 15 (CTZ-single *vs* CTZ-combined) (Table 1). On the contrary, significant differences were observed among salinities for CBZ-single (higher concentrations at salinities 15 and 25 than at 35) and CTZ-single (higher concentrations at salinities 15 and 35 than at 25) (Tables 1 and 2).

In Figure 1 are represented the drug concentrations in clams' tissues and BCF values. For drug concentration in clams' tissues (Figure 1A), no significant differences were observed between single and combined treatments for each salinity value (Figure 1A), similarly to the results described previously for drug concentrations in the exposure medium. Comparing treatments among salinity values, significant differences were observed for CTZ, where clams at salinity 15 showed lower concentrations than at salinities 25 and 35; and for CBZ-combined and CTZ-combined treatments, where clams at higher salinity (35) showed higher drug concentrations than at salinities 15 and 25 (Figure 1A and Table 2).

Figure 1B and Table 2 shows the results of BCF. No significant differences were observed between single and combined treatments at each salinity value (Figure 1B). However, significant differences were observed in all treatments among salinity values (Table 2). For CBZ and CTZ acting single, significant differences occurred among all the tested salinities. At CBZ-combined, BCF levels differed significantly between salinities 25 and 35. At CTZ-combined, significant differences were observed comparing the lowest salinity and salinities 25 and 35.

3.3. Biochemical analyses

3.3.1 Indicators of energy metabolism

The results of clams' energy metabolism indicators are shown in Figure 2 and Table 3.

Comparing treatments for each salinity level the ETS activity was: i) at salinity 15, significantly increased at CBZ+CTZ in comparison with the remaining treatments; ii) at salinity 25, significantly higher at CTZ in comparison to CTL and CBZ+CTZ treatments; iii) at salinity 35, significantly higher at CTZ and CBZ+CTZ in comparison to CTL (Figure 2A). Analysing responses among salinity values for each treatment, in general, significantly higher values were observed for clams under salinity 15 in comparison to salinities 25 and 35 (Figure 2A and Table 3).

The results of GLY content (Figure 2B) showed: i) at salinity 15, significantly higher values in clams exposed to CBZ in comparison to non-contaminated clams; ii) at salinity 25, significantly higher values at CBZ and CTZ treatments in comparison to CTL and CBZ+CTZ ones; iii) at salinity 35, significantly lower GLY content was observed in clams exposed to CBZ in comparison to clams exposed to the remaining treatments (Figure 2B). Comparing salinities at each treatment, at control significantly higher values were found at salinity 35; in clams exposed to CBZ significantly higher values were observed at salinity 25; in clams exposed to CTZ significant differences were observed between salinities 15 and 25, with higher values at salinity 25 (Figure 2B and Table 3).

PROT results (Figure 2C) showed no significant differences among treatments for each salinity level, except for salinity 15 where significantly higher PROT content was observed at CTZ and CBZ+CTZ treatments in comparison to clams exposed to CBZ (Figure 2C). Comparing salinities for each treatment, significantly lower values were observed in clams exposed to CBZ and salinity 15 in comparison to salinities 25 and 35 (Figure 2C and Table 3).

3.3.2 Oxidative stress-related enzymatic activity

The results of oxidative stress-related enzymatic activity are depicted in Figure 3 and Table 3.

Among treatments for each salinity level, it was observed that at: i) salinity 15, CBZ clams presented significantly lower SOD activity comparing with the remaining treatments; ii) for control salinity (25) and the highest salinity (35) SOD activity was significantly higher in clams

exposed to CTZ and CBZ+CTZ comparing with the remaining treatments (except to CBZ at salinity 25) (Figure 3A). The activity of SOD for the same treatment at the three salinity levels, in non-contaminated clams (CTL), SOD activity was significantly higher at salinities 15 and 35 than at salinity control (25). In CBZ and CBZ+CTZ treatments, SOD activity was significantly higher at salinity 35 (Figure 3A and Table 3).

The results of CAT activity, depicted in Figure 3B, showed no significant changes among treatments at salinity 15. However, for the salinity control (25), CAT activity was significantly increased in clams exposed to CTZ and CBZ+CTZ in comparison with non-contaminated clams (CTL). Also, at salinity 35, CAT activity was significantly higher in CTZ and CBZ+CTZ clams, comparing with the remaining treatments (Figure 3B). Among salinity levels for each treatment, it was observed a significantly higher CAT activity at salinities 15 and 35 in comparison to non-contaminated clams maintained at salinity 25. The same trend was observed for CBZ+CTZ. Also, at CBZ treatment, CAT activity was significantly higher at salinity 15 comparing with the remaining salinities (Figure 3B and Table 3).

The activity of GSTs (Figure 3C) at salinity 15, GSTs activity was significantly lower in CBZ treatment in comparison with the remaining treatments, except with CTZ. No significant changes were observed among treatments at salinity control (25). However, at salinity 35, GSTs activity was significantly higher in CTZ clams than in CTL and CBZ treatments (Figure 3C). Comparing responses among salinity levels in non-contaminated clams (CTL), the significantly higher GSTs activity was observed at salinity 15. In CTZ, GSTs activity was significantly higher at salinities 15 and 35. In clams exposed to CBZ+CTZ, significant differences were observed between all the salinities levels, with the highest activity at the lowest salinity (15).

3.3.3 Indicators of cellular damage

The results of LPO, PC and GSH/GSSG, chose to indicate the levels of cellular damage are depicted in Figure 4 and Table 3.

At low salinity (15), no significant changes in LPO results occurred among treatments (Figure 4A). However, at salinities 25 and 35, LPO levels were significantly higher in contaminated (at CBZ, CTZ, CBZ+CTZ for salinity 25; CTZ, CBZ+CTZ for salinity 35) than in non-contaminated clams. Among salinities for each treatment: for CTL clams, significantly lower

LPO levels were observed at salinity 25 in comparison to salinities 15 and 35; for CTZ and CBZ+CTZ exposed clams, significantly higher LPO values were observed at salinity (Figure 4A, Table 3).

Significant differences among treatments for each salinity value were found for PC results (Figure 4B). Lower PC levels were observed between CBZ clams with non-contaminated clams at extreme salinities (SAL 15 and 35). At salinity 25, PC levels were significantly higher in CTZ clams, comparing with the remaining treatments (Figure 4B). Among salinities, significantly lower PC levels were found in CTL clams at salinity 25 in comparison to salinity 15; at CBZ and CTZ exposed clams, significantly lower values were observed at salinity 35 in comparison with the remaining salinities; in clams exposed to both drugs significantly higher PC values were observed at salinity 15 comparing with the remaining salinities (Figure 4B and Table 3).

Figure 4C depicts the results of GSH/GSSG content. No significant changes in GSH/GSSG levels occurred among treatments for salinities 15 and 35. At salinity 25, clams exposed to CTZ and CBZ+CTZ presented lower GSH/GSSG levels than non-contaminated clams (CTL) (Figure 4C). For the same drug treatment, among salinity levels, significant differences were observed in CTL and CBZ treatments, with lower levels at salinity 15 comparing with salinity 25 in CTL, and with salinity 35 in CBZ (Figure 4C and Table 3).

