# Biochemical and physiological responses of two clam species to Triclosan combined with climate change scenario

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## ABSTRACT

Ocean acidification and warming are among the man-induced factors that most likely impact aquatic wildlife worldwide. Besides effects caused by temperature rise and lowered pH conditions, chemicals of current use can also adversely affect aquatic organisms. Both climate change and emerging pollutants, including toxic impacts in marine invertebrates, have been investigated in recent years. However, less information is available on the combined effects of these physical and chemical stressors that, in nature, occur simultaneously. Thus, this study contrasts the effects caused by the antimicrobial agent and plastic additive, Triclosan (TCS) in the related clams *Ruditapes philippinarum* (invasive) and *Ruditapes decussatus* (native) and evaluates if the impacts are influenced by combined temperature and pH modifications. Organisms were acclimated for 30 days at two conditions (control: 17 ºC; pH 8.1 and climate change scenario: 20 ºC, pH 7.7) in the absence of the drug (experimental period I) followed by a 7 days exposure under the same water physical parameters but either in absence (unexposed) or presence of TCS at 1 µg/L (experimental period II). Biochemical responses covering metabolic, oxidative defences and damage-related biomarkers were contrasted in clams at the end of experimental period II. The overall picture showed a well-marked antioxidant activation and higher TCS bioaccumulation of the drug under the forecasted climate scenario despite a reduction on respiration rate and metabolism in the exposed clams. Since clams are highly consumed shellfish, the consequences for higher tissue bioaccumulation of anthropogenic chemicals to final consumers should be alerted not only at present conditions but more significantly under predicted climatic conditions for humans but also for other components of the marine trophic chain.



**Keywords:** Drugs; Bivalves; Respiration rate; Metabolic capacity; Oxidative stress; *Ruditapes* clams.

## 1. INTRODUCTION

A significant worldwide increase in population and industrialization has been identified as a major catalyser of climate change (CC) (Hashmi and Alam, 2019; Ghazali and Ali, 2019). Climate change is already occurring nowadays as a result of the increasing carbon dioxide ($CO_2$) concentration in the atmosphere (Bove et al., 2019) that will, consequently, reach the aquatic environment. According to IPCC (2014) predictions, $CO_2$ increase is expected to cause a decrease of about 0.4 pH units at the end of the century. The importance of understanding the impacts of acidification is therefore irrefutable and well documented in the last few years regarding both physiological and biochemical responses in aquatic organisms (Almeida et al., 2018; De Marchi et al., 2017; Moreira et al; 2018a; Munari et al., 2016; 2018; Nardi et al., 2017; Range, 2014; Matoo et al., 2013). Combined with ocean acidification, earth surface temperature is also foreseen to rise up to 4 ºC by 2100 (IPCC, 2014), due to the extensive emissions of greenhouse gases (e.g., $CO_2$, methane ($CH_4$)) and nitrous oxide ($N_2O$) (Durack et al., 2018). This temperature increase will greatly influence ectotherms, especially those low-lying in aquatic coastal areas (e.g.: estuaries) as the most vulnerable ecosystems (Brown et al., 2016). Previous studies have already demonstrated the consequences of temperature rise on growth and reproduction in aquatic invertebrates (Fabbri et al., 2016; Moreira et.al, 2018b; 2018c) as well as related cellular processes (Almeida et al., 2018; Maulvault et al., 2018; Freitas et al.,2017; Matoo et al., 2013).

Industrialization has also been associated to water pollution, with a large variety of chemicals reaching coastal environments and posing at risk inhabiting organisms. For decades, inorganic (e.g. trace metals) and persistent organic pollutants (POPs) have been monitored worldwide, but in regard to emerging pollutants, such as personal care products (PCPs), the knowledge on their environmental occurrence concentrations and risks to aquatic organisms is still scarce. Nevertheless, the presence of these chemicals has increased worldwide, reaching concentrations up to µg/L (Beretta et al., 2014; Cantwell et al., 2010). Among PCPs, Triclosan (TCS) is an antimicrobial agent with higher consumption rates, used in both human and veterinary medicine (Jones et al., 2000). In January 2017 the European Union (EU) banned

84 TCS from all human hygiene biocidal products but still this compound remains, at high
85 concentrations, in other personal care products such as toothpaste, mouthwash, hand sanitizer,
86 surgical soaps and plastic additives (Johnson et al., 2016; NICNAS, 2009; Riley and Lamont
87 2013). Accurate measurements from a Swiss waste water treatment plant (WWTP) have
88 detailed the elimination process of TCS: 79% was biologically degraded, 15% was sorbed to
89 sludge and 6% was remained in the plant's final effluent at a concentration of 42 ng/L (Singer et
90 al., 2002). TCS has already been found in marine, coastal and estuarine waters of Portugal
91 (124.1 ng/L) (Neng and Nogueira, 2012), but also in effluent waste waters from other countries
92 in Europe, North America, Australia and China that confirm its widespread occurrence at
93 concentrations ranging between 40 and 2,000 ng/L (Bester, 2003; Halden and Paull, 2005;
94 Weigel et al., 2004; Ying and Kookana, 2007). Several studies have already highlighted the
95 negative impacts caused by TCS in aquatic organisms some even including warming conditions
96 (Freitas et al., 2019a, 2019b; Li et al., 2018; Pirone et al., 2019; Tastan et al., 2017). However,
97 no studies report on the consequences of environmental TCS concentrations under a
98 forecasted realistic CC scenario of temperature rise and lowered pH in combination. In fact, the
99 impacts of climate change related factors on the bioaccumulation and toxicity of pollutants,
100 including PCPs, as well as on the sensitivity of organisms towards these substances is still on
101 its infancy. Moreover, it is not well known how the effects caused by pollutants will modify
102 organism's responses to climate related factors. Bivalves, including clams, are good
103 bioindicators of marine pollution, and previous studies have already demonstrated their
104 usefulness to reflect TCS exposures (Binelli et al., 2009; Matozzo et al., 2012). TCS has proved
105 to alter biomarkers related to bivalve's metabolic capacity (measured by the activity of the
106 electron transport system) and energy reserves (including glycogen and protein content);
107 antioxidant and biotransformation defence mechanisms (such as superoxide dismutase,
108 catalase and glutathione S-transferases activities), cellular damage (lipid peroxidation, protein
109 carbonylation) and redox status (Freitas et al., 2019b; Matozzo et al., 2012; Pirone et al., 2019).
110 Thus, the aim of the present study was to evaluate the impact of an acute TCS exposure
111 (7 days) at a relevant environmental concentration (1 µg/L) upon two estuarine clam species

112 previously acclimated to actual conditions and those predicted for a CC scenario (for 30 days).
113 To this end, relevant biochemical markers related to clam's metabolic capacity, oxidative and
114 neurotoxic status were evaluated including respiration rate as physiological marker. Clams,
115 *Ruditapes decussatus* and *R. philipinarum* were selected as useful bioindicator species in
116 environmental monitoring, given their well-characterised oxidative, metabolic and neurotoxic
117 performance to fluctuations in temperature and seawater pH as independent variables (Munari
118 et al., 2016; Almeida et al., 2017; De Marchi et al., 2019). Likewise, it was also intended to
119 unveil which of the two clam species selected, the European clam *R. decussatus* (native) or the
120 Manila clam, *R. philippinarum* (invasive), would be more competitive under an upcoming climate
121 scenario.
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## 2. MATERIALS AND METHODS

### 2.1 Sample collection and experimentation

Clam species of *R. decussatus* and *R. philippinarum* of comparable biometrics, were collected in October of 2018 from the Ria de Aveiro (NW coast of Portugal). During the two weeks' adaptation to laboratory conditions: temperature (17 ºC), salinity (30) and photoperiod (12 light:12 dark) they were only fed in the second week as detailed recently (Costa et al., 2020).

