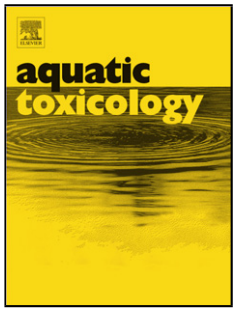

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Can ocean warming alter sub-lethal effects of antiepileptic and antihistaminic pharmaceuticals in marine bivalves?

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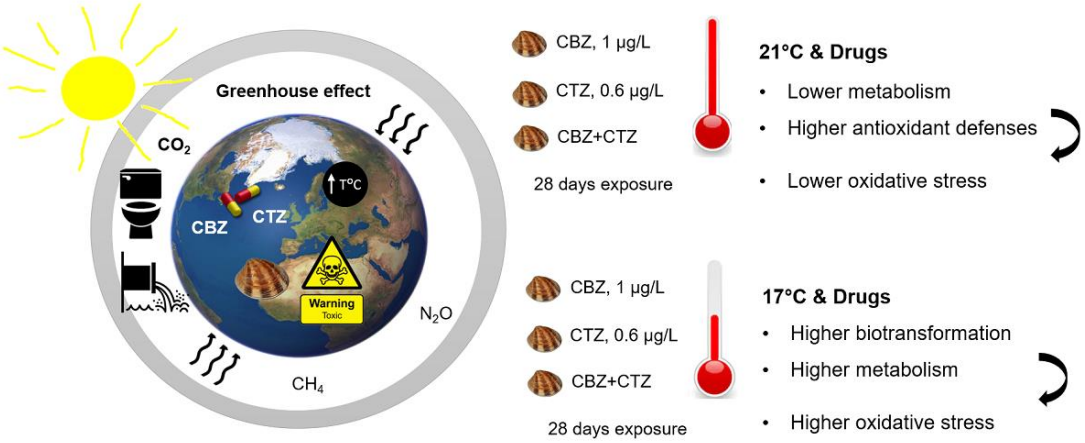
Graphical abstract



Highlights

- Limited impacts of drugs at control temperature and warming in clams
- No influence of warming on drug uptake and bioconcentration factor in clams
- Higher oxidative stress in contaminated clams at control temperature than warming
- Impacts of drugs acting together were lower than impacts of single exposures

Abstract

The negative effects induced in marine organisms by Climate Change related abiotic factors consequences, namely ocean warming, are well-known. However, few works studied the combined impacts of ocean warming and contaminants, as pharmaceutical drugs. Carbamazepine (CBZ) and cetirizine (CTZ) occur in the marine environment, showing negative effects in marine organisms. This study aimed to evaluate the impacts of ocean warming on the effects of CBZ and CTZ, when acting individually and combined (drug *vs* drug), in the edible clam *Ruditapes philippinarum*. For that, drugs concentration, bioconcentration factors and biochemical parameters, related with clam's metabolic capacity and oxidative stress, were evaluated after 28 days exposure to environmentally relevant scenarios of these stressors. The results showed limited impacts of the drugs (single and combined) at control and warming condition. Indeed, it appeared that warming improved the oxidative status of contaminated clams (higher reduced to oxidized glutathione ratio, lower lipid peroxidation and protein

carbonylation levels), especially when both drugs were combined. This may result from clam's defence mechanisms activation and reduced metabolic capacity that, respectively, increased elimination and limited production of reactive oxygen species. At low stress levels, defence mechanisms were not activated which resulted into oxidative stress. The present findings highlighted that under higher stress levels clams may be able to activate defence strategies that were sufficient to avoid cellular damages and loss of redox homeostasis. Nevertheless, low concentrations were tested in the present study and the observed responses may greatly change under increased pollution levels or temperatures. Further research on this topic is needed since marine heat waves are increasing in frequency and intensity and pollution levels of some pharmaceuticals are also increasing in coastal systems.

Capsule: Warming did not change the impacts induced by carbamazepine and cetirizine in *Ruditapes philippinarum* clams.

Keywords: Pharmaceutical drugs; climate change; bioconcentration; biomarkers; marine environment.

1. INTRODUCTION

The aquatic systems are increasingly subjected to a multitude of environmental stressors as Climate Change (CC) related abiotic factors. The increase of CO_2 (carbon dioxide) and other gases (e.g., methane) in the atmosphere, mostly derived from anthropogenic activities, are responsible for the "greenhouse effect" and consequent increase in ocean acidification and temperature (IPCC, 2019). These changes have already triggered significant effects on aquatic organisms, with most research on the effects of ocean warming (OW) and acidification at the organisms' individual level, as reviewed by Harley et al. (2006). Recently, the IPCC report on global warming (2018) confirms that CC is already exerting socio-economic and ecological consequences. This report further shows that without a sharp decline in greenhouse gas emissions by 2030, global warming will surpass 1.5°C in the following decades, leading to irreversible impacts on the most vulnerable ecosystems, where transitional systems as estuaries are included. Studies conducted with marine and estuarine species already demonstrated that temperature rise will exert changes from the molecular level to the ecosystem functioning, as reviewed by Yao and Somero (2014).

In the environment, CC related factors rarely occur isolated and generally aquatic systems are simultaneously affected by a range of stressors, including the presence of mixtures of pollutants. Organisms in a given environment are, therefore, rarely exposed to a single stressor. Pharmaceutical are an example of pollutants that have been detected in the aquatic environment (wastewater, surface water, groundwater, drinking water), including coastal systems, with concentrations ranging between ng/L up to several µg/L (e.g., Alygizakis et al., 2016; Fatta-Kassinos et al., 2011; Mezzelani et al., 2018; Patel et al., 2019). Carbamazepine (CBZ) and cetirizine (CTZ) are, respectively, an antiepileptic and an antihistaminic drug occurring in the aquatic environment with concentrations mainly in the ng/L range (e.g., Brumovskỳ et al., 2016; Calisto et al., 2011; Čelić et al., 2019; Ebele et al., 2017; Nödler et al., 2014;

Rehrl et al., 2020). However, higher concentrations (µg/L range) have also been found, especially in the freshwater ecosystem (e.g., Bahlmann et al., 2014, 2012, 2009). Although pharmaceuticals are considered a threat to aquatic organisms (Mezzelani et al., 2018), most of the research has been devoted to understand the occurrence and impacts of these compounds in freshwater ecosystem (e.g., Ebele et al., 2017) in comparison to estuarine and marine systems (e.g., Gaw et al., 2014). Marine bivalves have frequently high ecological and socio-economic value (Voultsiadou et al., 2010) being used by humans, shellfish consumers. Furthermore, bivalves are commonly used as bioindicator species of pollution in the coastal system since these filter-feeder organisms present a bioaccumulation potential, environmental resistance to different conditions, wide distribution, sedentary behaviour and low procurement costs (Helmholz et al., 2016; Strehse and Maser, 2020).

The effects of pharmaceutical drugs and CC related abiotic factors consequences as OW have been investigated in bivalves, showing individual and sub-individual impacts (tissue, cellular and molecular levels of biological organization) (e.g., Anacleto et al., 2014; Fabbri and Franzellitti, 2016; Matoo et al., 2013; Matozzo et al., 2013; Mezzelani et al., 2018; Michaelidis et al., 2005; Yao and Somero, 2014). However, studies that evaluated the interactive effects of CC related abiotic factors and pharmaceutical drugs in seawater environments are limited to a few marine species (fish, bivalves, protozoa) and drugs (e.g., Almeida et al., 2018a; Blewett et al., 2013; Campos et al., 2016; Correia et al., 2016; Costa et al., 2020; Franzellitti et al., 2020; Freitas et al., 2020, 2019a, 2019b, 2015; Gomiero and Viarengo, 2014; Gonzalez-Mira et al., 2018; González-Mira et al., 2016; Maulvault et al., 2019, 2018a, 2018b, 2018c, 2018d; Munari et al., 2019, 2018, 2016; Scott et al., 2019; Serra-Compte et al., 2018; Su et al., 2019). Under these conditions, CC can alter pharmaceutical drugs bioavailability, changing drugs physical-chemical properties and consequently their toxicity. Climate changes may also influence marine species

sensitivity, altering the drug uptake, detoxification and metabolism. This work intended to evaluate the bioconcentration and biochemical effects of two pharmaceutical drugs (CBZ and CTZ), when acting alone and combined under a scenario of OW, based on environmentally relevant conditions.