3.4. Ordination analysis

The results from the PCO analysis (Figure 5) showed that the first principal component (PCO1) axis explained 39% of the total variation among treatments, separating the non-contaminated clams under salinities 25 and 35 and clams contaminated with CBZ (at salinities 25 and 35) and combined with CTZ (at salinity 25) on the positive side from the contaminated clams with CTZ, single or combined with CBZ at the extreme salinities (15 and 35) on the negative side. The biochemical descriptors superimposed on the PCO showed that ETS and SOD activities and LPO levels were the variables that best explained PCO1 variation. PCO2 axis explained 27% of the total variation among treatments, separating the contaminated and non-contaminated clams at low salinity (15) and clams maintained at salinity 25 (CTL and CBZ+CTZ) on the positive side and contaminated clams at high salinity (35) on the negative

side. ETS activity was better correlated with clams at low salinity while LPO levels was better correlated with contaminated clams at high salinity.

4. DISCUSSION

4.1 Impacts of treatments, for each salinity level, on the bioconcentration of CBZ and CTZ (single and combined) by *R. philippinarum*

Overall, concerning treatments at the same salinity level, although no significant differences were observed comparing single and combined treatments, significant differences occurred when comparing CBZ and CTZ BCFs. Higher BCFs were observed for CTZ (single and combined) comparing with CBZ at salinities 25 and 35, while at salinity 15 the BCFs levels were, in general, similar among CBZ and CTZ treatments. In accordance with the present results, previous studies found a higher BCF of CTZ comparing with CBZ at control salinity conditions (25) (Almeida et al., 2021a, 2018a, 2018b, 2017). Herein, it seemed that at control salinity higher metabolic activity (ETS) in CTZ over CBZ clams may be responsible for increased BCF, while at higher salinity (35), although the activity of biotransformation enzymes (GSTs) was increased in CTZ exposed clams, this was not enough to lower the content of this drug. At low salinity, however, CTZ is possibly more excreted than CBZ, presenting a lower BCF value. This response is contrary to what happens at control salinity and in a previous study (Almeida et al., 2021a), however, it resembles the responses that occur in vertebrates in which CTZ is almost eliminated with limited metabolism, by opposition to CBZ that is extensively metabolized. Thus, although different mechanisms may underlie the uptake, detoxification, and excretion of CBZ and CTZ in clams, as suggested in previous studies (Almeida et al., 2021a, 2018, 2017), salinity may modulate these mechanisms, as observed here.

4.2 Impacts of salinities, for each treatment, on the bioconcentration of CBZ and CTZ (single and combined) by *R. philippinarum*

Overall, changing salinity values had effects on both drugs BCFs, however, marked effects were observed in CTZ clams (single and combined) (lower BCF at salinity 15 in comparison with salinities 25 and 35).

The results obtained for CTZ clams under low salinity (15) are possibly related to increased excretion (elimination), leading to a lower BCF value. Also, bivalves are known to protect themselves by closing the valves and consequently reducing the filtration activity when

exposed to changes in salinity (*e.g.*, Domínguez et al., 2020; Pourmozaffar et al., 2020) and pharmaceutical drugs (*e.g.*, Almeida et al., 2014; Chen et al., 2014; Solé et al., 2010). Indeed, it is known that, under low salinity values (salinity 15 and below), *R. philippinarum* close their valves for protection (Domínguez et al., 2020), thus, valve closure could result in lower CTZ BCF, but this only occurred in CTZ and not in CBZ clams. The evaluation of the salinity impacts on the uptake of pharmaceutical drugs by marine bivalves is limited to a few studies. For instance, Freitas et al. (2019) showed higher uptake of diclofenac (1.0 µg/L) in the mussel *Mytilus galloprovincialis* exposed for 28 days under extreme salinities (salinity 25: ~47 ng/g dry weight; salinity 35: ~40 ng/g dry weight) in comparison with salinity control (salinity 30: ~28 ng/g dry weight). This response was justified considering the high activity of detoxification enzymes (glutathione S-transferases) reducing diclofenac concentrations in tissues at salinity control (30) by comparison with salinities 25 and 35. For the same species, a higher BCF was observed in mussels exposed to salicylic acid under extreme salinities (25 and 35) comparing with the salinity control (30) (Freitas et al., 2020). Although testing the elimination of drugs and not the uptake, Chang et al. (2012) showed a higher rate of elimination of the antibiotic enrofloxacin in *R. philippinarum* held at salinity 30 than at low salinity (20) at the same temperature (22ºC). Comparing to the present data, these studies seemed to indicate that the impacts of salinity on drug uptake, detoxification and excretion are dependent on the salinity level and the drug type. Possibly, at low salinity levels (as 15) and in the presence of some drugs (as CTZ), clams activate defense mechanisms (*e.g.*, valve closure, metabolism and/or excretion) more efficiently than at high salinities (as 35), that, even with detoxification processes are not able to lower drug concentrations. However, due to the limited knowledge on this issue and to the different conditions applied (e.g., different salinity levels tested) it is difficult to properly assess these impacts.

4.3 Impacts of treatments, for each salinity level, on the biochemical alterations induced in *R. philippinarum* exposed to CBZ and CTZ (single and combined)

Overall, when comparing treatments for each salinity level, the results showed that the pharmaceutical drugs tested exerted oxidative stress on clams, with CTZ acting alone and in combination with CBZ being the most stressful conditions, causing cellular damage under high

salinity, while at low salinity despite increasing metabolic activity no cellular damage was observed. Although CTZ and its combination with CBZ presented the worst effects, CBZ had limited impacts on clams.

Regarding energy metabolism indicators, electron transport system (ETS) activity was the most affected parameter. Overall, regardless of the salinity level clams exposed to CTZ and CBZ+CTZ showed higher ETS activity, indicating that CTZ and the combination of drugs may act as the most stressful treatments leading to activation of clams' metabolism. Higher metabolic capacity in clams exposed to CTZ (at salinities control and 35) and to CBZ+CTZ (at salinities 15 and 35) may indicate that under these treatments' clams were able to activate their metabolism probably in an attempt to increase defense mechanisms. On the other hand, at control salinity (25) and in the presence of both drugs, clams were able to maintain their metabolism, highlighting that under stressful salinities this strategy was no longer valid. CBZ at all the tested salinities did not generate any metabolic activation. In accordance, a previous study performed with similar drug exposure conditions (Almeida et al., 2021a) demonstrated that, at salinity control, clams exposed to CBZ and CBZ+CTZ maintained their ETS activity at control levels while the exposure to CTZ activated clams' metabolism. Nevertheless, clams tended to maintain constant their energy reserves content regardless of the salinity level. However, changes were observed at salinity control (25), with the highest GLY values in CBZ and CTZ clams, which is associated with no activation of metabolism, but also when an increase in ETS activity was observed for CTZ. The activation of the metabolism in clams exposed to CTZ and CBZ+CTZ under salinity control and 35 may also be related to the increased BCF values at these conditions, as possibly clams increased the filter-feeding activity to obtain energy and, consequently, increase the drug uptake. Previous authors showed different metabolic responses comparing contaminated and non-contaminated bivalves under different salinity levels. For example, the previously referred study by Freitas et al. (2019) showed higher ETS activity in mussels contaminated with drugs (diclofenac, triclosan) compared with non-contaminated mussels at salinity 25, while ETS activity was decreased comparing contaminated and non-contaminated organisms at salinity 30 (control) and 35. On the same mussel species, Freitas et al. (2020) showed an increase in ETS activity comparing

mussels exposed for 28 days to salicylic acid with non-contaminated mussels at salinity 30 (control), while at extreme salinities (25 and 35) no differences were observed.