After these two initial weeks, clams were submitted to two scenarios for 30 days (experimental period I): A) actual field conditions at the time of sampling (pH 8.1, 17ºC) and B) mimicking forecasted CC conditions of pH decrease and temperature increase (pH 7.7, 21 ºC). After this period, the experiment continued for 7 additional days which included TCS exposure in half of the acclimated specimens at the two conditions and for each species making a total of eight possible combinations (2 climate factors x 2 chemical exposures x 2 species). During these further 7 days that included TCS treatment (experimental period II) water from all tanks (control and spiked) was renewed every 2 days, followed by TCS concentration reestablishment in those corresponding to TCS exposure conditions.

Along the experimental period II, water samples were collected weekly immediately after spiking to confirm targeted nominal TCS concentration in water.

Temperature (T (°C)), pH and salinity (S) were measured periodically during both experimental phases and water samples (50 mL) were collected to determine total alkalinity (TA) by potentiometric titration. Generated TA values, temperature and salinity were used to determine $CO_2$ partial pressure ($pCO_2$), bicarbonate ($HCO^3$) and carbonate ($CO_3^2$) ions concentrations, and the saturation states of calcite (ΩCal) and aragonite (ΩAg) as described in more detail in Costa et al (2020).

A total of 9 individual clams per species and condition (3 individuals per aquaria) were sampled after each experimental phase (I and II),immediately frozen in liquid nitrogen and maintained at -80 ºC until analysis. The clams used for biochemical analysis and TCS quantification (3 individuals per aquarium) were individually pulverized with liquid nitrogen,

divided into 0.3 g fresh weigh (FW) aliquots and stored at -80 ºC. Two additional organisms per aquarium were used to evaluate the respiration rate (RR) and lipid content (LIP).

### 2.2 Water parameters and TCS presence in water and clams

Concentrations of TCS were measured in water and clams soft tissue by high performance liquid chromatography-ultraviolet detection (HPLC-UV) method. Water samples were analyzed (in duplicate) following Madikizela et al. (2017) protocol while soft tissues samples were extracted according to Gatidou et al. (2007) (for more details see Freitas et al., 2019a). No matrix effect was observed after triplicate measures in different matrices. The recovery was >80% for water samples and >77% for soft tissues. The detection limit, calculated as a signal-to-noise ratio of 3:1, was 0.008 µg/L for water samples and 0.13 ng/g dry weight for soft tissues. Limits of Quantification (LOQ) are 0.025 µg/L (water) and 0.40 ng/g dry weight (tissue).

### 2.3 Physiological responses

At the end of experimental period II, Respiration rate (RR) was measured in 2 individual clams from each triplicate aquaria, (n=6 per condition), considering contaminated and non-contaminated clams at actual and CC conditions that makes a total of 24 individuals per species. Measurements were performed by simple static respirometry (see for complete details Costa et al., 2020). Respiration rate was recorded as a function of declining $O_2$ concentration (mg/L) over time recorded every 15 min during 2 h, using a multi-channel fibre optic oxygen meter (Multi channel oxygen meter, PreSens GmbH, Regensburg, Germany) for simultaneous read-outs.

### 2.4 Biochemical responses

Biochemical parameters were performed in 9 frozen individuals per condition and species. Specimens were individually frozen and pulverized with liquid nitrogen and stored in 0.3 g aliquots. Extractions were made using a 1:2 (w/v) ratio of specific buffers (see for details Andrade et al., 2018). Homogenization was carried out after 30 s sonication and centrifugation for 10 min at 10,000 g (or 3,000 g for ETS) at 4 ºC. Supernatants were either stored at -80 ºC or immediately used. Determinations were conducted at room temperature and each sample replicate was read at least in duplicate. In the present study were considered the metabolic (ETS, electron transport system), energy reserves content (GLY, glycogen; PROT, protein; LIP, lipid), enzymatic (SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GST, glutathione S-transferases) and non-enzymatic (LPO, lipid peroxidation; PC, protein carbonylation; reduced (GSH) and oxidized (GSSG) glutathione contents) markers and a neurotoxicity parameter (AChE, acetylcholinesterase activity). Biomarker measurement are detailed in Costa et al. (2020).

**2.5 Data analysis**

Data analysis was performed by PERMANOVA+add-on in PRIMER v6 using multivariate analysis of variance. Different null hypotheses were tested: i) for same species and CC scenario, no significant differences existed between non-contaminated and TCS contaminated organisms (represented by an asterisk, *); for the same species and TCS treatment (presence/absence), no significant differences existed between CC scenarios (represented with the symbol #); for the same CC scenario and TCS treatment (presence/absence), no significant differences existed between species, identified in figures with lower case letters (for non-contaminated organisms) and an upper case letters (for TCS exposed ones). The Euclidean distance similarity matrix was calculated and, posteriorly, simplified when submitted to ordination analysis by Principal Coordinates (PCO) by measuring the distance between centroids based on all tested conditions. Person correlation vectors (correlation higher than 80%) for all data obtained were superimposed on the PCO graph.

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## 3. RESULTS & DISCUSSION

### 3.1 Water parameters and TCS presence in water and clams

A relation of relevant physicochemical water characteristics during experimental periods I and II is detailed in Table 1. Concentrations of TCS in water soon after spiking were below the targeted nominal concentration (1 μg/L) regardless of condition (Table 2). Lower TCS concentrations soon after seawater contamination may suggest the quick precipitation of this compound due to its low solubility that combined with Aranami and Readman (2007) observation in which TCS had high photodegradation rates in saltwater. The authors also recognized the need to assess the effect of water parameters such as pH or temperature as potential variables in the TCS interactions with water. Scarce information is available in literature about the TCS behaviour in sea water but Freitas et al. (2019a) observed a similar trend when spiking at the same concentration.

In ascertain the impact of TCS upon aquatic organisms, knowledge on its bioaccumulation and metabolism is necessary. However, only few studies have recently addressed this issue in marine organisms under predicted CC scenario (Maulvault et al., 2018; Serra-Compte et al., 2018). The study by Serra-Compte et al. (2018) used the mussel *Mytilus galloprovincialis* exposed to a mixture of contaminants including TCS (15.7 μg/L) under warming and acidification conditions. The authors concluded that the acidification condition alone led to a lower elimination rate of TCS (higher bioaccumulation) which is in accordance with the present results in both clam's species. However, it is not possible to discard other mechanisms controlling the interaction between the compound's electrochemistry properties and the biotic matrix (clam's tissues), including metabolite formation and associated physiological responses, highlighting the importance of further research on this topic.

### 3.2 Physiological responses

A mortality rate of 1.5% *(R. philippinarum)* and of 9.6% *(R. decussatus)* was registered at the end of the 30-day acclimation at actual pH and temperature conditions (17 ºC and pH 8.1).

When acclimated to CC conditions for the same time period, only *R. decussatus* specimens died (22.8%). No mortality was observed after 37 days, even after TCS administration.

The respiration rate (RR) decreased in *R. philippinarum,* exposed to TCS under actual but also CC conditions, although it did not reach significance in the latter case due to large variability of the responses (Figure 1). A decrease in RR was also seen in the same clam species under similar pH and temperature conditions but exposed to the drug diclofenac (Costa et al., 2020); in *R. philippinarum* when exposed to a combination of a nanoparticles and ocean acidification (Marchi et al. 2019); or in bivalves reared in an acidified ambient (Range et al., 2014). Also Zhao et al. (2017) observed lower RR under an ocean acidification scenario in the marine bivalve *Tegillarca granosa*, stressing the important role not only of contaminants but also of the physical variables alone. Also Bielen et al. (2016) reported lower respiration estimations under thermal and Zn pollution stressors in competing freshwater bivalves. Overall, the literature suggests that bivalves, inhabiting estuarine, intertidal and subtidal areas, have developed physiological and biochemical strategies to cope with the simultaneous presence of multiple environmental stressors (i.e. contaminants, pH and/or temperature fluctuations (Lannig et al., 2010). Such ability may be compromised by a time factor, i.e., species resilience to stress being committed for longer and continuous periods of exposure (Belivermis et al., 2016). Belivermis et al. (2016) verified that the oyster *Crassostrea gigas* adapted differently to metals. The author registered a metal or species specificity for different exposure periods responses.