2. MATERIALS AND METHODS

2.1 Experimental conditions

The clam *Ruditapes philippinarum* (Adams & Reeve, 1850) (mean length: 4.2±0.3 cm; mean width: 3.0±0.2 cm), collected in Mira channel (Aveiro, Portugal), a southern arm of the Ria de Aveiro, was used as bioindicator species in this study.

After collection, clams were maintained in the laboratory for eight days, for depuration and acclimation to laboratory conditions, following the procedure of Almeida et al. (2018b). Salinity 25 was selected based on clam's natural habitat conditions and limitations on drugs quantification procedure (Almeida et al., 2014).

After this period, half of the organisms was maintained at the control temperature (17ºC) and the other half was submitted to a slow temperature rise (1ºC per day) until 21ºC was reached. This temperature level was selected based on the expected effects of CC in seawater temperature (predicted temperature rise and extreme weather events) (IPCC, 2019).

The experimental setup consisted of a chronic exposure (28 days) to carbamazepine (CBZ) and cetirizine (CTZ), acting alone and combined (drug *vs* drug), under control (CTL) temperature (17ºC) and OW scenario (21ºC), with the following conditions for each temperature tested: control (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 work were selected based on the concentrations found in Ria de Aveiro as well as in other aquatic systems (e.g., Bahlmann et al., 2012; Calisto et al., 2011) and in previous studies that revealed biological effects at these levels (e.g., Almeida et al., 2018b).

Three aquaria per condition, filled with 6 L of artificial seawater (salinity 25±1 g/L) and with six individuals in each were used. Along the 28 days of exposure containers were submitted to the conditions of aeration, room temperature, photoperiod, food, and water renewal following Almeida et al. (2018b). Mortality was checked daily.

Blanks (two aquaria per condition, with the same drug concentrations used in the assay and exposed to the same conditions but without organisms) were also prepared following Almeida (2018b).

To confirm drugs concentration in the exposure medium and blanks, in all conditions, along the entire assay, aliquots of water were collected each week immediately after spiking (beginning of the exposure week) and immediately before the water renewal (end of the exposure week) (here identified as sampling moments).

After 28 days of exposure, organisms were frozen and mechanically pulverized with liquid nitrogen. Aliquots of 0.3 g fresh weight (FW) were prepared from each homogenized clam (six organisms per condition), to be used for biochemical analyses and drug quantification. The fresh tissue aliquots of the same organisms were used in both biochemical and drug determination.

2.2. Determination of drugs concentrations

The concentrations of CBZ and CTZ were quantified in clam's tissues (0.3 g FW aliquots) and water from the exposure medium and blanks, by a direct competitive ELISA (Enzyme-Linked Immunosorbent Assay), according to Almeida et al. (2018b).

2.3 Biochemical analyses

Biochemical analyses were determined in the whole soft tissues of *R. philippinarum*, using protocols already described previously (Almeida et al., 2018b, 2017; Costa et al., 2020). For each biochemical analyses, 0.3 g FW of soft tissue per organism was used, to assess: indicators of energy metabolism (electron transport system activity, ETS; total protein content, PROT; glycogen content, GLY); cellular damage (lipid peroxidation levels, LPO; protein carbonylation levels, PC); redox homeostasis (ratio between reduced (GSH) and oxidized (GSSG) glutathione); antioxidant and biotransformation capacity (activities of the enzymes: superoxide

dismutase, SOD; glutathione peroxidase, GPx; glutathione S-transferases, GSTs) and neurotoxicity (acetylcholinesterase activity, AChE).

2.4 Data and statistical analyses

The bioconcentration factor (BCF) (Arnot and Gobas, 2006), for each condition, was calculated according to Almeida et al. (2018b), using an average of the concentrations in the exposure medium, obtained immediately before the water renewal (end).

Drug concentrations in clam's soft tissues, from the exposure medium and blanks, BCF's and biochemical parameters, obtained from each condition, 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 temperature level (17ºC, 21ºC) and sampling moment (beginning of exposure weeks (beginning); end of exposure weeks (end)), no significant differences exist between the concentrations in the exposure medium and blanks of each drug treatment (e.g., 17ºC, beginning, control *vs* 17ºC, beginning, CBZ-single; b) for each temperature and drug treatment, no significant differences exist between the concentrations in the exposure medium and blanks at the beginning and end of all weeks of exposure (e.g., 17ºC, beginning, CBZ-single *vs* 17ºC, end, CBZ-single); c) for each sampling moment and drug treatment, no significant difference exist in the concentrations in the exposure medium and blanks between temperature levels (e.g., 17ºC, beginning, CBZ-single *vs* 21ºC, beginning, CBZ-single); d) for each temperature level, no significant differences exist in drug concentrations in clams tissues and BCF between drug treatments (CBZ-single, CTZ-single, CBZ-combined, CTZ-combined) (e.g., 17ºC, CBZ-single *vs* 17ºC, CTZ-single); e) for each drug treatment, no significant differences exist in drug concentrations in clams tissues and BCF between temperature levels (e.g., 17ºC, CBZ-single *vs* 21ºC, CBZ-single); f) for each temperature and biochemical marker, no significant differences exist in clams

responses between drug treatments (CTL, CBZ, CTZ, CBZ+CTZ) (e.g., 17ºC, CTL *vs* 17ºC, CBZ); g) for each drug treatment and biochemical marker, no significant differences exist in clams responses between temperature levels (17ºC, CTL *vs* 21ºC, CTL). The significant differences ($p \leq 0.05$), when existing, were represented in the figures with a different letter (for hypotheses a, d, f), with an asterisk (for hypotheses b, e, g) or with a cardinal (for hypothesis c). The *p* values for the previously comparisons made are present in Tables 2 (for drug concentrations in exposure medium), 3 (for drug concentrations in blanks), 4 (for drugs concentrations in clams' tissues and BCF) and 5 (for biochemical results).

3. RESULTS

3.1 Mortality

After 28 days of exposure dead clams were observed in two conditions: CBZ 17ºC (6% of mortality) and CTZ 21ºC (11% of mortality).

3.2 Drugs concentrations in the exposure medium

Drug concentrations in the exposure medium (Table 1) and blanks (data not shown), for each drug (single and combined), at the same sampling moment (immediately after spiking and immediately before water renewal) and temperature level, were similar to the nominal concentrations. For the same drug treatment, at a similar sampling moment and temperature level, no significant differences were observed comparing single and combined drug treatments (CBZ-single *vs* CBZ-combined; CTZ-single *vs* CTZ-combined) both in exposure medium and blanks. However, exceptions were observed in the exposure medium, between CBZ-single and CBZ-combined as well as between CTZ-single and CTZ-combined, both at 17ºC, at the end of the exposure weeks. Moreover, for the exposure medium and blanks, comparing sampling moments for each drug treatment at temperature level, no significant changes occurred between the beginning and end of the exposure weeks, except in the exposure medium for CBZ-single at 21ºC. Also, no significant differences were observed comparing temperature levels (17ºC *vs* 21ºC) for each drug treatment at the same sampling moment in the exposure medium and blanks, except at CTZ-combined (beginning) in blanks. The previous results validate the spiking methodology due to the constant drug concentrations in the exposure medium and blanks along the 28 days of exposure and similarity with the nominal concentrations.

3.3 Drugs concentrations in the clams' tissues and bioconcentration factor

The results of drug tissues concentrations and BCFs are shown in Figure 1. At each temperature condition (17ºC and 21ºC), a significantly higher concentration of CTZ in clams' tissues comparing with CBZ tissue concentrations was observed, both in single and combined treatments (Figure 1A). Concerning the differences among single and combined treatments for the same drug condition, CBZ tissue concentration decreased comparing single and combined treatments (CBZ *vs* CBZ-combined) at 17ºC but remained similar in both conditions at 21ºC. For CTZ, no significant differences were observed comparing single and combined treatments (CTZ *vs* CTZ-combined) for both temperatures tested. For each drug treatment tested, no significant differences were found comparing the two temperatures (17ºC and 21ºC). Similar responses to the previously reported were found for BCF results (Figure 1B).