In what regards clam's antioxidant and biotransformation defenses, in general for the 3 salinities tested, greater enzymes activation was observed in clams exposed to CTZ and the combination of both drugs. Such activation is associated with clams' metabolism induction (higher ETS) and with this, higher reactive oxygen species (ROS) generation in mitochondria or in drug detoxification processes (higher GSTs activity), that need to be converted into less reactive species by the antioxidant enzymes. In addition, and mainly for SOD activity at control salinity (25), a lower enzyme activation was observed in CBZ+CTZ treatment comparing with CTZ, being related to the low metabolism activation referred previously. Despite the activation of metabolism and antioxidant machinery, cellular damage occurred in drug contaminated clams for salinity control and 35, especially at CTZ and CBZ+CTZ at salinity 35. This response may be related to the high BCF of CTZ (single and combined) under these conditions, leading to a higher biotransformation (GSTs) activity and ROS generation that was not effectively removed by the antioxidant defenses (SOD, CAT). At salinity control, despite the increased BCF levels, as GSTs were not activated and possibly no additional contribution of ROS occurred, the antioxidant defense system (SOD) was enough to avoid even higher cellular damage. Also, no cellular damage occurred in all drug treatments at low salinity possibly due to the low BCF and insufficient drug concentrations (possibly due to excretion). Previous studies with *R. philippinarum* exposed to the pharmaceutical drugs tested herein under control salinity (25) showed CBZ to cause cellular damage on the lipid membranes while CTZ induced oxidative stress, without exerting damage (*e.g.*, Almeida et al., 2018, 2017, 2015). Although it was suggested in a previous study (Almeida et al., 2021a) that CTZ may suffer low biotransformation compared with CBZ in *R. philippinarum*, both at the same concentrations used in this study, the results obtained herein seem to show that, in the presence of high salinity (35), CTZ biotransformation is stimulated in clams. Possibly, because of that, CTZ concentrations in clams under salinity 35 did not differ statistically from CBZ, as occurs at salinity control (25) in this study and in Almeida et al. (2021a). In addition to the lipid membranes damage, for low and control salinity, PC levels, an indicator of protein oxidative damage, were increased in

contaminated clams, mainly for CTZ, which is related to the metabolic activation under these conditions.

In general, and as confirmed in the PCO analysis, the pharmaceutical drugs herein tested exerted oxidative stress, with CTZ acting alone and in combination with CBZ causing cellular damage under high salinity, while at low salinity despite increasing metabolic activity no cellular damage occured. Previous studies showed that the metabolic activity of marine bivalves exposed to salinity changes usually is restored to normal conditions after a brief period (*e.g.*, 24 h in Chen et al., 2021). Thus, in this study, we could hypothesize that the combination of pharmaceutical drugs (CBZ+CTZ) and low salinity values, although without exerting cellular damage, caused an increase in metabolic activity that was not restored to control levels, even after a 28-days exposure period. In addition, it seemed that, under control salinity, CBZ can act as a buffer of the effects of CTZ, but this capacity is lost at extreme salinities. Nonetheless, CTZ and its combination with CBZ were the worst treatments for the clams, while CBZ had limited impacts on clams. Exposure tests using mixtures of pharmaceutical drugs and other contaminants have been performed, showing variable results (Di Poi et al., 2018; Juhel et al., 2017). Di Poi et al. (2018), among other tests, assessed the individual and combined (mixtures, binary and ternary) effects of CBZ and other contaminants (a biocide, methylparaben and a pesticide degradation product, aminomethylphosphonic acid) in oysters *Crassostrea gigas* using acute and sub-chronic parameters (embryotoxicity and metamorphosis test). The authors found that the toxicity of binary and ternary mixtures was not different, but the mixtures were more toxic than each compound acting single, resulting in synergistic or antagonistic interactions.

4.4 Impacts of salinities, under each treatment, on the biochemical alterations induced in *R. philippinarum* exposed to CBZ and CTZ (single and combined)

Overall, when comparing salinities for each treatment applied, the results showed that salinity *per se* led to impacts (oxidative stress) on the biomarker responses, with high intensity at low salinity levels. In the presence of pharmaceutical drugs, the responses were altered, depending on the salinity tested.

When exposed to different salinities, in general, and for non-contaminated clams (CTL), high intensity of responses was observed in clams under low salinity, followed by high salinity

and with lower values for control salinity. This indicates that salinity *per se* is able to change biomarker responses in non-contaminated clams, causing itself oxidative stress as observed by higher metabolic activity (ETS), antioxidant and biotransformation enzymes activities (SOD, CAT and GSTs), and consequently cellular damage (LPO and PC). The high metabolic activity in marine bivalves exposed to low salinity values was already demonstrated to be related to a metabolic adjustment to the osmotic stress (Freitas et al., 2017; Kim et al., 2001; Moreira et al., 2016; Velez et al., 2016). In accordance, Velez et al. (2016), after a 28-day assay, showed higher ETS activity in *R. philippinarum* exposed to low salinity levels (14), decreasing with the increase of salinity (28-control, 35). Besides the increased metabolic capacity at low salinity, the authors also showed higher cellular damage (LPO), antioxidant (SOD, CAT) and biotransformation (GSTs) enzymes activities in clams exposed to low salinity comparing to control and high salinity. Also, Kim et al. (2001) showed higher metabolic activity (as oxygen consumption rate, OCR) in *R. philippinarum* exposed to low salinities. In fact, the OCR at salinity 15 increased by 23.5-38.1% compared to the OCR at salinity 31.2 (control). According to Kim et al. (2001), salinities between 15 and 10 can be regarded as the lower tolerance range for this clam, below that, clams don't recover normal metabolic activities.