### 3.3 Biochemical responses

#### *3.3.1 Metabolic related biomarkers*

Closely related to the physiological marker RR, the measure of the mitochondrial Electron transport system (ETS) activity estimates energetic production. ETS was only significantly higher in non-contaminated native clams, *R. decussatus,* at actual conditions (Figure 2A). Under the present experimental conditions of considering both physical variables together as a more realistic approach, no significant alteration of metabolism measured as ETS activity was related to any of the tested stressors. By contrast, clams exposed to diclofenac under the same

264 CC conditions showed an elevation in ETS activity (Costa et al., 2020). Formerly, Schiedek et
265 al. (2007) observed under independent but also combined warming and acidification conditions,
266 a reduction in metabolism in calcifying organisms, mainly driven by pH variations. Mussels
267 exposed to TCS combined with extreme weather shifts and/or other drugs also experienced a
268 decrease in ETS activity (Pirone et al. 2019; Freitas et al. 2019a; 2019b). Thus, metabolic shifts
269 under stressful conditions are diverse and species dependent.

270 Biochemical composition of reserves suggests how organisms deal with energetic
271 demanding processes and its usage was particularly affected by the 3 factors: TCS exposure,
272 climatic condition and species. Glycogen (GLY) content (Figure 2B) decreased after 7 day TCS
273 exposure in *R. philippinarum* under CC conditions. GLY content also revealed species
274 differences with the native clam always reflecting higher polysaccharide content regardless of
275 climate scenarios. Protein (PROT) content (Figure 2C) decreased significantly in *R.*
276 *philippinarum* exposed to TCS even at actual conditions. Also unexposed *R. decussatus*
277 specimens, decreased their PROT content at CC conditions. However, when exposed to both
278 stressors combined (TCS + CC scenario) native clam specimens showed an elevation in PROT
279 content. Species contrasts confirmed consistently lower PROT reserves in *R. decussatus* at
280 both scenarios, as opposed to GLY content observations. Lipid (LIP) reserves (Figure 2D)
281 showed differences between non-contaminated and contaminated organisms of both species
282 under actual conditions but in opposed ways. Moreover, for *R. decussatus*, the forecasted
283 environmental conditions combined with TCS exposure lowered their lipid reserves. As far as
284 species contrasts concerns, significantly higher LIP content was observed in *R. decussatus*
285 more clearly under the current climatic situation and in the same sense as GLY reserves.

286 Overall, clam's metabolic capacity (measured as ETS activity) remained practically
287 unaltered after exposure to TCS alone or combined with the additional stress caused by a
288 challenging CC scenario. Former studies with the same physical water variables but with clams
289 exposed to diclofenac displayed a coincident pattern (Costa et al., 2020). Unaltered ETS was
290 also revealed in the Manila clam when exposed to lower range concentrations of caffeine (0.3-
291 18.0 µg/L) (Cruz et al. 2016). Unaltered metabolic rates were previously described by Freitas et

al. (2016) and by Bamber (1990) in several bivalve species facing seawater acidification. Thus, it seems that the physical and chemical modifications from the actual conditions were not strong enough to alter clam's metabolic activity. Furthermore, traditional energy reserves (GLY, PROT and LIP) did not follow a clear usage pattern in both species as a consequence of chemical and physical stressors but GLY and LIP natural reserves were consistently higher in the European native clam while PROT content was elevated in the Manila clam. This same species pattern in terms of energy reserves consumption was formerly seen in clams exposed to diclofenac (Costa et al., 2020). A reduction in energy reserves (carbohydrates, GLY and PROT but not LIP) was seen in *R. decussatus* exposed to elevated $CO_2$ (acidification conditions) combined with other ambient stressors but after longer exposures (75 days) (Range et al., 2014). However, in *R. philippinarum* LIP content was reduced under lower pH conditions (Velez et al. 2016a) and GLY content decreased due to exposure to the pharmaceutical drug paracetamol (Nunes et al. (2017). Recent literature regarding the effects of TCS on the biochemical composition of *M. galloprovincialis,* reported an increase in GLY content when exposed to TCS alone or combined with high salinity (Freitas et al., 2019a; 2019b) and a decrease in PROT content when exposed to TCS and high temperature (Pirone et al. 2019). Overall, both clam species did not clearly respond in terms of energetic parameters, and it seems that existing baseline species-particularities under actual conditions were maintained under a CC scenario. The biochemical composition in terms of energy reserves (PROT, GLY and fatty acids) in the two contrasted clam species was also formerly described and their lipid profile being affected by warming mainly in the native species (Anacleto et al., 2014).

*3.3.2 Oxidative stress biomarkers*

The antioxidant responses were selected an unspecific defence mechanism to face stress.

In the present study, Superoxide dismutase (SOD) activity (Figure 3A) significantly increased in TCS exposed clams although it only reached significance for *R. decussatus* under actual climate situations due to data dispersion. Differences between climatic scenarios were

also confirmed in *R. decussatus* contaminated clams, with depressed activities at the forecasted conditions. When comparing species, *R. decussatus* consistently showed lower antioxidant SOD activity. The antioxidant defence Catalase (CAT) activity (Figure 3B) was also significantly elevated in TCS exposed *R. decussatus* under both climate scenarios. This increase also took place in *R. philippinarum* but only under warming and acidified conditions, while the opposite trend was experienced by *R. decussatus* when under a CC scenario. Concerning species contrasts, significantly higher CAT activity was seen in *R. decussatus* regardless the climate scenario and chemical condition. The antioxidant measure of Glutathione peroxidase (GPx) activity (Figure 3C) was elevated in TCS exposed *R. decussatus* in the actual climate situation but it decreased in contaminated specimens of the same species under a CC scenario. Species differences were also confirmed under comparable situations with the native clam displaying higher antioxidant capacity. The activity of the biotransformation and antioxidant enzyme Glutathione-S-transferases (GSTs) (Figure 3D) was significantly elevated in contaminated organisms of both species at CC conditions. GSTs activity in *R. philippinarum* increased also as a response to predicted climate conditions in those non-exposed. More significantly, species differences were confirmed with the native clam also presenting a more significant defence capacity.

Overall the enzymatic antioxidant defences in clam's exposure to TCS and/or CC scenario were activated in both clam species with *R. decussatus* showing greater sensitivity to chemical and physical stressors. Moreover, the European clam displayed higher baseline enzymatic activities than *R. philippinarum*, for all antioxidant defences except SOD. The activities of CAT and GPx followed a similar trend at the contrasted situations in the two species but GPx was more inhibited in co-exposed organisms. It is recognised that CAT is more effective under specific higher $H_2O_2$ pressure while GPx also catalyses other organic peroxides (Regoli and Giuliani., 2014). GSTs responded under multiple stressors pressure confirming activation of the antioxidant and detoxification processes.

A similar trend in activation of antioxidant defences was seen in the same clam species exposed to 1 μg/L diclofenac (Costa et al., 2020). Other studies with other bivalve species,

reported enhanced SOD activity when the mussel *M. galloprovincialis* was exposed to combined TCS and lead (Pb) (Pirone et al. 2019) or to TCS alone (Freitas et al. 2019a). The recent studies by Freitas et al. (2019a, 2019b) also revealed that mussels enhanced CAT activity under TCS exposure alone and/or in combination with high salinity. Alteration in GSTs enzymes were also observed in mussels under combined exposure to TCS and Pb (Pirone et al., 2019) and TCS alone (Freitas et al., 2019a, 2019b). In regard to species differences in terms of basal enzymatic levels, the present study goes along with previous studies when comparing the same parameters in the clam species exposed to As and Hg (Velez et al., 2016b) or acetaminophen (Antunes et al., 2013). Moreover, the enzymatic inhibition observed under co-exposure in bivalves seems to potentially compromise their defences and they showed a higher adaption capacity when facing independent stressors.