3.4. Biochemical analyses

3.4.1 Indicators of energy metabolism

The results of the biochemical analyses related with energy metabolism are depicted in Figure 2. ETS activity (Figure 2A) increased significantly in clams exposed to CTZ 17ºC comparing with the remaining conditions, except at CBZ 17ºC. No significant differences were observed at 21ºC comparing contaminated (CBZ, CTZ, CBZ+CTZ) clams with the control, although a significant increase occurred comparing CTZ with CBZ. Comparing control temperature and OW for the same drug treatment, significant differences were observed at CBZ and CTZ exposed clams, where ETS activity was significantly lower at OW comparing with the control temperature.

Regarding GLY content (Figure 2B), no significant differences were found among treatments. However, CBZ exposed clams at 17ºC showed higher values. At OW, GLY levels significantly decreased at CBZ+CTZ comparing with the remaining conditions. Comparing the same drug exposure condition at the two temperatures, significantly higher GLY content was observed at control temperature than at 21ºC in clams exposed to CBZ+CTZ.

At control temperature, PROT content (Figure 2C) was significantly higher in CBZ exposed clams comparing with the remaining conditions except with CTZ. No significant differences were observed at 21ºC among the treatment conditions. Comparing temperature levels, significantly higher PROT content was observed at 17ºC comparing with OW in clams exposed to CBZ and CBZ+CTZ.

3.4.2 Indicators of cellular damage

The results of the parameters indicators of cellular damage are represented in Figure 3. At 17ºC significant differences in LPO levels (Figure 3A) were observed comparing CBZ and CTZ, acting individually and the combined condition, CBZ+CTZ. At OW, significantly lower LPO levels occurred comparing CBZ and CBZ+CTZ with the control condition. Significant differences between temperatures for the same drug treatment were observed for CBZ, CTZ and CBZ+CTZ, with higher LPO levels at 17ºC than at 21ºC.

Concerning PC results (Figure 3B), at 17ºC, significantly lower levels were observed at CBZ+CTZ comparing with the non-contaminated clams. At OW, significantly higher PC levels occurred in clams exposed to CBZ and CTZ (both acting single) comparing with the remaining conditions. Comparing the same drug treatment at both temperatures, significantly higher values were found at CTL 17ºC comparing with CTL 21ºC.

GSH/GSSG levels (Figure 3C) at 17ºC showed significantly lower values at CTZ exposed clams compared with clams under control conditions. At 21ºC significantly lower values were observed at CTZ compared with CTL and CBZ+CTZ conditions. Comparing temperatures, significant differences were observed at CBZ+CTZ, with higher values at 21ºC comparing with 17ºC.

3.4.3 Oxidative stress-related enzymatic activity

The results of oxidative stress-related enzymatic activity are depicted in Figure 4. At 17ºC, SOD activity (Figure 4A) was significantly increased at drug exposure treatments (CBZ, CTZ, CBZ+CTZ) comparing with the non-contaminated clams. The same response was observed at 21ºC. Comparing temperatures for the same drug treatment, significant differences were observed for CTZ and CBZ+CTZ, with higher levels at OW comparing with temperature control.

For the temperature control, the activity of GPx (Figure 4B) was, in general, similar or higher (significantly only CBZ) comparing the control and the remaining conditions. No significant differences were observed comparing all conditions at OW. Higher GPx activity was observed in non-contaminated clams at 21ºC than at 17ºC.

The activity of GSTs (Figure 4C) was significantly higher in contaminated clams comparing with non-contaminated organisms at 17ºC. At 21ºC no significant differences were observed comparing all the conditions. Comparing the same drug condition at the two temperatures, significantly higher GSTs activity occurred at CBZ+CTZ at 17ºC compared with 21ºC.

The activity of AChE (Figure 4D) was similar among conditions (contaminated and non-contaminated clams) exposed at temperature control and OW. No significant differences were observed comparing the same drug treatment at the two temperatures.

4. DISCUSSION

The impacts of CC related abiotic factors (e.g., OW) and contaminants (e.g., pharmaceutical drugs) to the aquatic organisms are well known. However, the combined impacts of CC and pharmaceutical drugs in seawater environments are scarce and limited to a few marine species and drugs. Thus, the impacts of OW on the effects of CBZ and CTZ on the bioconcentration and biochemical endpoints in *R. philippinarum* related to energy metabolism and oxidative stress were evaluated in the present study.

4.1 Impacts of OW on the bioconcentration of CBZ and CTZ by *R. philippinarum*

The results of drugs tissue concentration and BCF in clams showed higher accumulation of CTZ (both acting alone or combined with CBZ), comparing with CBZ, even lower CTZ exposure concentration was used (CTZ: 0.6 µg/L; CBZ: 1 µg/L). These results are in accordance with previous studies that showed higher uptake and BCF of CTZ over CBZ by marine clams (Almeida et al., 2018a, 2018b, 2017). These results may indicate that different mechanisms can be associated with the uptake, detoxification and excretion of CBZ and CTZ in clams. In vertebrates, CTZ is primarily eliminated with limited metabolism of the parent drug while CBZ is extensively metabolized through the action of CYP450 enzymes system (Church and Church, 2013; Marvanova, 2016). Thus, the observed lower tissue levels of CBZ in comparison with CTZ may reflect higher degree of clams CBZ detoxification (e.g., phase I biotransformation) or/and transformation in metabolites, which are not detected by the quantification method applied. On the other way, CTZ may present low transformation into metabolites, being mostly present as the parent compound. The occurrence of CBZ metabolites in marine bivalves exposed to the drug was already reported in literature (Abdelhafidh et al., 2018; Boillot et al., 2015). However, for CTZ, there are no studies on metabolite identification in marine bivalves. Also, comparing with previous

studies, only for CTZ a species-specific drug uptake in bivalves was observed. Indeed, lower BCF (1.6 L/kg FW) was observed in mussels (*Mytilus galloprovincialis*) exposed for 28 days at a similar CTZ exposure concentration (0.3 µg/L) (Teixeira et al., 2017) comparing with the present study (BCF 4 L/kg FW in *R. philippinarum*). Concerning CBZ exposure, similar BCFs occurred in both clams and mussels exposed at similar concentrations and assay length: BCF of 1.2 L/kg FW in *R. philippinarum* and 1.5 L/kg FW in *M. galloprovincialis* at CBZ 3 µg/L and 28 days of exposure (Almeida et al., 2015; Oliveira et al., 2017). Also, in mussels exposed to similar conditions (0.3 µg/L, 28 days exposure), the BCF was identical for CBZ (BCF: 1.5 L/kg FW) and CTZ (BCF: 1.6 L/kg FW) (Oliveira et al., 2017; Teixeira et al., 2017). The present study further revealed that when clams were submitted to the combination of both drugs, generally, the uptake was slightly decreased for CBZ and slightly increased for CTZ, despite not always accompanied by significant changes. Previous studies demonstrated that CBZ is generally more stressful than CTZ to marine bivalves at environmental concentrations (e.g., Almeida et al., 2017, 2015). Thus, organisms may attempt to restrict more the uptake of CBZ (e.g., limiting the feeding rate) comparing with CTZ in the presence of even higher stressful condition (CBZ+CTZ).