When exposed to pharmaceutical drugs at different salinities, in general, the biological response was enhanced, as observed for CBZ and especially for CTZ and CBZ+CTZ under extreme salinities. Indeed, for energy metabolism indicators, regardless of the treatment applied, higher ETS activity was observed at low salinity comparing with control and high salinity. Bivalves are osmoconformer organisms and to regulate the extracellular fluid concentration and the external osmotic pressure, the transfer of energy is involved (Pourmozaffar et al., 2020), possibly causing the differences observed in ETS activity comparing salinity values. As a consequence of high metabolic activity in clams exposed to low salinity, GLY content was, in general, lower in clams at salinity 15 compared with salinities 25 and 35. This response possibly indicates the use of glucose to produce energy to fuel up biologic defense strategies (*e.g.*, antioxidant defenses, osmoconformation process) under the low salinity values. Freitas et al. (2020), referred previously, showed no significant differences in ETS of mussels exposed to salicylic acid among the tested salinities, revealing no clear impacts of salinity on metabolic activity.

Also, in general, higher antioxidant and biotransformation enzymatic activity was observed in clams exposed to extreme salinities, comparing with salinity control, being related to the higher metabolic activity (ETS) and oxidative injury (LPO, PC) under these conditions. However, the results of cellular damage seemed to indicate different "targets" at extreme salinities, with proteins being more prone to oxidative injury at the lowest salinity (high PC at CTZ and CBZ+CTZ) and membrane lipids at the highest salinity (high LPO at CTZ and CBZ+CTZ at salinity 35), with higher cellular damage at salinity 35 as highlighted in PCO graph. Nonetheless, despite the high metabolic activity, salinity 15, *per se*, is responsible for oxidative stress (high SOD and CAT), but the presence of pharmaceutical drugs does not stimulate this response and no cellular damage occurred. The impairment of biological responses in marine bivalves exerted by salinity *per se*, having more impact than the combination with pharmaceutical drugs was already reported. On mussels, Freitas et al. (2020) showed higher cellular damage in *M. galloprovincialis* exposed to high salinity (35), irrespectively the presence of salicylic acid, with no additive or synergistic effects. The authors indicated that the concentrations tested were not high enough to induce cellular damage, neither salinity exerted impacts on the sensitivity of mussels to the presence of the drug. For the same species, Freitas et al. (2019), showed that salinity alone modulated several biochemical responses (low metabolic activity, increase in energy reserves content, low cellular damage), especially at the low salinity tested (25). The responses to pharmaceutical drugs under different salinities were less significant than the responses to salinity acting itself, especially for the metabolic capacity. However, Correia et al. (2016) showed that salinity changes (14, 28, 35) were not responsible for significant impacts on cellular damage (LPO) in *R. philippinarum* exposed to acetaminophen for 96 hours.

5. Conclusions

The present study aimed to fill the gap regarding the impacts of Climate Change abiotic factors (specifically, salinity changes) on the effects exerted by pharmaceutical drugs (single and combined) on marine organisms. This study shows that salinity *per se*, mainly low salinity, is a challenge to *R. philippinarum* (*e.g.*, cellular damage), however, the responses of clams changed under the combination with pharmaceutical drugs and depending on the salinity value.

Indeed, no cellular damage was observed in contaminated clams at low salinity while contaminated clams, specifically CTZ and CBZ+CTZ at higher salinities, presented the highest negative impacts (*e.g.*, higher levels of cellular damage). This study reinforces that the species sensitivity to drugs can be changed by the presence of Climate Change abiotic factors, causing negative impacts.

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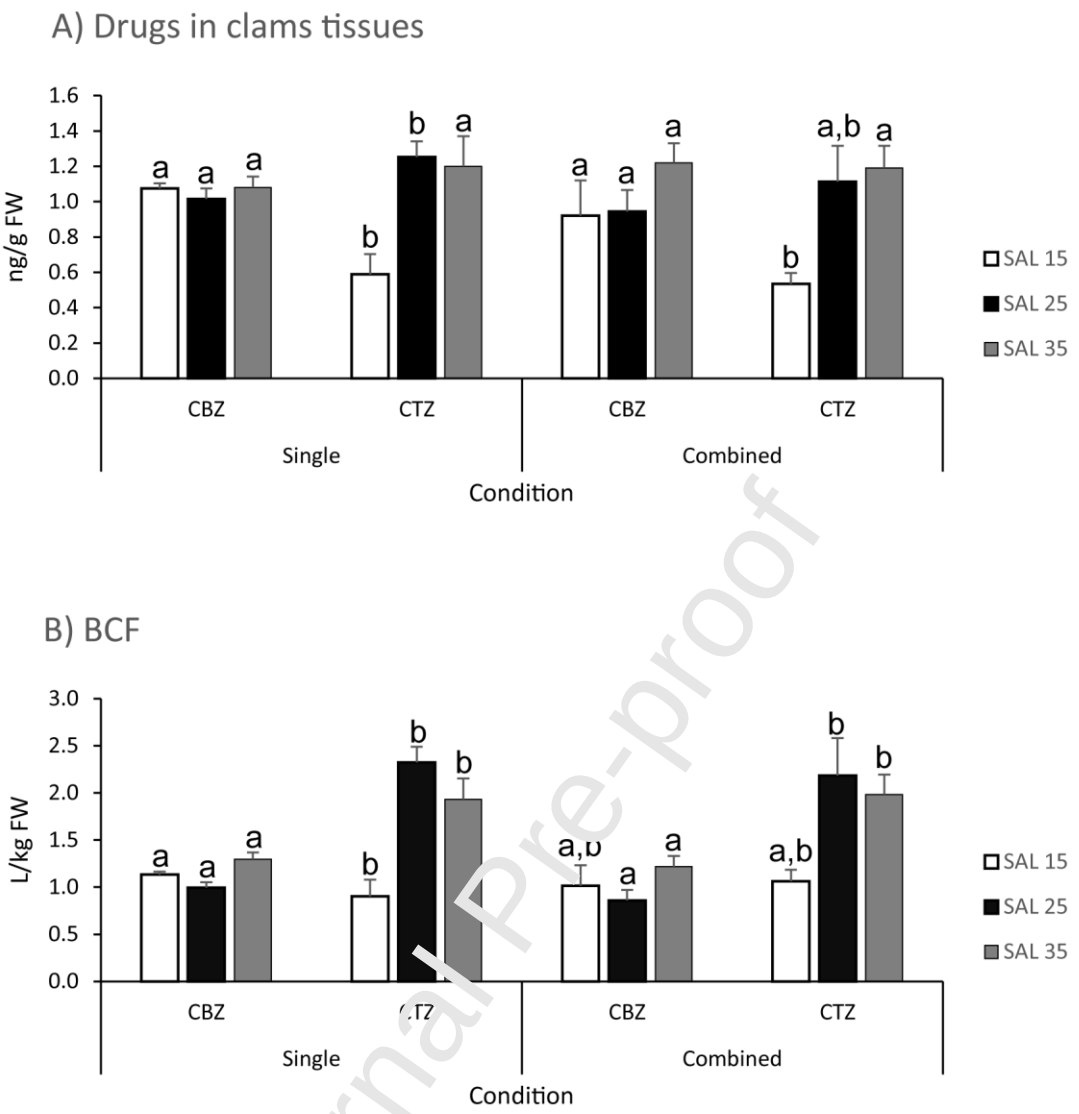