*3.3.3 Oxidative stress damage measures*

As a consequence of an overwhelming oxidative stress challenge, damage to biological components: lipids (measured as LPO occurrence), proteins (presence of enhanced carbonyl proteins) and endogenous antioxidant molecules (such as depleted GSH) can occur.

Higher LPO values (Figure 4A) were observed in TCS contaminated clams but it was only significant in *R. decussatus* under actual conditions. In terms of oxidized protein formation (Figure 4B), although small but significantly higher PC values were also observed in unexposed *R. philippinarum* clams under CC conditions, but this trend was inverted in the European clam reared under a CC situation. Consistently higher PC levels were always measured in *R. decussatus,* regardless contamination level and climate conditions. The quantification of oxidized forms of glutathione (GSSG) can also be used as a marker of oxidative damage and the balance GSH/GSSG is as a well adopted effect biomarker. Opposite responses were recorded for TCS exposed *R. philippinarum* individuals, depending if they were reared under the present (depressed ratio) or under a forecasted climate scenario (enhanced ratio). Contradicting opposite responses were also revealed when Manila clams were facing the CC parameters as a function of their former chemical condition (unexposed or TCS exposed). That is, a high

GSH/GSSG ratio for non-contaminated Manila clam organisms at the actual situation was depressed under CC while in those exposed to TCS once subjected to predicted climate conditions this ratio increased. Such findings could suggest TCS was the main trigger responsible for the response (Figure 4C). As a function of this, species differences on this parameter were not so clear and dependent on the chemical scenario.

Overall, baseline and induced antioxidant defences seemed to be sufficient to prevent LPO and PC occurrence in both clam species. Although the manila clam consistently revealed higher LPO levels and the European clam presented higher levels of PC in the whole tissue, this characteristic could be a reflection of their natural biochemical composition and not a deleterious sign since oxidised by-products formation was not induced by exposure to TCS alone or combined with physical stressors. Moreover, in accordance to a higher antioxidant pool (CAT, GPx and GSTs activities) in *R. decussatus*, LPO levels were consistently lower in this species as formerly pointed out (Costa et al., 2020; Velez et al., 2016c). However, remarkable higher PC values were observed in the native clam at both scenarios, despite the total PROT content did not follow this species trend. Although some hypothesis can be pointed out, those relations should be taken with some caution since it is already described in literature that energy content usage of endogenous fuels can change along year within a very wide range (Ngo et al, 2018). Nevertheless, the particular enzymatic traits between clam species give support to the different sensitivities of bivalves to pharmaceuticals (Lacaze et al., 2015; Martín-Diaz et al., 2009).

Since LPO and PC levels remained almost unchanged in both clam species, and a positive GSH/GSSG balance was maintained in the forecasted scenario it can be assumed that the observed higher TCS bioaccumulation in clam's tissues at this predicted situation may not be responsible for an oxidative stress condition. The present results in terms of damage evaluation are highly similar to those formerly described under the same climate conditions but to the drug diclofenac (Costa et al., 2020) which suggests that responses to the chemical insults are not due to the particular mode of action of each drug and supporting responses to CC conditions. Effect markers in other bivalves than clams, revealed that mussels, *M.*

404 *galloprovincialis,* exposed to TCS and elevated temperature did not experience LPO and PC
405 variations but a combination of including and additional Pb factor was responsible for PC
406 elevation (Pirone et al. 2019). Also Freitas et al. (2019a) observed no LPO differences, but
407 elevated PC levels, in mussels exposed to TCS at higher temperature and no LPO occurrence
408 was due to TCS and salinity variations (Freitas et al. 2019b). Overall, bivalves seemed to be
409 able to cope with environmental chemical exposures and physical variables modifications and
410 prevent the occurrence of tissue damage.
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413 *3.3.4 Marker of neurotoxicity*
414 Significant differences between non-contaminated and contaminated clams in AChE
415 activity (Figure 5) were only identified in *R. decussatus* under a CC situation. TCS exposed
416 clams of both species significantly elevated this esterase activity under forecasted conditions.
417 Species particularities were confirmed at all conditions with higher activity always found in the
418 European clam which was consistent with former observations in terms of most enzymatic
419 defenses.
420 Increase in AChE activity in both species was more likely a response to increased
421 temperature and decreased water pH than to TCS exposure. A similar trend was revealed in
422 diclofenac exposed clams with the concomitant confirmation of the same species trends (Costa
423 et al., 2020). In this regard, Durieux et al. (2011) also observed a strong relationship between
424 temperature and this enzymatic activity in fish. Although AChE inhibition is a well-established
425 biomarker of neurotoxicity in vertebrates, increases in this esterase activity may be associated
426 to generalized cell disrupting processes such as apoptosis (Zhang et al., 2002). In fact, the
427 study by Rosa et al. (2016) assessing the neuro-oxidative damage of sharks' brain cells under
428 climate change conditions indicated an elevation of AChE activity in this organ. In the present
429 but also in a former clam study by Costa et al (2020), the observed increases in AChE activity
430 may result from two factors: a temperature increase and/or a degenerative condition in clams
431 exposed to combined chemical and physical stressors, including temperature. The first one

seems more plausive as damage to biological components was not evidenced. Nevertheless, AChE activity measures in *Ruditapes* spp. clams are controversial since the hydrolysis rates are very low if compared to other bivalves (Valbonesi et al 2003; Solé et al., 2018) but potentially responsive at higher substrate concentrations. In fact, a recent study of another B-esterase (carboxylesterase activity) was inducible in clams exposed to the retroviral drug Tamiflu® (Dallares et al., 2019).

### 3.4 Integrative biomarker analysis

Principal coordinates analysis (PCO) was selected as an integrative tool due to the comprehensive set of parameters and conditions considered (Figure 6A). A clear separation of the species in the axes corroborated species differences with higher cellular damage recorded in the European clam (driven by PC levels) while in the Manila clam it was due to the elevated LPO levels together with greater responses due to TCS exposure under a CC scenario. Given the unmistakable large species differences here confirmed, an independent PCO analysis for each clam was performed in order to identify the biomarkers responsible for the observed particularities. Overall, LIP, CAT, AChE, PC, GSTs and GLY were the biomarkers positively correlated to PCO 1 ($r > 0.80$) in *R. decussatus* organisms, while SOD, PROT, LPO, GSH, GSSG and the respective ratio showed a high negative correlation ($r > -0.80$) in the same axis, being closely related to *R. philippinarum* organisms.

In Figure 6B (*R. philippinarum)* the PCO1 clearly separated clams exposed to TCS under actual conditions from those exposed to the CC scenario regardless of TCS presence. The markers: LPO, ETS, GSH, GSSG and GPx presented higher correlation with clams exposed to TCS even at present conditions. PCO2 separated non-contaminated clams in the positive side from those contaminated.

In Figure 6C (*R. decussatus*) the PCO1 clearly separated, on the positive side of the axis, TCS exposed organisms, regardless of physical parameter conditions, from those reared under a CC scenario on the negative side. PCO2 showed a clear separation between clams subjected

459 to both stressors (chemical and physical) in the positive side from the remaining conditions at
460 the negative side of the axis.