Concerning the impacts of OW on the tissue concentration levels and BCF of CBZ and CTZ, the present findings showed that OW had no influence on these, neither when the drugs were acting individually nor combined. Few works addressed the impacts of OW on the uptake of pharmaceutical drugs by marine bivalves, with different patterns of responses, which turns difficult to discuss this issue. For example, similarly to what was observed in the present study, Serra-Compte et al. (2018) showed no significant effects exerted by OW on the BCF of CBZ in *M. galloprovincialis* exposed for 20 days to 15.7 µg/L (~ 30 L/kg DW (7.5 L/kg FW) at both 18ºC and 22ºC). These authors also tested other pharmaceutical drugs (sotalol, sulfamethoxazole, venlafaxine and citalopram), reporting either increases or decreases on the uptake of drugs in mussels, promoted by OW. These responses were related with the increase of

organism's metabolic activity, i.e., by increasing feeding adsorption leading to higher uptake of contaminants or by enhancing drug depuration conducting to their elimination. Despite using a different drug, Freitas et al. (2019b) showed that OW (17ºC, control temperature; 21ºC) didn't change the BCF of diclofenac (1 µg/L) (~39 ng/g DW at 17ºC and ~44 ng/g DW at 21ºC) in *M. galloprovincialis* mussels exposed for 28 days. Other studies found variable results concerning the impacts of different CC related factors in bivalve's tissue drugs concentrations. For example, Almeida et al. (2018a) observed no impacts of acidification (pH 7.8 *vs* pH 7.5, ΔpH -0.3) on CBZ uptake and BCF in *R. philippinarum* exposed for 28 days to 1 µg/L. Freitas et al. (2016, 2015) found that acidification (pH 7.8 *vs* pH 7.1, ΔpH -0.7) decreased the uptake of CBZ (3 µg/L) in *Scrobicularia plana* after 96 h of exposure, but no changes occurred after 28 days of exposure. Costa et al. (2020) found a decrease in the tissue concentration levels of diclofenac (1 µg/L) in *R. philippinarum* and *R. decussatus* under the combination of both acidification (pH 7.7, ΔpH -0.4) and warming (21ºC, ΔTºC + 4ºC), comparing with the control conditions (17ºC and pH 8.1). Also, Su et al. (2019) found that ocean acidification (pH 7.8 and pH 7.4, ΔpH -0.4) decreased the accumulation of chloramphenicol (100 ng/L) and nitrofurazone (150 µg/L) in *Tegillarca granosa*, comparing with the control pH (8.1). However, it has been reported that acidification, more than warming, is the dominant factor when both stressors are combined (Serra-Compte et al., 2018). In general, the existing studies reported different patterns of responses (uptake, detoxification) depending on the compound, marine species and CC (e.g., Almeida et al., 2018a; Freitas et al., 2020, 2019b; Maulvault et al., 2018b; Scott et al., 2019; Su et al., 2019). It was observed that OW either increased or decreased pharmaceutical drugs uptake by marine organisms, particularly when both stressors (drug, acidification and warming) were acting in combination. However, more research on this topic is necessary for a proper assessment of the combined impacts of CC and pharmaceutical drug contamination in marine organisms.

4.2 Impacts of OW on the biochemical alterations induced in *R. philippinarum* exposed to CBZ and CTZ

The combined impacts of OW and pharmaceutical drugs on the biological endpoints of marine organisms was studied in a few works, referred previously (e.g., Costa et al., 2020; Serra-Compte et al., 2018).

Concerning the effects of CBZ and CTZ on the energy metabolism indicators, the main parameter altered was the electron transport system activity (ETS). Under temperature control (17ºC), clams showed higher metabolic activity only in the presence of CTZ with a tendency to decrease ETS activity at CBZ+CTZ. Such response may be related with clams "perception" of an increased threat at this condition, in comparison with the risk induced by each drug acting alone. With this, the closure of bivalve's valves under the most threatened condition to reduce their filtration could avoid the accumulation of contaminants (Freitas et al., 2019b). On the other hand, one may also hypothesize that under higher stress levels clams were able to actively limit their metabolism, in an attempt to reduce their filtration capacity and avoid drugs accumulation. However, the slight decrease in the metabolic activity was not enough to limit drugs tissues concentrations and BCF at the combined exposure. Although previous studies conducted by Almeida et al. (2018b, 2017, 2015) reported a decrease or lack of changes in the metabolic activity in *R. philippinarum* exposed to CTZ and CBZ under similar concentrations and assay length, no studies are known on the combined effects of these two drugs.

When combining OW with CBZ or CTZ (CBZ 21ºC; CTZ 21ºC) exposure, a clear decrease on clams metabolic capacity was observed. These results suggest again a slowdown in the metabolism to cope with a possibly higher stressful condition. Higher temperatures can provide benefits (e.g., enhanced animal fitness) but can also exert negative impacts on the energy metabolism (e.g., additional energetic costs) when it's not in the optimal level for a species or additional stressors are present (Maulvault et

al., 2018d). However, Matoo et al. (2013) showed that an exposure for 15 weeks to temperature levels (22ºC to 27ºC), as expected in the global CC, had minimum impacts on the energy metabolism in clams *Mercenaria mercenaria*. The authors related this response with the adaptation of clams to fluctuations of temperature that occur naturally in their environment. When studying the impacts of OW on the effects of diclofenac to *M. galloprovincialis*, Freitas et al. (2019b) showed that the temperature increase (from 17 to 21ºC) had no impact on the contaminated mussel's energy metabolism (ETS activity).

Despite the changes in the metabolic activity observed in the present study, energy reserves were maintained among conditions revealing reduced impacts on PROT and GLY contents due to drugs exposure and temperature increase. Nevertheless, GLY levels decreased significantly under CBZ+CTZ at 21ºC comparing with the remaining conditions, suggesting the expense of the cellular reserves, possibly to obtain energy to fuel up the antioxidant system defense.

Concerning the effects of drugs on the indicators of cellular damage, it was observed that neither CBZ nor CTZ, acting individually or combined exerted cellular damage on cellular membranes (LPO), although a tendency for an increase in LPO levels was observed in CTZ and CBZ-exposed clams at 17ºC. Besides the lack of oxidative injury to the cellular membranes, no oxidative damage to proteins (PC) occurred at 17ºC. Despite no significant cellular damage was observed, higher LPO levels in CBZ and CTZ clams acting alone may be related with higher reactive oxygen species (ROS) production associated with higher metabolic activity (ETS) at these conditions. Since in mitochondria takes place ROS production, higher metabolic activity may result in a higher ROS production, ultimately causing oxidative stress. Previous studies, performed with similar exposure concentrations and assay length, showed that CBZ is responsible for the induction of cellular damage in marine bivalves (Almeida et al., 2018b, 2015; Freitas et al., 2016). On the other way, CTZ, while inducing oxidative stress does not exert damage to cellular membranes or proteins at environmental

concentrations (Almeida et al., 2018b, 2017; Teixeira et al., 2017). Indeed, cellular damage (LPO) was only observed at higher non-environmental concentrations (3-12 µg/L) (Teixeira et al., 2017). Also, it seemed that under the combination of both drugs at temperature control, LPO and CP levels were lower comparing to the compounds acting individually, possibly being related with the slowdown in the metabolism to cope with the suggested higher stressful condition.

Temperature rise has been related with oxidative stress due to an imbalance between the production and elimination of ROS (Abele et al., 2002). In the present study, OW, although exerting no oxidative damage itself, seemed to have a protective role when combined with pharmaceutical drugs. Indeed, CBZ and CTZ contaminated clams presented, in general, lower LPO levels, that were even lower in clams exposed to the combination of drugs at 21ºC. Accordingly, the GSH/GSSG levels increased at this condition (CBZ+CTZ) comparing OW with temperature control, indicating a better oxidative status. Furthermore, the increase of temperature resulted in mechanisms to avoid oxidative damage to proteins (PC) at control clams, but when combined with drugs (CBZ 21ºC; CTZ 21ºC) an increase in oxidized proteins content was observed.