Figure 1

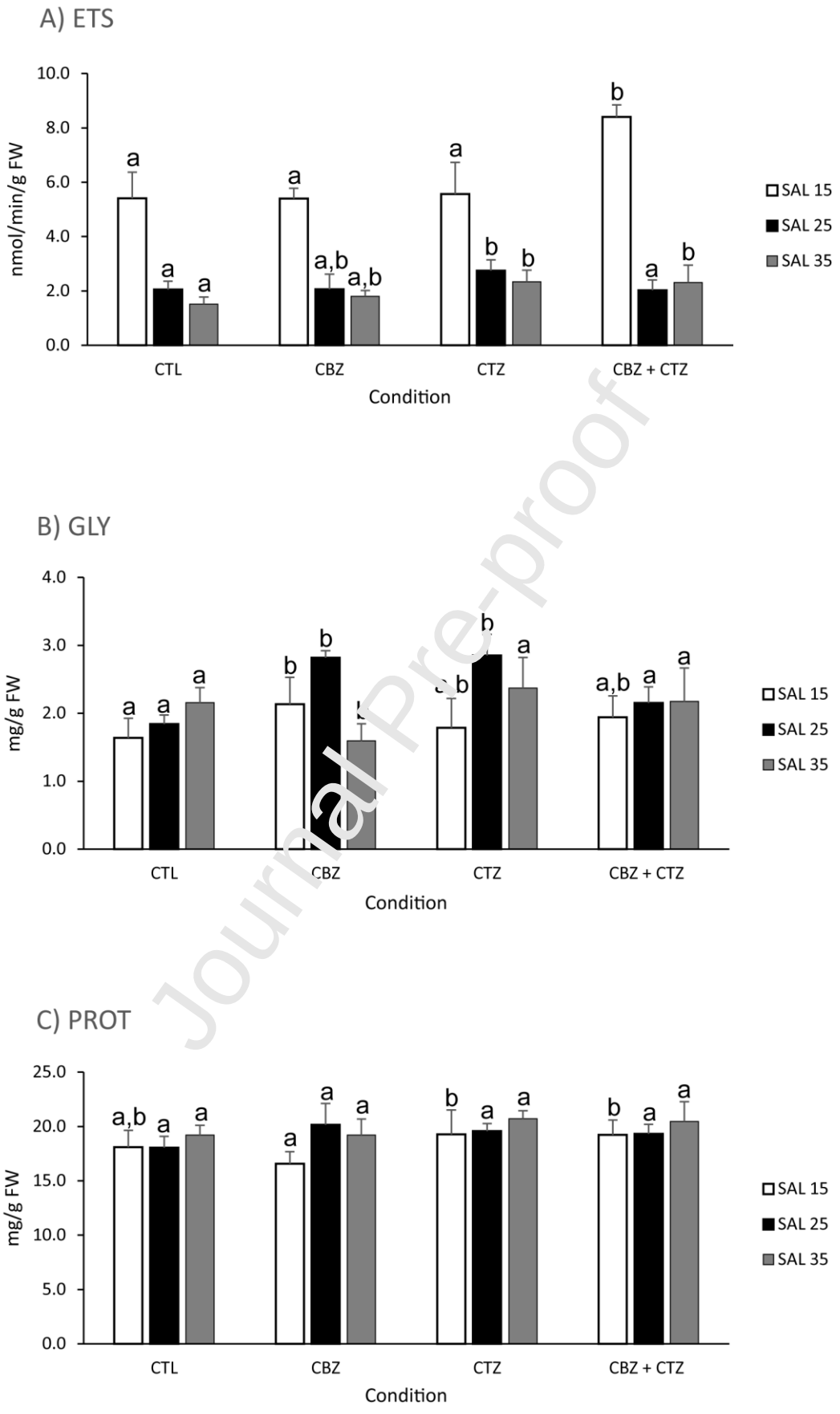
A) ETS
nmol/min/g FW
10.0
8.0
6.0
4.0
2.0
0.0
a
a
a
a
a,b
a,b
a
b
b
b
a
b
CTL
CBZ
CTZ
CBZ + CTZ
Condition
SAL 15
SAL 25
SAL 35
B) GLY
mg/g FW
4.0
3.0
2.0
1.0
0.0
a
a
a
b
b
a,b
b
a
a,b
a
a
CTL
CBZ
CTZ
CBZ + CTZ
Condition
SAL 15
SAL 25
SAL 35
C) PROT
mg/g FW
25.0
20.0
15.0
10.0
5.0
0.0
a,b
a
a
a
a
a
b
a
a
b
a
a
CTL
CBZ
CTZ
CBZ + CTZ
Condition
SAL 15
SAL 25
SAL 35