461 In addition to the evident species differences, the individual PCO analysis for each clam
462 suggested: 1) a greater response of the Manila clam to TCS exposure combined with
463 forecasted CC conditions and 2) biomarkers in the native species did not clearly discriminate
464 between actual and CC conditions and TCS exposure. The greater responses of *R.*
465 *philippinarum* to combined environmental stressors, together with its commercial interest,make
466 of the Manila clam a potential better sentinel. The native clam was already naturally well
467 equipped with antioxidant baseline defences that also responded to stressing challenges.
468 However, the discriminatory potential of biomarkers in the European clam was less evident from
469 the PCO results.

470

471 **CONCLUSION**

472 The present results clearly revealed a species-dependent response of clams towards
473 TCS contamination and environmental variable fluctuations. Antioxidant defences, rather than
474 energetic and reserves pathways, were seen as the most sensitive responses to TCS exposure
475 regardless of the climatic scenario. When comparing both species, the Manila clam showed
476 higher baseline LPO levels and SOD antioxidant defence while the European clam presented
477 higher baseline PC values, GSTs and AChE activities. The baseline LIP and PC levels
478 differences could be associated to a different biochemical composition of LIP and PROT in the
479 two species. Despite both clams are close in phylogenetic terms, the enzymatic and
480 biochemical discrepancies observed are sound and consistent. Moreover, when exposed to
481 stressful conditions, both species activated defensive mechanisms in order to prevent cellular
482 damage although the Manila clam biomarker responses were more closely related to chemical
483 and physical stressing factors.

484

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**Table**



**Table 1-** Carbonate system physicochemical parameters. Mean values (mean values of 2 individuals per aquarium, 6 per condition) ± standard errors of pH and total alkalinity (AT) from weekly water sampling (temperature 17 °C and 21 °C). Partial $CO_2$ pressure ($pCO_2$), carbonate ion concentrations ($CO_3^{2-}$), saturation states of calcite (ΩCal) and aragonite (ΩAra) were calculated with $CO_2$ SYS software (Robbins et al., 2010).

| *Temperature (ºC)* | pH | AT (µmol/kg) | pCO2 (µatm) | $CO_3^{2-}$ (µmol/kg) | Ω Cal | Ω Ar |
|---|---|---|---|---|---|---|
| *17* | 8.12 ± 0.03 | 2042 ± 196 | 458 ± 43.2 | 110.6 ± 14.6 | 2.81 ± 0.37 | 1.76 ± 0.23 |
| *21* | 7.72 ± 0.07 | 1780 ± 139 | 1189 ± 148 | 44.10 ± 5.55 | 1.13 ± 0.14 | 0.71 ± 0.01 |

**Table 2-** Triclosan concentrations in water (µg/L) (± standard deviation), collected immediately after spiking (mean of the 1st, 2nd and 3rd water samplings) and in clams tissues (ng/ g dry weight) at the end of the experimental period II (37 days). Results are the means (mean values of 3 individuals per aquarium, 9 per condition) ± standard errors. Different letters denote statistical significance. LOD: 0.008 µg/L (water) and is 0.13 ng/ g dry weight (tissue). LOQ: 0.025 µg/L (water) and 0.40 ng/g dry weight (tissue).

| | Species | Water | Clams tissues |
|---|---|---|---|
| *17 °C, pH 8.1* | *R. philippinarum* | 0.74 ± 0.09a | 7.66 ± 0.8a |
| | *R. decussatus* | 0.92 ± 0.11b | 7.75 ± 2.1a |
| *21 °C, pH 7.7* | *R. philippinarum* | 0.64 ± 0.006c | 12.6 ± 2.5b |
| | *R. decussatus* | 0.85 ± 0.02b | 12.7 ± 3.0b |

Figure 1
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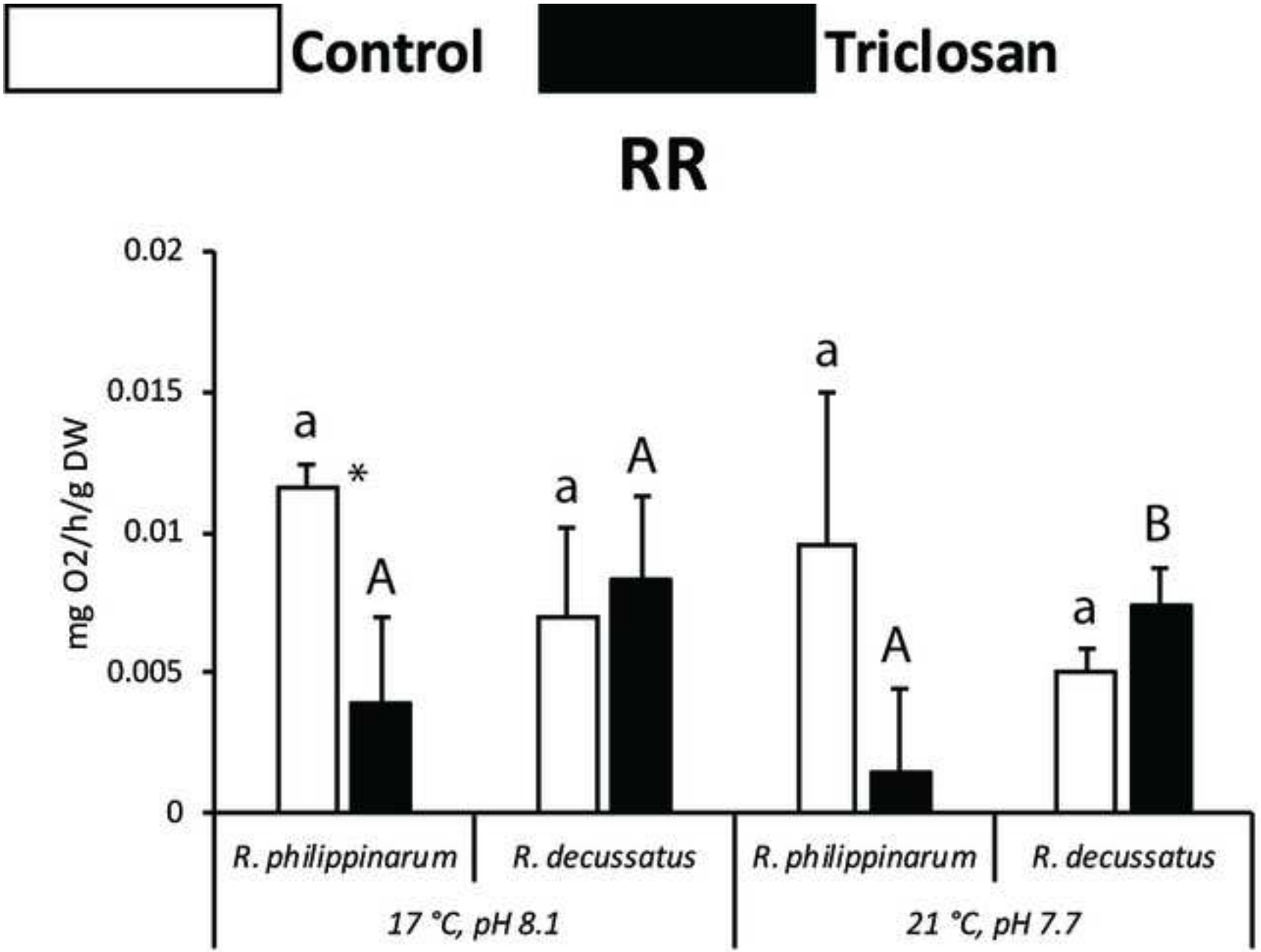