Thus, the results obtained seem to reveal a different impact of temperature on the drug's effects on oxidative stress, especially when acting individually. At 17ºC, higher impacts were generated on cellular membranes through a general increase in LPO, while at increased temperature higher impacts were exerted on proteins, through increased PC. The present findings also indicate that clams exposed to the combination of both drugs at 21ºC, presented better oxidative status (reduced LPO and PC levels and increased GSH/GSSG) than at 17ºC. Such results may be associated with clam's defense mechanisms. In particular, the activities of SOD and GSTs, enzymes involved in ROS elimination (besides biotransformation, GSTs are also capable of inactivating lipoperoxidation products), were altered by OW, drug exposure or the combination of both. In fact, the results obtained indicate that these enzymes had different responses in accordance to the temperature of exposure. At 17ºC and in

the presence of CBZ and CTZ (single exposures), the increased activity of GSTs and ETS possibly resulted in higher ROS content, formed during the detoxification process and in the mitochondria, thus, leading to the LPO levels observed. However, the same was not observed for the combination of both drugs (CBZ+CTZ). Indeed, at temperature control, although GSTs activity was increased, ETS activity was similar to the control levels, thus, possibly, lower content of ROS was produced as a consequence of the energy metabolism slowdown, resulting into lower levels of LPO, comparing with action of single drugs. At warming conditions, the slowing down of the metabolism was more evident, and the activity of SOD was enough to eliminate the produced ROS, resulting into lower LPO levels, especially at the combination of both drugs. In accordance, Freitas et al. (2020) observed that, under warming conditions (21ºC) and in the presence of salicylic acid (4 mg/L, 28 days of exposure), the antioxidant defense mechanisms (e.g., SOD) of *M. galloprovincialis* were enough to avoid increased LPO levels compared with contaminated mussels exposed to the temperature control (17ºC).

In what regards to neurotoxicity, in the present study, no changes in AChE activity was observed in clams exposed to CBZ and/or CTZ, at both temperatures, indicating that the conditions used were not enough to cause neurotoxicity. However, previous studies showed that CBZ is neurotoxic to marine bivalves when a decrease of 20% or more in AChE activity is observed. Possibly, CBZ-ROS impacts may also lead to the development of neurotoxicity due to, among others, the high rate of oxygen consumption by the central nervous system (Sayre et al., 2008). Aguirre-Martínez et al. (2016) showed a decrease of AChE activity at all exposure concentrations (0.1-50 µg/L) in *R. philippinarum* exposed for 14 days, being related with the action of CBZ as a mood stabilizer (strong inhibitors of AChE). Using the same species, Trombini et al. (2019) observed, however, an increase of AChE activity in clam's gills after 7 days of exposure to CBZ 15 µg/L that was restored to control levels by the end of the exposure

period (14 days). Also, in mussels *Perna viridis* hemolymph CBZ was found to inhibit AChE activity at 96 ng/L (7 days exposure) (Juhel et al., 2017).

5. CONCLUSIONS

The present work addressed the impacts of OW on the effects of pharmaceutical drugs (CBZ and CTZ), acting alone or combined, to the marine clam *R. philippinarum*. The results obtained showed limited effects on the uptake and biochemical analyses related with oxidative stress caused by CBZ, CTZ or the combination, regardless the temperature. Furthermore, the present study showed that warming did not change the capacity of clams to uptake the drugs, and it seemed to improve clam's oxidative status, especially when exposed to the combination of both drugs. Indeed, warming led to a slowdown of metabolism (reduced ETS), being enough for the antioxidant defences to avoid cellular damage and improve oxidative status.

CRediT author statement

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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.

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Figure 1. A) Drugs concentrations in tissues (CBZ, CTZ, ng/g FW) and B) Bioconcentration factor (BCF), in *R. philippinarum* exposed under control temperature (17ºC) and ocean warming condition (21ºC) to single and combined drug treatments (CBZ, 1 µg/L; CTZ, 0.6 µg/L; CBZ, 1 µg/L + CTZ, 0.6 µg/L) for 28 days. Results represent the mean + standard deviation. Significant differences ($p \leq 0.05$) between drug treatments for each temperature level (17ºC, 21ºC) are presented with letters. Significant differences ($p \leq 0.05$) between temperature levels for each drug treatment are presented with an asterisk.

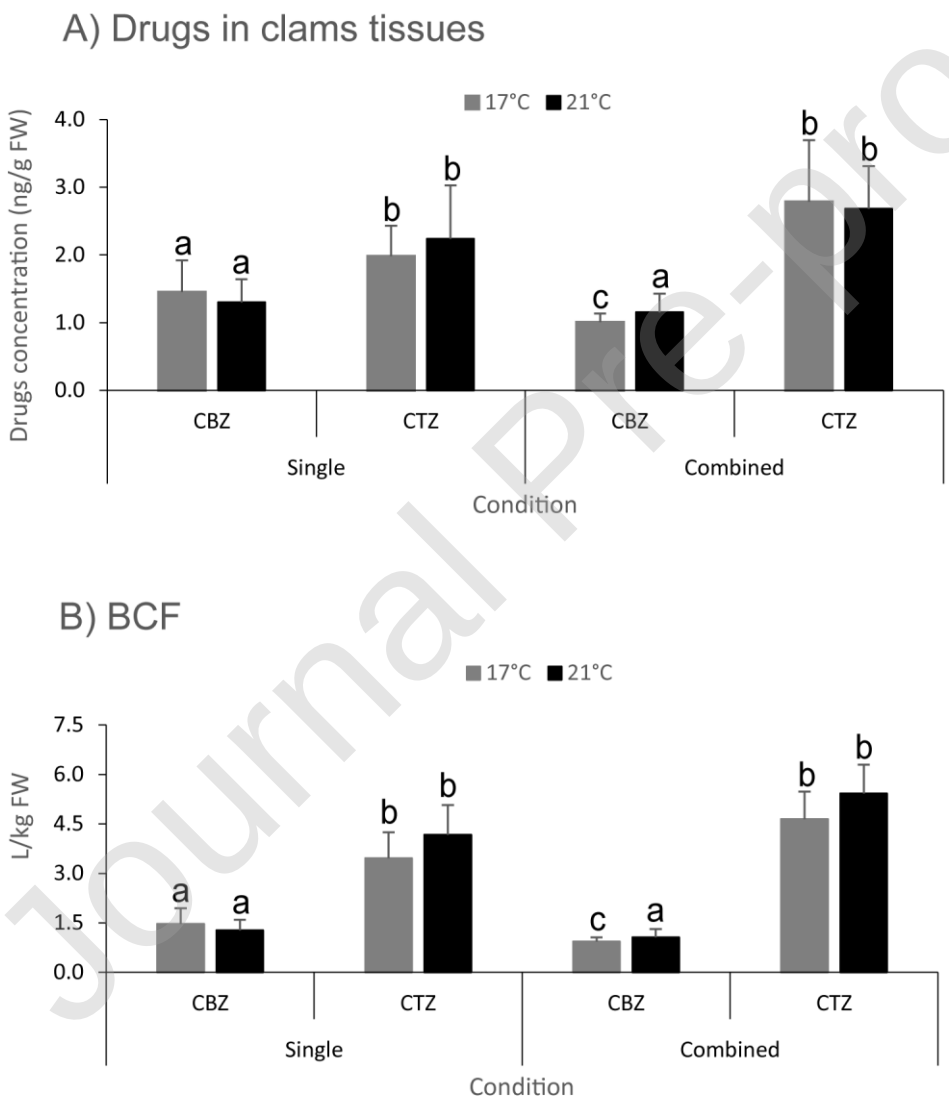


Figure 1

Figure 2. Energy-related parameters: A) ETS, energy transport system activity; B) GLY, glycogen content; C) PROT, protein content in *R. philippinarum* exposed under control temperature (17ºC) and ocean warming condition (21ºC) to single and combined drug treatments (CBZ, 1 µg/L; CTZ, 0.6 µg/L; CBZ, 1 µg/L + CTZ, 0.6 µg/L) for 28 days. Results represent the mean + standard deviation. Significant differences ($p \leq 0.05$) between drug treatments for each temperature level (17ºC, 21ºC) are presented with letters. Significant differences ($p \leq 0.05$) between temperature levels for each drug treatment are presented with an asterisk.

