**Oxidative effects of the pharmaceutical drug paracetamol on the edible clam
Ruditapes philippinarum under different salinities**

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Abstract

Paracetamol, a drug with analgesic and antipyretic properties, is one of the most used substances in human therapeutics, being also frequently detected in aquatic environments. Recent studies report its toxicity towards aquatic species, but the overall amount of data concerning its effects is still scarce. Global changes, likely alterations in abiotic conditions, including salinity, can modulate the interactions of contaminants with biota, conditioning the toxicological responses elicited also by pharmaceuticals. The present article describes the oxidative toxic effects posed by paracetamol on the clam species *Ruditapes philippinarum* under different salinity conditions. The results demonstrated the establishment of an oxidative-based effect, with significant alteration of several parameters, such as superoxide dismutase (SOD) and the ratio of reduced / oxidized glutathione (GSH/GSSG). Water salinity influenced the response of clams exposed to different paracetamol concentrations, showing the importance of studying physiological traits under realistic test conditions, which are likely to vary in great extent as a result of climate change.

Keywords: clams, paracetamol, salinity, oxidative stress, biomarkers, climate change

1. Introduction

Contamination of the aquatic environment by pharmaceutical drugs is an emerging issue in ecotoxicology. Due to the ever-increasing human population with access to healthcare services (including medication) and to intense animal feeding and veterinary practices, the aquatic environment may be at risk from potential contamination with a large number and quantity of pharmaceutical products (Kim et al., 2007). A considerable number of studies have already shown effects of pharmaceutical contamination on aquatic organisms (Owen et al., 2009; Gelsleichter and Szabo, 2013; Brodin et al., 2013). Fent et al. (2006) suggested that residues of pharmaceuticals in aquatic systems are unlikely to pose an immediate risk in terms of acute toxicity (except for extreme scenarios, such as large volume spills); however, chronic exposures can exert deleterious effects in exposed biota. Pharmaceuticals, by being routinely

discharged into the aquatic ecosystem in amounts that roughly equal the environmental elimination rates, are considered pseudo-persistent pollutants (Zenker et al., 2014), and chronic effects are possible. Pharmaceuticals are known to have biological activity, which is the most important parameter when evaluating their toxicological impact in the wild (Halling-Sørensen et al., 1998; Daughton and Ternes, 1999; Jones et al., 2002; Miao et al., 2002; Brandão et al., 2014). These contaminants are specifically designed to resist metabolic degradation, and, albeit being polar molecules, are lipophilic enough to be absorbed by target organisms (Brandão et al., 2014). Due to the conservative nature of physiological processes, many aquatic species possess similar target molecules/receptors to those the drugs are intended to interact with in humans (Owen et al., 2007; Gunnarsson et al., 2008), favoring the exertion of pharmacological and toxicological effects on most aquatic organisms. Even if only present in surface waters at trace concentrations, typically ng – µg/L (Nikolaou et al., 2007), some pharmaceuticals cause adverse effects even at lower concentrations, <1ng/L in some cases (Parrot and Blunt, 2005; Garric et al. 2007; Zeilinger et al., 2009). The largest contributing sources of drugs into the environment are the sewage treatment and disposal systems, which are generally ineffective or otherwise inappropriate to deal with recalcitrant substances such as pharmaceutical drugs. On the other hand, an additional source of drugs in the environment comes from the release of raw, untreated sewage into the aquatic ecosystem. Even considering the wide use of sewage treatment processes, which should theoretically remove the majority of anthropogenic compounds, the reality shows that these processes are not effective to cope with pharmaceutical drugs. Pharmaceutically active compounds have been detected previously in sewage-treatment plants (STPs) (Hignite and Azarnoff, 1977).

One of the most used pharmaceutical drugs is paracetamol (also designated as acetaminophen) (Sebastine and Wakeman 2003; Yang et al. 2008; Lourenção et al. 2009; Solé et al. 2010) due to its antipyretic and analgesic properties (Xu et al., 2008), especially for pediatric purposes (Pandolfini and Bonati, 2005). It is reported as one of the most frequently detected pharmaceuticals in sewage treatment plant effluents, drinking water, and also surface water (Kim et al., 2007). Paracetamol has also been found in marine waters (namely in the Mediterranean Sea), in levels up to 3 µg/L (Nödler et al., 2014). Additional studies demonstrated that paracetamol is promptly accumulated by marine organisms, being one of the most common micropollutants