**Figure 2**

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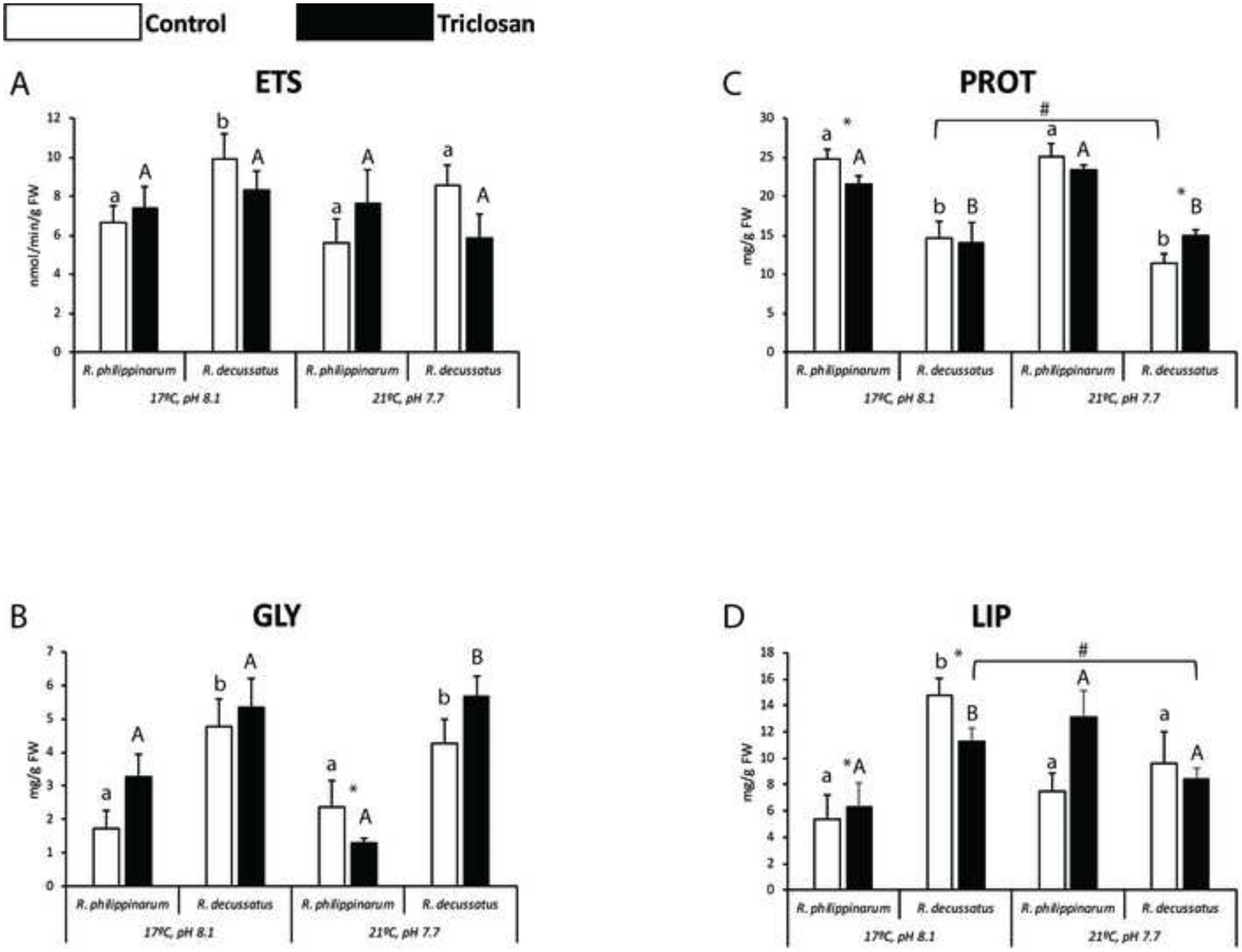

**Figure 3**

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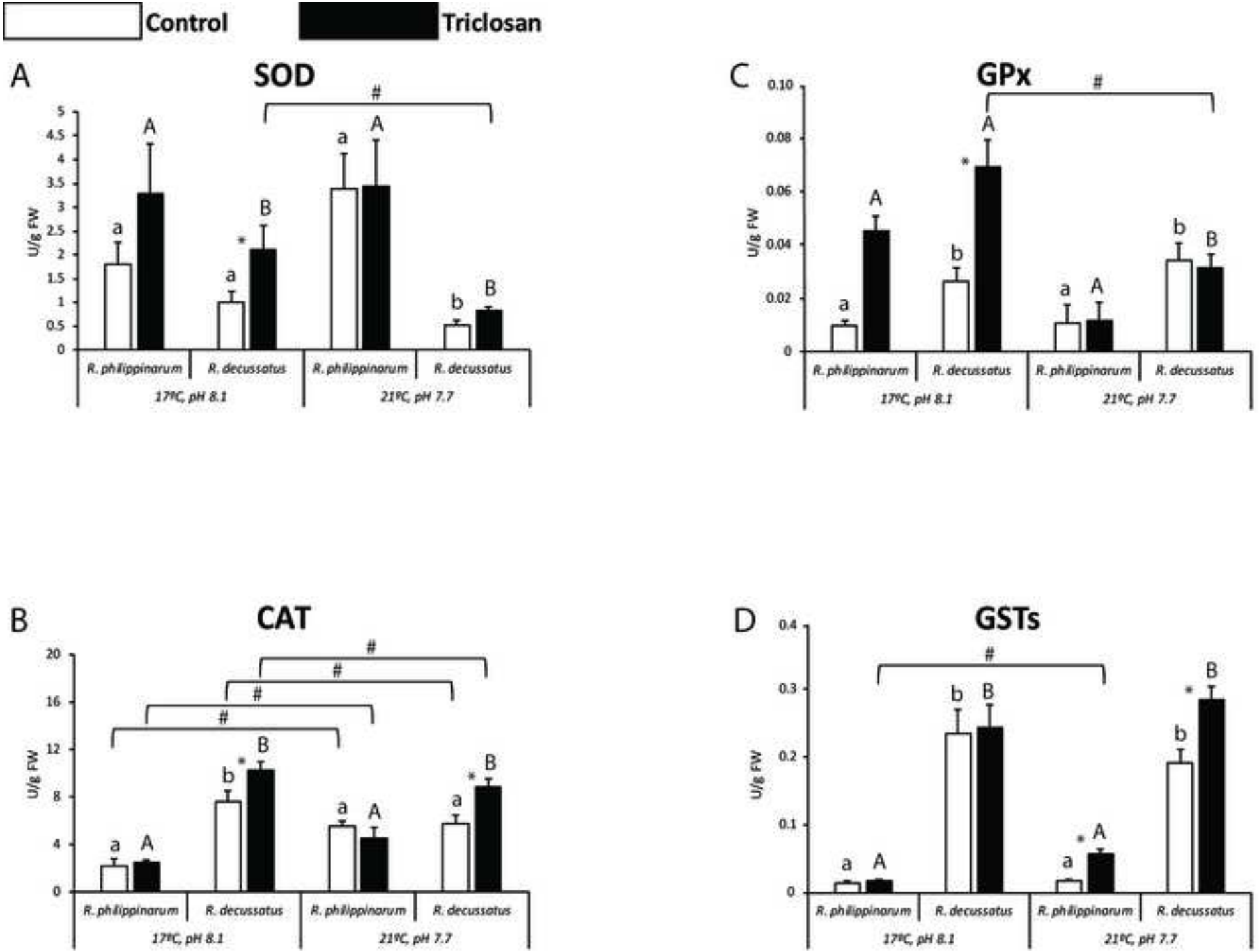