Figure 2

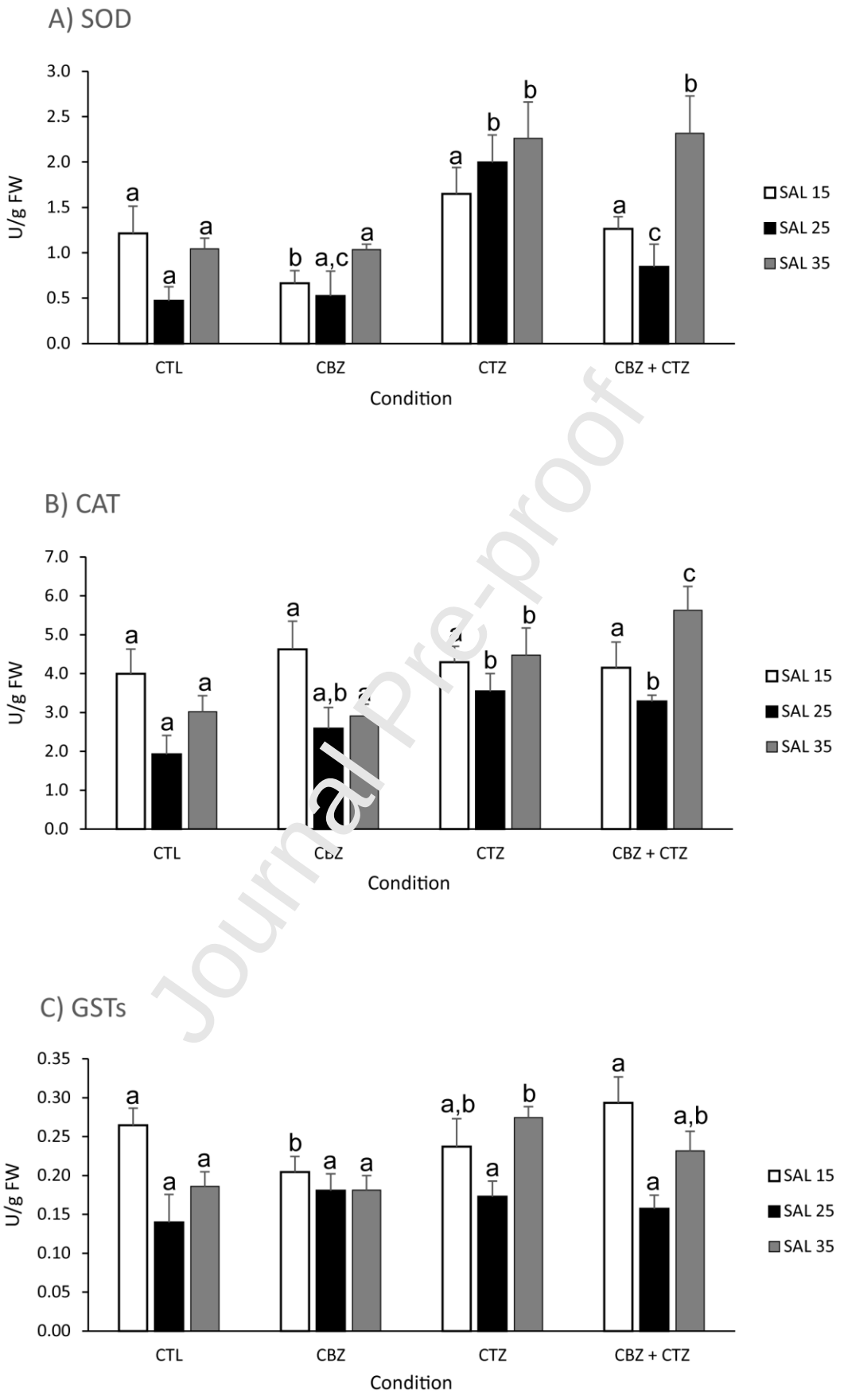
A) SOD
U/g FW
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
a
a
b
a,c
a
a
b
b
a
c
b
CTL
CBZ
CTZ
CBZ + CTZ
Condition
SAL 15
SAL 25
SAL 35
B) CAT
U/g FW
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
a
a
a
a
a,b
a
b
b
a
b
c
CTL
CBZ
CTZ
CBZ + CTZ
Condition
SAL 15
SAL 25
SAL 35
C) GSTs
U/g FW
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
a
a
a
b
a
a
a,b
a
b
a
a
a,b
CTL
CBZ
CTZ
CBZ + CTZ
Condition
SAL 15
SAL 25
SAL 35

Figure 3

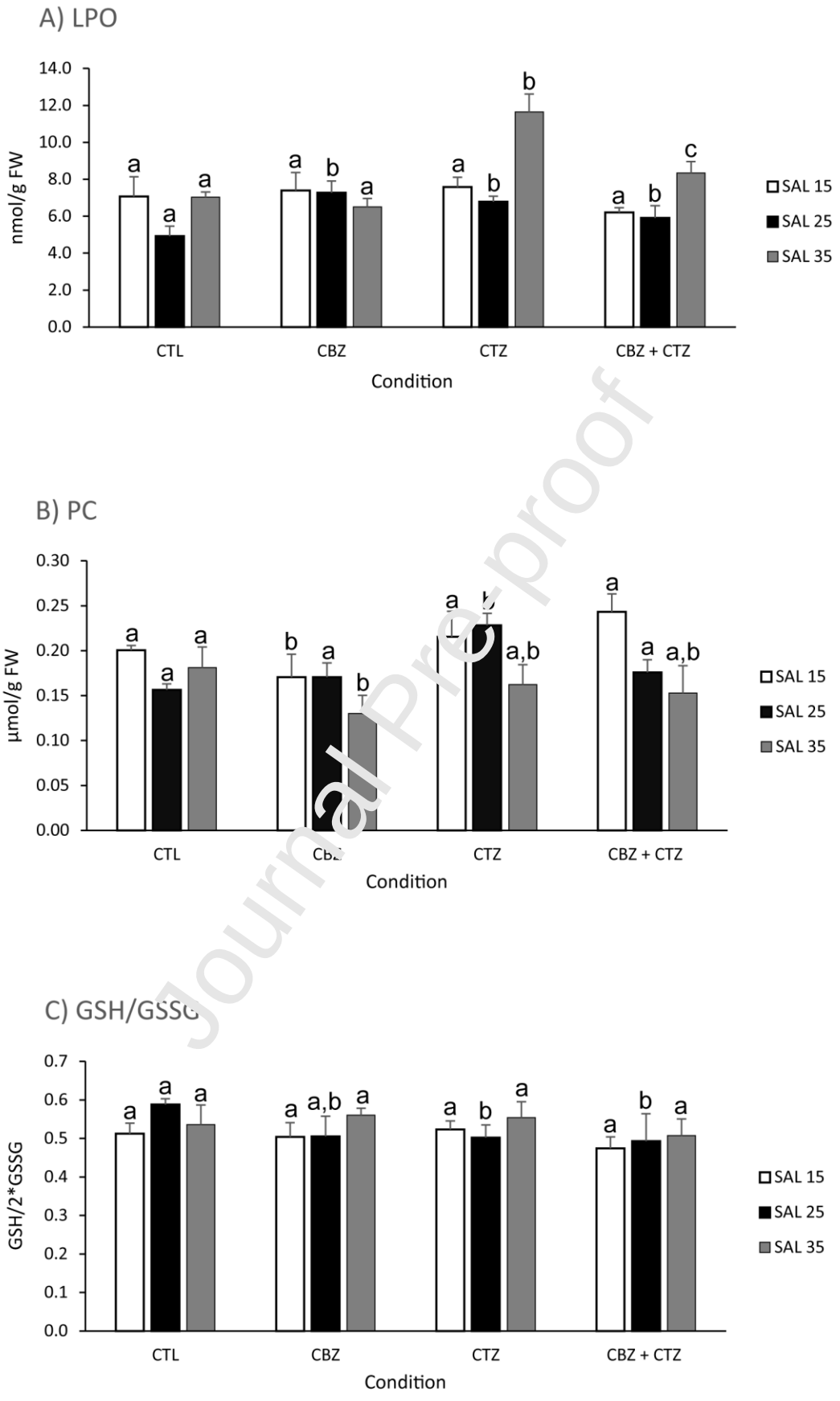
A) LPO
nmol/g FW
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
CTL
CBZ
CTZ
CBZ + CTZ
Condition
SAL 15
SAL 25
SAL 35
B) PC
µmol/g FW
0.30
0.25
0.20
0.15
0.10
0.05
0.00
CTL
CTZ
CBZ + CTZ
Condition
SAL 15
SAL 25
SAL 35
C) GSH/GSSG
GSH/2*GSSG
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
CTL
CBZ
CTZ
CBZ + CTZ
Condition
SAL 15
SAL 25
SAL 35