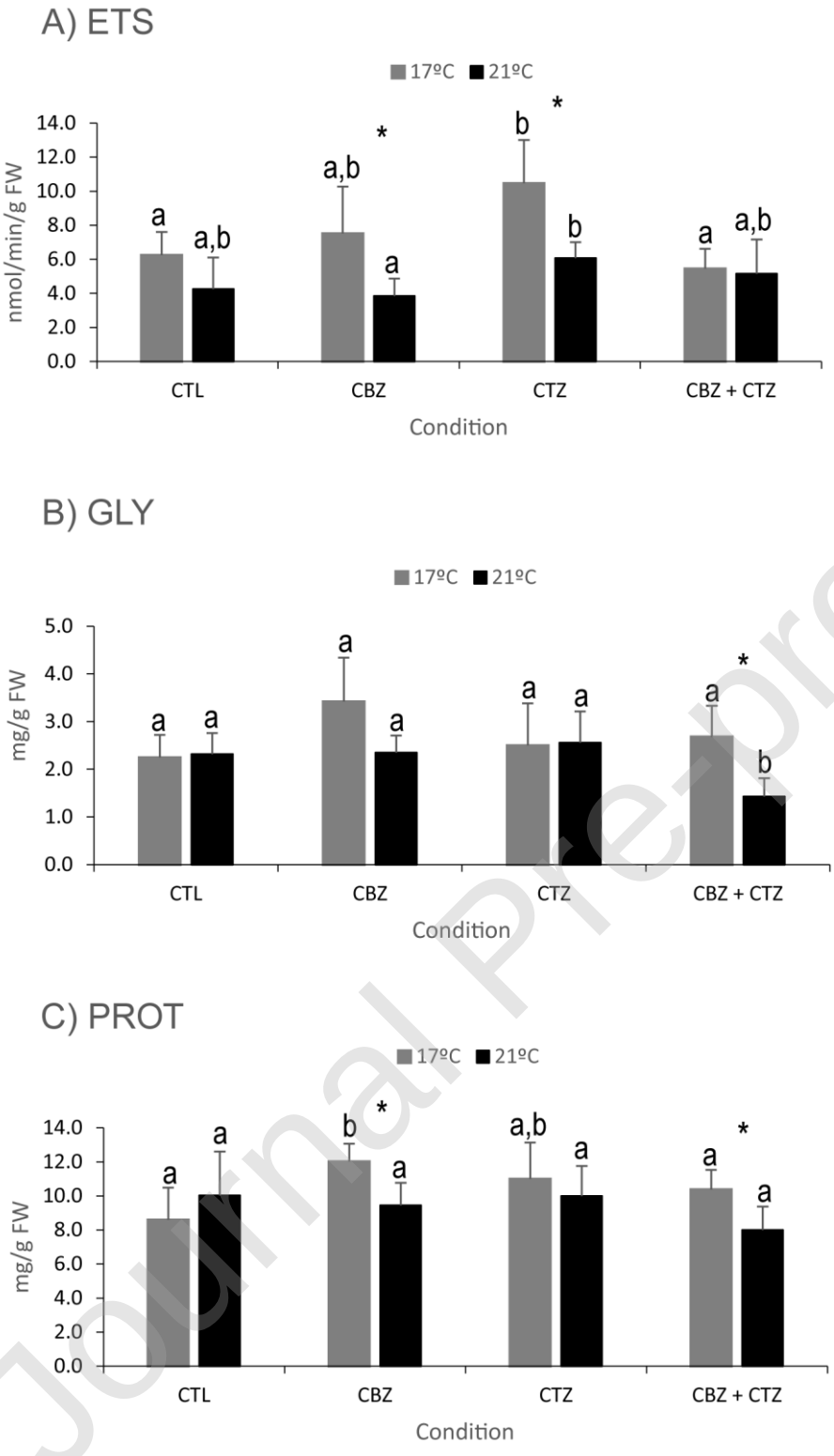


Figure 2

Figure 3. 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 under control temperature (17ºC) and ocean warming condition (21ºC) to single and combined drug treatments (CBZ, 1 µg/L; CTZ, 0.6 µg/L; CBZ, 1 µg/L + CTZ, 0.6 µg/L) for 28 days. Results represent the mean + standard deviation. Significant differences ($p \leq 0.05$) between drug treatments for each temperature level (17ºC, 21ºC) are presented with letters. Significant differences ($p \leq 0.05$) between temperature levels for each drug treatment are presented with an asterisk.

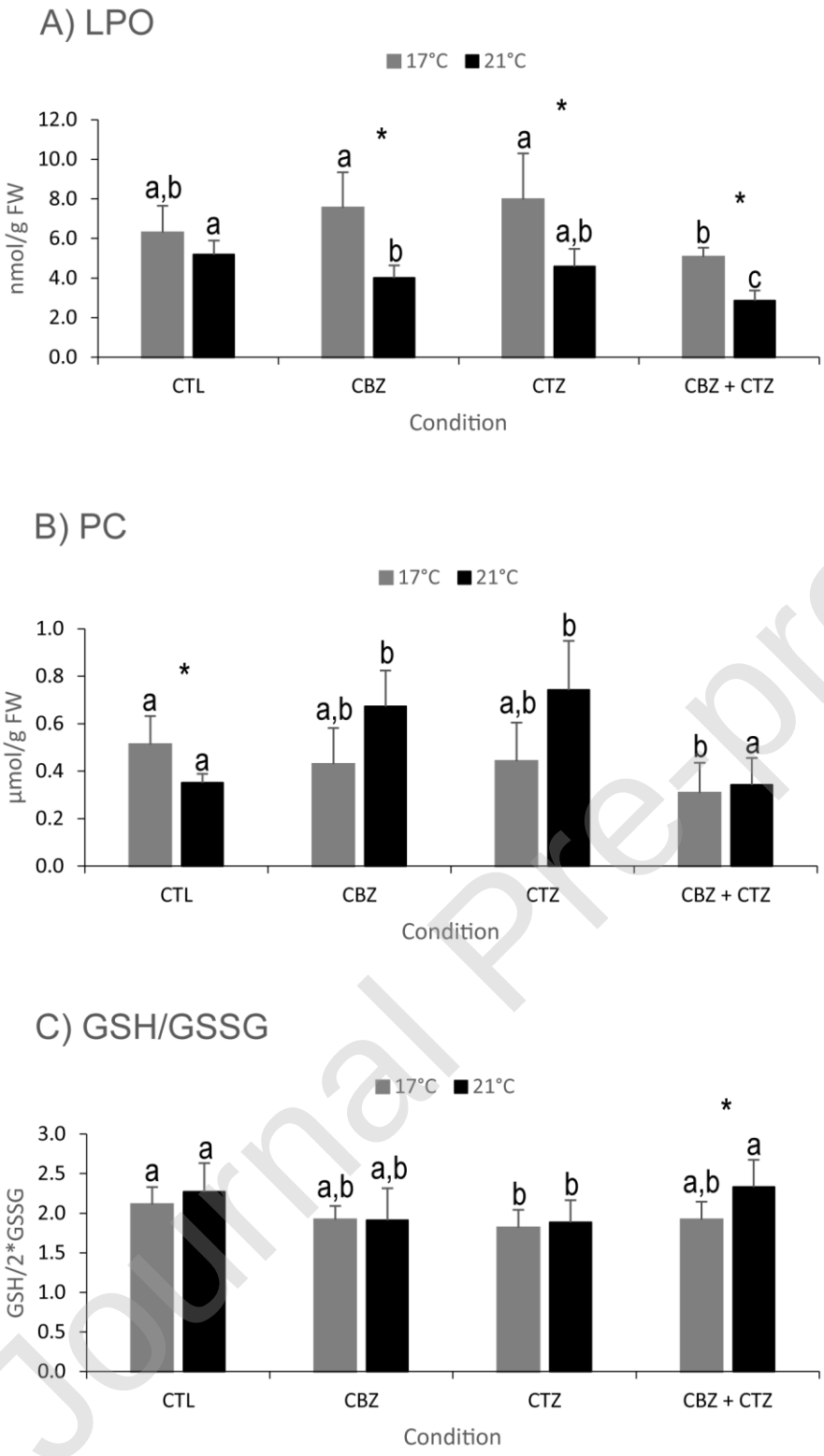
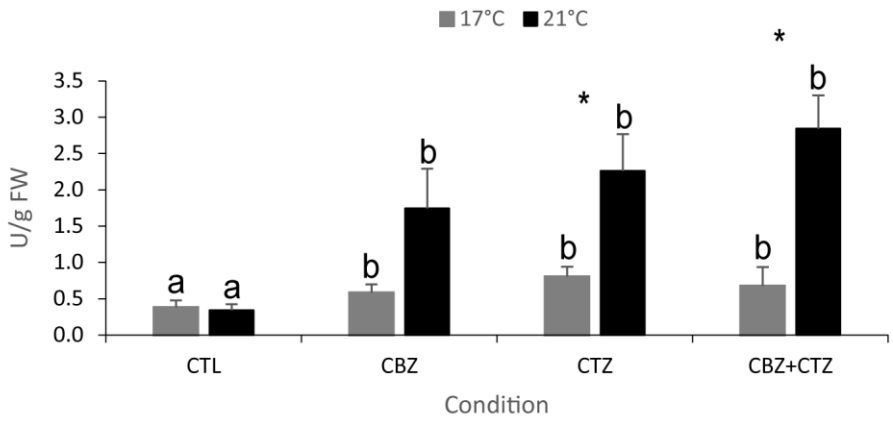