**Figure 4**

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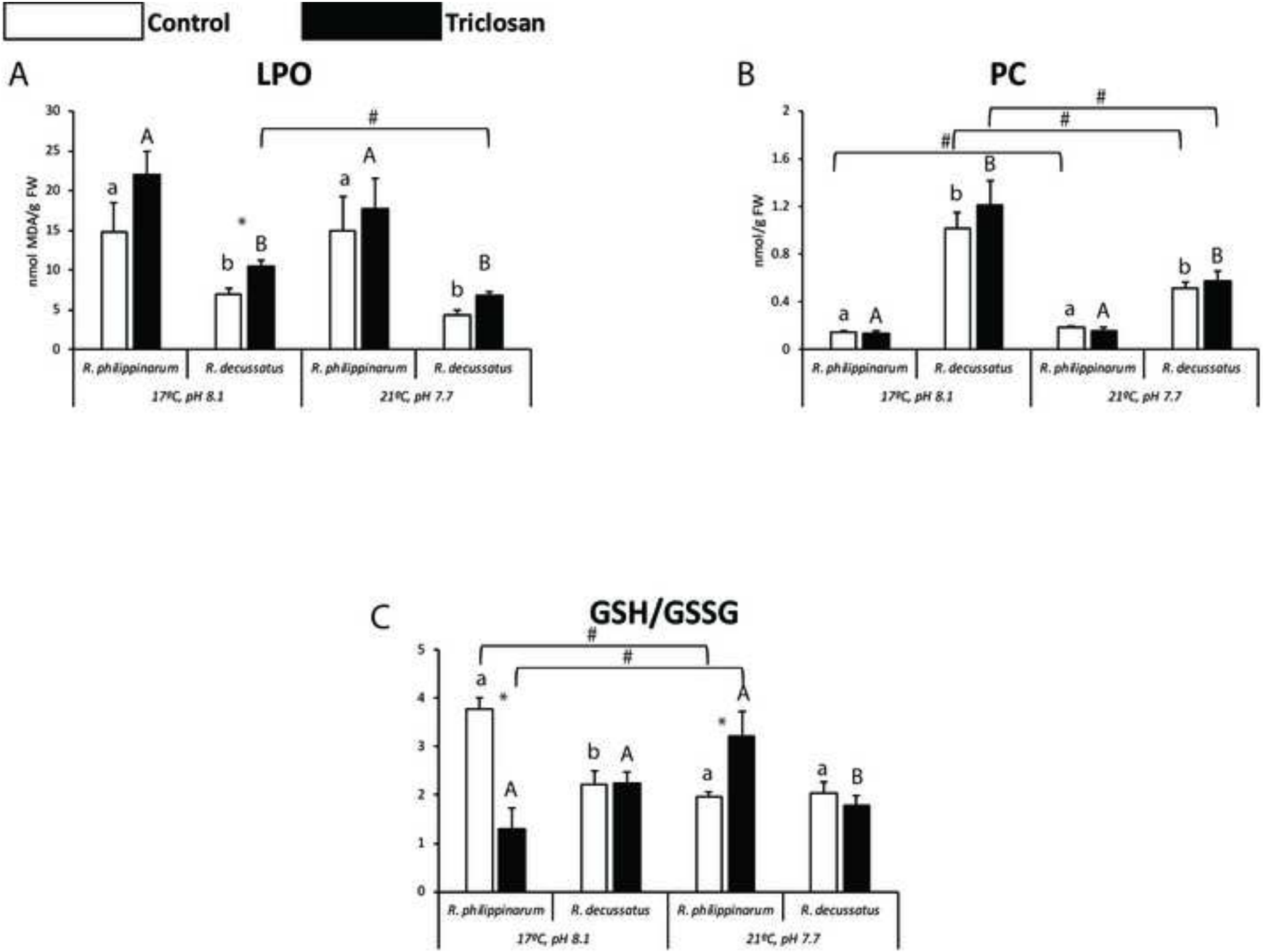

Figure 5

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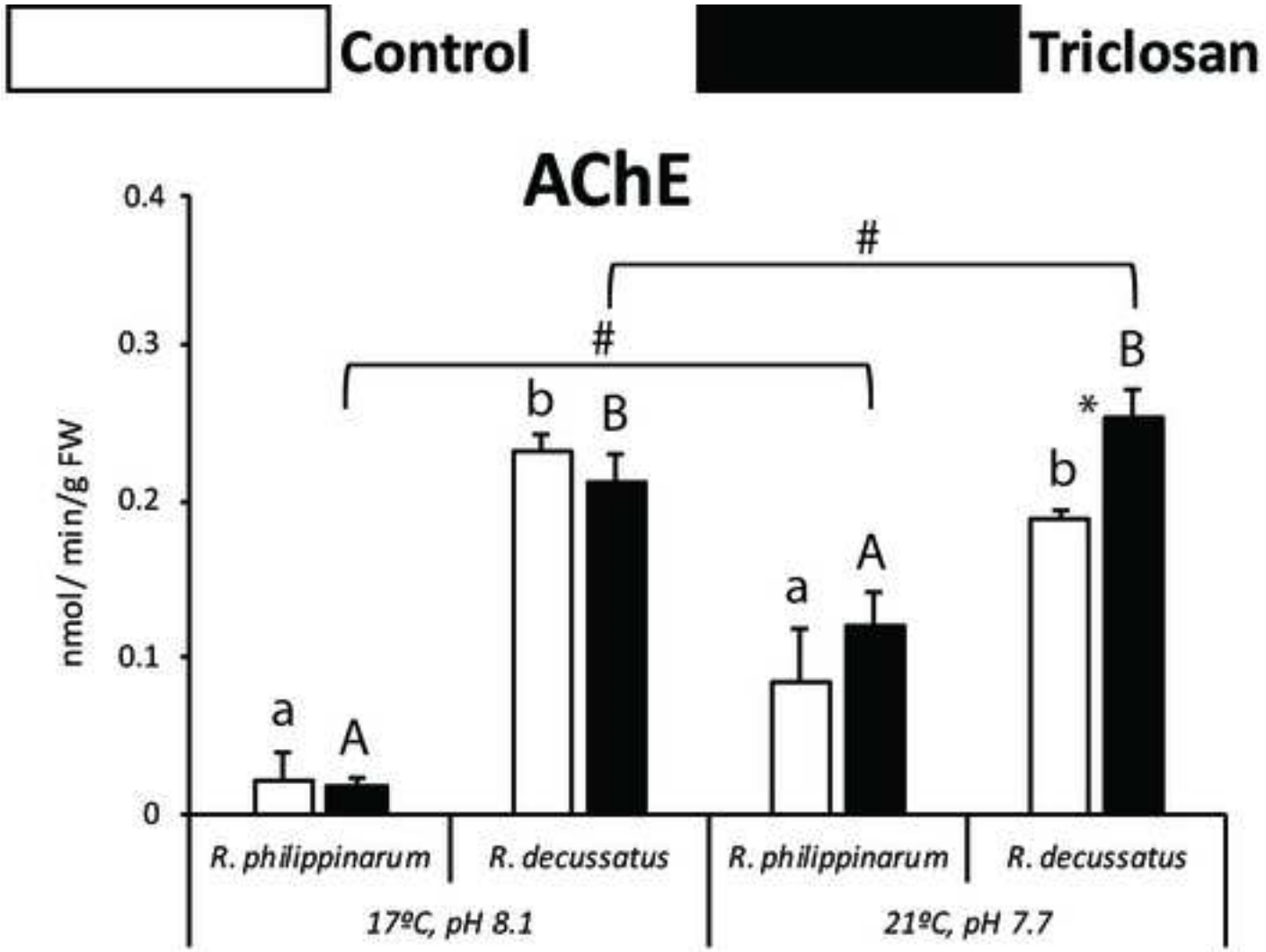

Figure captions

**Figure 1.** Respiration Rate (RR), in *Ruditapes decussatus* and *Ruditapes philippinarum* (for each species, 6 individuals per condition, 2 per aquarium) maintained for 30 days to actual conditions (17ºC, pH 8.1) and predicted climate change conditions (21ºC, pH 7.7), in the absence and presence of Triclosan (TCS, 1 µg/L). Results are the means +standard errors. White bars represent non-contaminated organisms and black bars represent the contaminated ones. Significant differences ($p<0.05$) between non- and TCS-exposed clams are represented in the figures with an asterisk (*); between climatic scenarios are represented with a cardinal (#) and between species with a lower case letter (non-contaminated) and an upper case letter (contaminated).