Figure 4

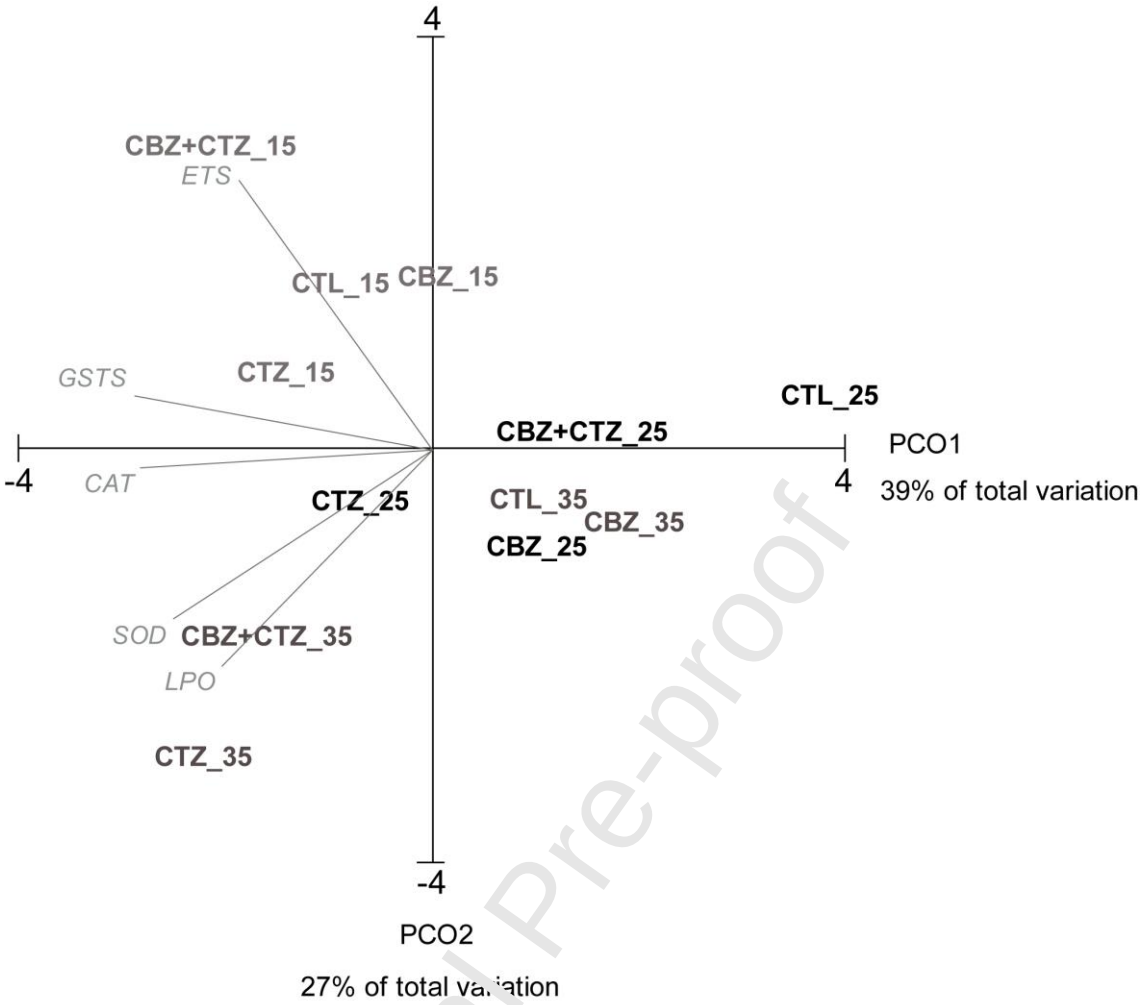

Figure 5

Figure 1. A) Drugs concentrations in tissues (CBZ, CTZ, ng/g FW) and B) Bioconcentration factor (BCF), in *R. philippinarum* exposed to single and combined treatments (CBZ-single; CTZ-single, CBZ-combined and CTZ-combined) under different salinities (SAL: 15, 25-control, 35) for 28 days. Results are the means (± standard deviation). Significant differences ($p \leq 0.05$) between treatments for the same salinity level are present with letters.

Figure 2. Energy-related parameters: A) ETS, energy transport system activity; B) GLY, glycogen content; C) PROT, protein content in *R. philippinarum* exposed to single and combined treatments (CTL, CBZ, CTZ and CBZ+CTZ) under different salinities (SAL: 15, 25-control, 35) for 28 days. Results are the means (± standard deviation). Significant differences ($p \leq 0.05$) between treatments for the same salinity level are present with letters.

Figure 3. Oxidative stress related enzymatic activity: A) SOD, superoxide dismutase; B) CAT, catalase; C) GSTs, glutathione S-transferases in *R. philippinarum* exposed to single and combined treatments (CTL, CBZ, CTZ and CBZ+CTZ) under different salinities (SAL: 15, 25-control, 35) for 28 days. Results are the means (± standard deviation). Significant differences ($p \leq 0.05$) between treatments for the same salinity level are present with letters.

Figure 4. Indicators of cellular damage: A) LPO, lipid peroxidation levels; B) PC, protein carbonylation levels; C) GSH/GSSG (ratio between reduced and oxidized glutathione) in *R. philippinarum* exposed to single and combined treatments (CTL, CBZ, CTZ and CBZ+CTZ) under different salinities (SAL: 15, 25-control, 35) for 28 days. Results are the means (± standard deviation). Significant differences ($p \leq 0.05$) between treatments for the same salinity level are present with letters.

Figure 5. Principal coordinated analyses (PCO) based on biochemical parameters obtained in *R. philippinarum* exposed to single and combined treatments (CTL, CBZ, CTZ and CBZ+CTZ) under different salinities (SAL: 15, 25-control, 35) for 28 days. Pearson correlation vectors are superimposed as supplementary variables, namely biochemical data ($r \geq 0.85$).

TABLE 1: DRUG CONCENTRATIONS IN µG/L FROM THE EXPOSURE MEDIUM OF R. PHILIPPINARUM SUBMITTED TO DRUG TREATMENTS (CBZ-SINGLE; CTZ-SINGLE, CBZ-COMBINED AND CTZ-COMBINED) UNDER DIFFERENT SALINITIES (15, 25-CONTROL, 35). RESULTS ARE THE MEANS OF CONCENTRATIONS IN THE EXPOSURE MEDIUM, OBTAINED IMMEDIATELY BEFORE EACH WATER RENEWAL DURING THE 28 DAYS OF EXPOSURE (± STANDARD DEVIATION, N=3). SIGNIFICANT DIFFERENCES (P < 0.05) BETWEEN TREATMENTS FOR THE SAME SALINITY LEVEL ARE PRESENTED WITH LETTERS. N.A., NOT AVAILABLE

DRUG TREATMENTS	SALINITY 15 (LOW)	SALINITY (CONTROL)	SALINITY 35 (HIGH)
CBZ-SINGLE	**0.95 (±0.05)^{A}**	**1.0 (±0.2)^{A}**	**0.83 (±0.06)^{A}**
CTZ-SINGLE	**0.65 (±0.04)^{B}**	**0.54 (±0.06)^{B}**	**0.62 (±0.05)^{B}**
CBZ-COMBINED	**0.91 (±0.07)^{A}**	**1.1 (±0.2)^{A}**	**N.A.**
CTZ-COMBINED	**0.50 (±0.04)^{C}**	**0.51 (±0.09)^{B}**	**N.A.**

Table 2: Pairwise comparisons (*p* values) among salinity levels (SAL: 15, 25-control, 35) for each treatment (CBZ-single; CTZ-single, CBZ-combined and CTZ-combined) for drug concentration (in exposure medium and clams) and BCFs results. Significant values ($p < 0.05$) are in bold. N= 6.