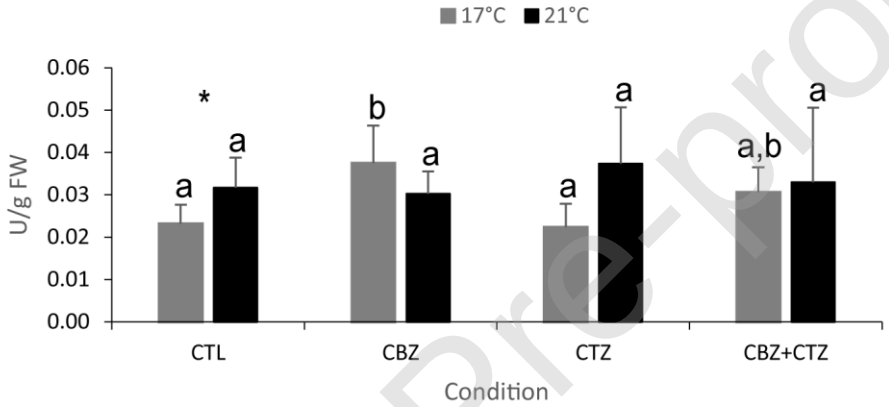
Figure 3

Figure 4. Oxidative stress related enzymatic activity: A) SOD, superoxide dismutase; B) GPx, glutathione peroxidase; C) GSTs, glutathione S-transferases; D) AChE, acetylcholinesterase in *R. philippinarum* exposed under control temperature (17ºC) and ocean warming condition (21ºC) to single and combined drug treatments (CBZ, 1 µg/L; CTZ, 0.6 µg/L; CBZ, 1 µg/L + CTZ, 0.6 µg/L) for 28 days. Results represent the mean + standard deviation. Significant differences ($p \leq 0.05$) between drug treatments for each temperature level (17ºC, 21ºC) are presented with letters. Significant differences ($p \leq 0.05$) between temperature levels for each drug treatment are presented with an asterisk.

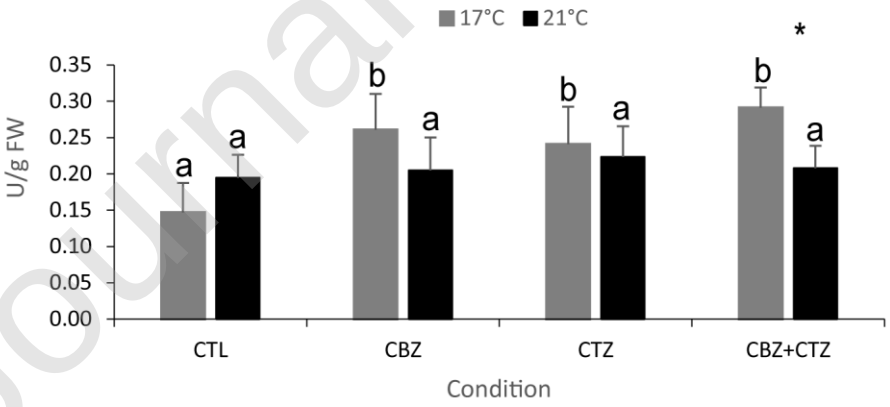
A) SOD



B) GPx



C) GSTs



D) AChE

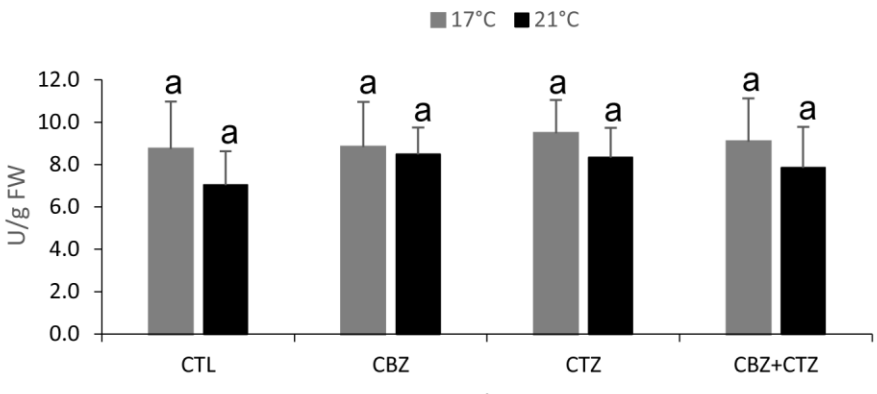


Table 1: Drugs concentrations (CBZ, CTZ, single and combined) from the medium exposure of *R. philippinarum* submitted to control temperature (17ºC) and ocean warming condition (21ºC) to single and combined drug treatments (CBZ, 1 µg/L; CTZ, 0.6 µg/L, CBZ, 1 µg/L + CTZ, 0.6 µg/L) for 28 days. Results are the means + standard deviation. Significant differences ($p \leq 0.05$) between drug treatments for each temperature level (17ºC, 21ºC) and sampling moment (beginning of exposure weeks, end of exposure weeks) are presented with letters. Significant differences ($p \leq 0.05$) between sampling moments for each drug treatment and temperature level are presented with an asterisk.

Conditions	**17°C**		**21°C**	
	Beginning	End	Beginning	End
Control	< LOQ	< LOQ	< LOQ	< LOQ
CBZ, single	1.1 (±0.1)^{a}	0.99 (±0.09)^{a}	1.11 (±0.07)^{a,*}	1.0 (±0.1)^{a}
CTZ, single	0.52 (±0.06)^{b}	0.57 (±0.06)^{b}	0.55 (±0.08)^{b}	0.51 (±0.07)^{b}
CBZ, combined	1.1 (±0.09)^{a}	1.07 (±0.08)^{c}	1.1 (±0.1)^{a}	1.1 (±0.1)^{a}
CTZ, combined	0.52 (±0.09)^{b}	0.50 (±0.04)^{d}	0.51 (±0.08)^{b}	0.49 (±0.06)^{b}

Table 2: Pseudo-F and *p* values for the comparisons done in the exposure medium drug concentrations results of *R. philippinarum* submitted to control temperature (17ºC) and ocean warming condition (21ºC) to single and combined drug treatments (CBZ, 1 µg/L; CTZ, 0.6 µg/L, CBZ, 1 µg/L + CTZ, 0.6 µg/L) for 28 days. The results significantly different ($p \leq 0.05$) are in bold.