**Figure 2. A:** Electron transport system (ETS) activity; **B:** Glycogen (GLY); **C:** Protein (PROT) and D: Lipids (LIP) concentrations, in *Ruditapes decussatus* and *Ruditapes philippinarum* (for each species, 9 individuals per condition, 3 per aquarium), maintained for 30 days to actual conditions (17ºC, pH 8.1) and predicted climate change conditions (21ºC, pH 7.7), in the absence and presence of Triclosan (TCS, 1 µg/L). Results are the means + standard errors. White bars represent non-contaminated organisms and black bars represent the contaminated ones. Statistical contrasts as in Figure 1.

**Figure 3. A:** Superoxide dismutase (SOD); **B:** Catalase (CAT); **C:** Glutathione peroxidase (GPx); **D:** Glutathione-S-transferases (GSTs) activities, in *Ruditapes decussatus* and *Ruditapes philippinarum* (for each species, 9 individuals per condition, 3 per aquarium), maintained for 30 days to actual conditions (17ºC, pH 8.1) and predicted climate change conditions (21ºC, pH 7.7), in the absence and presence of Triclosan (TCS, 1 µg/L). Results are the means + standard errors. White bars

represent non-contaminated organisms and black bars represent the contaminated ones. Statistical contrasts as in Figure 1.

**Figure 4. A:** Lipid peroxidation (LPO); **B:** Protein carbonylation (PC) levels; **C:** Reduced/Oxidised Glutathione (GSH/GSSG) ratio in *Ruditapes decussatus* and *Ruditapes philippinarum* (for each species, 9 individuals per condition, 3 per aquarium), maintained for 30 days to actual conditions (17ºC, pH 8.1) and predicted climate change conditions (21ºC, pH 7.7), in the absence and presence of Triclosan (TCS, 1 µg/L). Results are the means + standard errors. White bars represent non-contaminated organisms and black bars represent the contaminated ones. Statistical contrasts as in Figure 1.

**Figure 5.** Acetylcholinesterase (AChE) activity, in *Ruditapes decussatus* and *Ruditapes philippinarum* (for each species, 9 individuals per condition, 3 per aquarium), maintained for 30 days to actual conditions (17ºC, pH 8.1) and predicted climate change conditions (21ºC, pH 7.7), in the absence and presence of Triclosan (TCS, 1 µg/L). Results are the means + standard errors. White bars represent non-contaminated organisms and black bars represent the contaminated ones. Statistical contrasts as in Figure 1.

**Figure 6. A:** Centroids ordination diagram (PCO) based on TCS concentrations, physiological and biochemical parameters, measured in *Ruditapes decussatus* and *Ruditapes philippinarum* (for each species, 9 individuals per condition, 3 per aquarium), maintained for 30 days to actual conditions (17ºC, pH 8.1) and predicted climate change conditions (21ºC, pH 7.7), in the absence and presence of Triclosan (TCS, 1 µg/L). **B:** results for *Ruditapes philippinarum*; **C:** results for *Ruditapes decussatus.* Pearson correlation vectors ($r > 0.80$) of physiological and biochemical descriptors

were provided as supplementary variables being superimposed on the top of the PCO graph ETS, GLY, PROT, LIP, LPO, PC, GSH/GSSG, SOD, CAT, GPx, GSTs, AChE.

**Figure 6**

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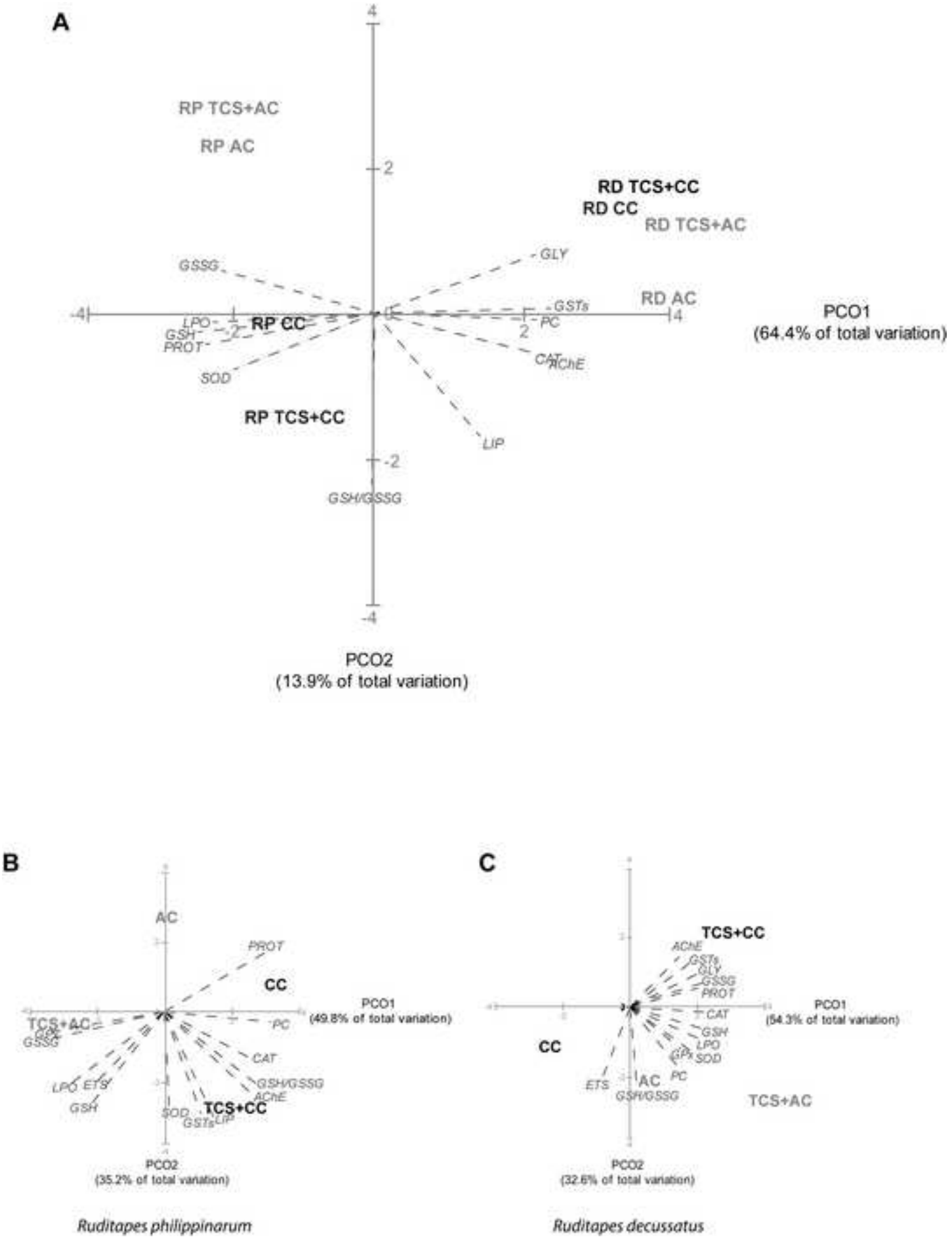

## Conflict of Interest

The Authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affi liations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

***Credit Author Statement**

Rosa Freitas and Montserrat Solé supervised the students (Silvana Costa and Francesca Coppola) in all biochemical assays and funded all biochemical analyses. Carlo Pretti, Luigi Intorre, Valentina Meucci were responsible for all pollutants quantifications analyses and costs. Amadeu M.V.M. Soares is the leader of Rosa Freitas group at CESAM. Rosa Freitas and Montserrat Solé gave the idea of this study to the students that accepted this challenge and performed all the analyses.