Comparisons	**Drugs in exposure medium**	**Drugs in clams tissues**	**BCF**
CBZ-single 15 *vs* CBZ-single 25	0.22	0.27	**0.023**
CBZ-single 15 *vs* CBZ-single 35	**0.0018**	0.93	**0.020**
CBZ-single 25 *vs* CBZ-single 35	**0.017**	0.20	**0.00010**
CTZ-single 15 *vs* CTZ-single 25	**0.0017**	**0.00010**	**0.00010**
CTZ-single 15 *vs* CTZ-single 35	0.22	**0.00010**	**0.00010**
CTZ-single 25 *vs* CTZ-single 35	**0.012**	0.53	**0.018**
CBZ-combined 15 *vs* CBZ-combined 25	0.12	0.81	0.13
CBZ-combined 15 *vs* CBZ-combined 35	--	**0.0080**	0.058
CBZ-combined 25 *vs* CBZ- combined 35	--	**0.0080**	**0.0012**
CTZ-combined 15 *vs* CTZ- combined 25	0.86	**0.00010**	**0.00010**
CTZ-combined 15 *vs* CTZ- combined 35	--	**0.00010**	**0.00010**
CTZ-combined 25 *vs* CTZ- combined 35	--	0.39	0.25

Table 3: Pairwise comparisons (*p* values) among salinity levels (15, 25-control, 35) for each treatment (CTL, CBZ, CTZ, CBZ+CTZ) for biochemical results (ETS, electron transport system activity; GLY, glycogen content; PROT, protein content; SOD, superoxide dismutase activity; CAT, catalase activity; GSTs, glutathione S-transferases activity; LPO, lipid peroxidation; PC, protein carbonyl levels; GSH/GSSG, ratio between reduced and oxidized glutathione). Significant values ($p \leq 0.05$) are in bold. N= 6.

Comparisons	ETS	GLY	PROT	SOD	CAT	GSTs	LPO	PC	GSH/GSSG
CTL 15 *vs* CTL 25	**0.0010**	0.20	0.97	**0.0040**	**0.00010**	**0.0010**	**0.00010**	**0.018**	**0.0010**
CTL 15 *vs* CTL 35	**0.00010**	**0.010**	0.37	0.40	0.063	**0.0010**	0.93	0.31	0.45
CTL 25 *vs* CTL 35	**0.037**	**0.025**	0.25	**0.00010**	**0.026**	0.080	**0.00010**	0.066	0.090
CBZ 15 *vs* CBZ 25	**0.00010**	**0.015**	**0.0030**	0.30	**0.0010**	0.22	0.85	0.99	0.97
CBZ 15 *vs* CBZ 35	**0.00010**	**0.023**	**0.0060**	**0.0010**	**0.0010**	0.15	0.14	**0.029**	**0.015**
CBZ 25 *vs* CBZ 35	0.36	**0.00010**	0.33	**0.0030**	0.49	0.97	0.23	**0.050**	0.18
CTZ 15 *vs* CTZ 25	**0.0010**	**0.0010**	0.78	0.30	0.18	**0.013**	0.24	0.37	0.58
CTZ 15 *vs* CTZ 35	**0.00010**	0.053	0.30	0.051	0.71	0.18	**0.00010**	**0.019**	0.30
CTZ 25 *vs* CTZ 35	0.20	0.14	0.38	0.51	0.080	**0.00010**	**0.00010**	**0.0030**	0.21
CBZ + CTZ 15 *vs* CBZ + CTZ 25	**0.00010**	0.48	0.90	0.061	**0.018**	**0.00010**	0.65	**0.010**	0.60
CBZ + CTZ 15 *vs* CBZ + CTZ 35	**0.00010**	0.37	0.22	**0.0010**	**0.0080**	**0.036**	**0.0050**	**0.0010**	0.18
CBZ + CTZ 25 *vs* CBZ + CTZ 35	0.40	0.95	0.22	**0.00010**	**0.00010**	**0.037**	**0.0010**	0.27	0.70

Credit author statement

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Rudolf J. Schneider: funding

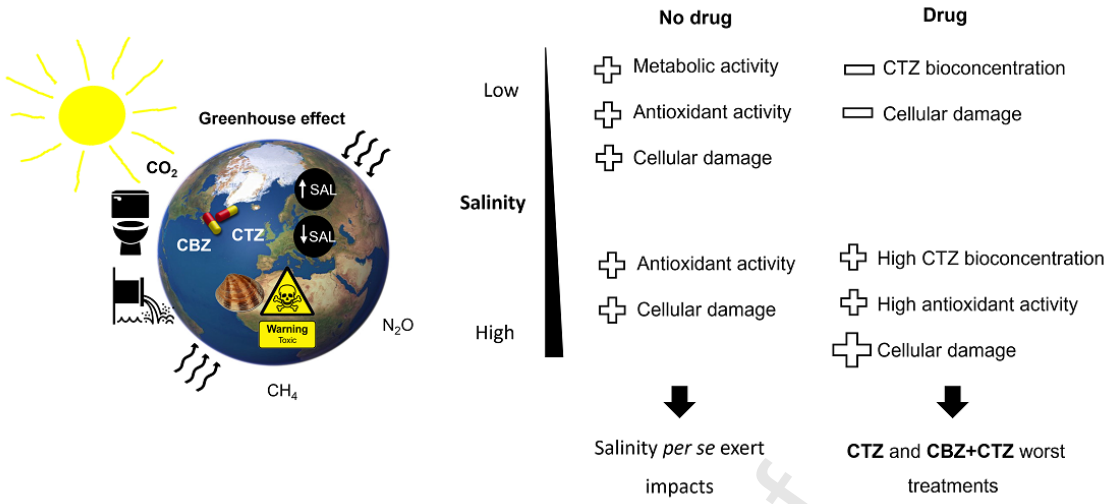
Amadeu M. V. M. Soares: funding

Rosa Freitas: Conceptualization; supervision; Writing - Review & Editing; funding

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



Graphical abstract

Highlights

- Salinity changes alter the impact of pharmaceutical drugs in marine bivalves
- Regardless salinity level, CTZ alone and combined with CBZ were the worst treatments
- Salinity *per se,* especially low salinity caused an impact
- Low salinity under the presence of pharmaceutical drugs exerted limited impacts
- CTZ and CBZ+CTZ with the higher impacts at higher salinity levels (35)