Table 3:

Drugs in exposure medium (pseudo-F value: 86.635)					
	Comparisons	17ºC, beginning	21ºC, beginning	17ºC, end	21ºC, end
Hypothesis: Spike	CBZ-single *vs* CTZ-single	**0.0001**	**0.0001**	**0.0001**	**0.0001**
	CBZ-single *vs* CBZ-combined	0.3424	0.564	**0.0411**	0.2428
	CBZ-single *vs* CTZ-combined	**0.0001**	**0.0001**	**0.0001**	**0.0001**
	CTZ-single *vs* CBZ-combined	**0.0001**	**0.0001**	**0.0004**	**0.0001**
	CTZ-single *vs* CTZ-combined	0.4398	0.209	**0.0232**	0.1351
	CBZ-combined *vs* CTZ-combined	**0.0001**	**0.0001**	**0.0001**	**0.0001**
	Comparisons	17ºC, CBZ-single	17ºC, CTZ-single	17ºC, CBZ-combined	17º, CTZ-combined
Hypothesis: Time	Beginning *vs* end	0.4379	0.9435	0.7498	0.7676
		21ºC, CBZ-single	21ºC, CTZ-single	21ºC, CBZ-combined	21ºC, CBZ-combined
	Beginning vs end	**0.0209**	0.3399	0.7618	0.817
	Comparisons	CBZ-single, beginning	CTZ-single, beginning	CBZ-combined, beginning	CTZ-combined, beginning
Hypothesis: Temperature	17ºC *vs* 21ºC	0.2521	0.0748	0.6681	0.4348
		CBZ-single, end	CTZ-single, end	CBZ-combined, end	CTZ-combined, end
	17ºC *vs* 21ºC	0.4744	0.0797	0.873	0.3594

Pseudo-F and *p* values for the comparisons done in the blanks drug concentrations

results of *R. philippinarum* submitted to control temperature (17ºC) and ocean warming condition (21ºC) to single and combined drug treatments (CBZ, 1 µg/L; CTZ, 0.6 µg/L, CBZ, 1 µg/L + CTZ, 0.6 µg/L) for 28 days. The results significantly different are in bold ($p \leq 0.05$).

Drugs in Blanks (pseudo-F value: 75.631)					
	Comparisons	17ºC, beginning	21ºC, beginning	17ºC, end	21ºC, end
Hypothesis: Spike	CBZ-single *vs* CTZ-single	**0.0001**	**0.0001**	**0.0002**	**0.0001**
	CBZ-single *vs* CBZ-combined	0.1153	0.7172	0.2882	0.1945
	CBZ-single *vs* CTZ-combined	**0.0001**	**0.0001**	**0.0001**	**0.0001**
	CTZ-single *vs* CBZ-combined	**0.0001**	**0.0001**	**0.0001**	**0.0014**
	CTZ-single *vs* CTZ-combined	0.0892	0.1701	0.2379	0.2325
	CBZ-combined *vs* CTZ-combined	**0.0001**	**0.0001**	**0.0001**	**0.0004**
Hypothesis: Time	**Comparisons**	17ºC, CBZ-single	17ºC, CTZ-single	17ºC, CBZ-combined	17ºC, CTZ-combined
	Beginning *vs* end	0.27	0.8011	0.1168	0.0837
		21ºC, CBZ-single	21ºC, CTZ-single	21ºC, CBZ-combined	21ºC, CTZ-combined
	Beginning *vs* end	0.1846	0.3421	**0.024**	0.6647
Hypothesis: Temperature	**Comparisons**	CBZ-single, beginning	CTZ-single, beginning	CBZ-combined, beginning	CTZ-combined, beginning
	17ºC *vs* 21ºC	0.514	0.9195	0.1014	0.8474

		CBZ-single, end	CTZ-single, end	CBZ-combined, end	CTZ-combined, end
	17ºC *vs* 21ºC	0.885	0.3602	0.9175	0.1945

Table 4: Pseudo-F and *p* values for the comparisons done in the drug concentrations in clams tissues and BCF results of *R. philippinarum* submitted to control temperature (17ºC) and ocean warming condition (21ºC) to single and combined drug treatments (CBZ, 1 µg/L; CTZ, 0.6 µg/L, CBZ, 1 µg/L + CTZ, 0.6 µg/L) for 28 days. The results significantly different ($p \leq 0.05$) are in bold.

Comparisons		**Drugs in clams' tissues (pseudo-F value: 14.988)**	**BCF (pseudo-F value: 36.909)**
17ºC	CBZ-single *vs* CTZ-single	**0.0016**	**0.0001**
	CBZ-single *vs* CBZ-combined	**0.0098**	**0.0011**
	CBZ-single *vs* CTZ-combined	**0.0014**	**0.0001**
	CTZ-single *vs* CBZ-combined	**0.0001**	**0.0001**
	CTZ-single *vs* CTZ-combined	0.1957	0.3155
	CBZ-combined *vs* CTZ-combined	**0.0001**	**0.0001**
21ºC	CBZ-single *vs* CTZ-single	**0.0032**	**0.0001**
	CBZ-single *vs* CBZ-combined	0.3182	0.097
	CBZ-single *vs* CTZ-combined	**0.0001**	**0.0001**
	CTZ-single *vs* CBZ-combined	**0.0027**	**0.0002**
	CTZ-single *vs* CTZ-combined	0.1966	0.6658
	CBZ-combined *vs* CTZ-combined	**0.0001**	**0.0001**
CBZ-single	17ºC *vs* 21ºC	0.4208	0.2768
CTZ-single	17ºC *vs* 21ºC	0.9201	0.1279
CBZ-combined	17ºC *vs* 21ºC	0.1717	0.1391
CTZ-combined	17ºC *vs* 21ºC	0.7916	0.2686

Table 5: Pseudo-F and *p* values for the comparisons done with the biochemical results of the exposure of *R. philippinarum* submitted to control temperature (17ºC) and ocean warming condition (21ºC) to single and combined drug treatments (CBZ, 1 µg/L; CTZ, 0.6 µg/L, CBZ, 1 µg/L + CTZ, 0.6 µg/L) for 28 days. The results significantly different (*p* ≤ 0.05) are in bold.

Biochemical results		**ETS**	**GLY**	**PROT**	**LPO**	**PC**	**GSH/GSSG**	**SOD**
Comparisons		pseudo-F value: 5.018	pseudo-F value: 1.8889	pseudo-F value: 2.58	pseudo-F value: 12.242	pseudo-F value: 3.7356	pseudo-F value: 3.3978	pseudo-F value: 6.3639
17ºC	CTL *vs* CBZ	0.3772	0.1274	**0.0158**	0.1353	0.354	0.2949	**0.0171**
	CTL *vs* CTZ	**0.0159**	0.8242	0.1273	0.0895	0.8987	**0.0364**	**0.0459**
	CTL *vs* CBZ+CTZ	0.6092	0.4824	0.2558	0.0697	**0.0278**	0.1889	**0.0181**
	CBZ *vs* CTZ	0.2115	0.3452	0.373	0.7091	0.687	0.3541	0.2324
	CBZ *vs* CBZ+CTZ	0.2741	0.1949	**0.040**	**0.0053**	0.1933	0.861	0.4399
	CTZ *vs* CBZ+CTZ	**0.0173**	0.825	0.514	**0.0045**	0.2587	0.4322	0.3989
21ºC	CTL *vs* CBZ	0.5541	0.9499	0.5388	**0.0379**	**0.0215**	0.0596	**0.0173**
	CTL *vs* CTZ	0.1352	0.9052	0.9923	0.3998	**0.001**	**0.0162**	**0.0001**
	CTL *vs* CBZ+CTZ	0.663	**0.0174**	0.0719	**0.0001**	0.9934	0.7155	**0.0058**
	CBZ *vs* CTZ	**0.0105**	0.9194	0.5672	0.5328	0.6962	0.8598	0.3135
	CBZ *vs* CBZ+CTZ	0.2421	**0.0011**	0.1706	**0.0043**	**0.0182**	0.0385	0.3093
	CTZ *vs* CBZ+CTZ	0.2635	**0.0282**	0.0837	**0.0347**	**0.0004**	**0.0109**	0.7267
CTL	17ºC *vs* 21ºC	0.1815	0.8806	0.3555	0.0557	**0.0362**	0.2103	0.4799
CBZ	17ºC *vs* 21ºC	**0.0311**	0.0803	**0.002**	**0.0005**	0.0646	0.8828	0.0889
CTZ	17ºC *vs* 21ºC	**0.0103**	0.9507	0.4751	**0.0042**	0.1559	0.5782	**0.0031**
CBZ+CTZ	17ºC *vs* 21ºC	0.6463	**0.0012**	**0.0347**	**0.0005**	0.6982	**0.0178**	**0.0246**