found in sessile species, such as mussels (Wille et al., 2011). Considering that its main toxicological outcomes in mammals result from oxidative stress (Moore et al., 1985; Letelier et al., 2011), it is likely that a similar response may occur in the exposed aquatic macroinvertebrates. Paracetamol may be metabolized by conjugation with co-factors, forming the non-toxic conjugated metabolites paracetamol glucuronide and paracetamol sulphate (Patel et al., 1993; Klaassen 2001; Jaeschke and Bajt 2006; Xu et al. 2008). This pathway corresponds to a detoxification process, since the majority (circa 90%) of ingested paracetamol is converted into these two conjugate forms, which are promptly excreted. The remaining portion of absorbed paracetamol is oxidized via cytochrome P450 enzymes (primarily CYP 2E1, 1A2, and 3A4), into a highly reactive, oxidant, and electrophilic intermediate, N-acetyl-p-benzoquinoneimine (NAPQI; Xu et al. 2008), which in turn is usually detoxified by conjugation with intracellular glutathione (Prescott 1980; Patel et al. 1993; Klaassen 2001; Xu et al. 2008). However, higher paracetamol dosages are responsible for the exhaust of required cofactors for ulterior conjugation (both of paracetamol and, ultimately, NAPQI) and NAPQI is accumulated, exerting multiple toxic effects, such as covalent modifications of thiol groups on cellular proteins (Xu et al. 2008), DNA and RNA damage, and oxidation of membrane lipids, resulting in necrosis and cellular death (Prescott 1980; Jaeschke et al. 2003; Hinson et al. 2004; Jaeschke and Bajt 2006). Despite the absence of metabolic studies that corroborate this hypothesis, evidences point to the involvement of a similar oxidative pathway in aquatic organisms (Antunes et al., 2013; Nunes et al., 2014a,b; Ramos et al., 2014; Nunes et al., in press).

When an organism is exposed to oxidative stress conditions, a defensive cascade of reactions is triggered in order to cope with overproduction of reactive chemical species that may act as oxidants. This process aims to defend cells and tissues from the oxidative insult, preventing the establishment of severe and widespread pro-oxidative damage. The first line of the enzymatic defense mechanism is superoxide dismutase (SOD) that catalyzes the dismutation of superoxide (O_2^-) into oxygen and hydrogen peroxide (McCord and Fridovich 1969). The hydrogen peroxide formed due to the activity of SOD is nevertheless a harmful byproduct, which requires to be eliminated or degraded. To prevent further cellular and tissue damage, a second enzymatic mechanism is activated, catalase (CAT), which is involved in the conversion of hydrogen peroxide into water (Aebi, 1984). By registering significant increases of

specific enzymes (e.g. CAT and SOD activities), one can infer about the most likely occurrence of oxidative stress.

Considering that a vast amount of scientific data is still required to address the issue of pharmaceutical contamination of the aquatic environment, more realistic approaches are required for testing and assessing potential effects of these substances on biota. Furthermore, it is also important to test the sensitivity and responsiveness of distinct tests organisms when exposed to pharmaceuticals, to address the type, extent and profile of biological response elicited, namely by higher, albeit not environmentally realistic, doses. Additionally, the toxicological effects of pharmaceutical substances may be influenced by alterations of abiotic conditions, including salinity, as a result of global change (IPCC, 2007). It has been demonstrated that warmer temperatures and lower rainfall increase seawater salinity, while extreme rainy events decrease seawater salinity. Both situations will promote species physiological, biochemical and metabolomic responses (among others, Matozzo et al., 2007; Carregosa et al., 2014a and b; Freitas., 2015). According to Widdows and Shick (1985) salinity is one of the dominant environmental factors that most affect marine organisms, limiting their spatial distribution in the environment, and having high impact in the fishery and culture of bivalves (Matozzo et al., 2007). In this particular sense, modifications in sensitive geographic areas, such as estuaries, are a major concern for ecotoxicologists (Booij, 2005; Kay et al., 2006), since biological responses elicited by anthropogenic contamination may be difficult to predict and characterize in a continuously changing environment.

To attain the general purpose of studying its major impacts on a species of mollusk, the present article describes the deleterious effects of paracetamol on the clam species *Ruditapes philippinarum*, in terms of oxidative stress defense/metabolism, under varied conditions of salinity. Test organisms (*Ruditapes philippinarum*) were manually collected at the Ria de Aveiro (40°45.00'36"N; 8°41.45'58"W), a shallow lagoon in the Atlantic coast of the Central Region of Portugal. The Ria de Aveiro is of considerable regional importance, with a wide range of biotopes used as nursery areas for many economically and biologically valuable species, including polychaetes, bivalves, crustaceans, fish and birds (Lopes et al., 2007; Rodrigues et al., 2011). It is characterized by high salinity fluctuations mainly due to inputs of freshwater from rivers, and also due to the direct influence of the seawater (Dias et al., 1999) that enters

directly from the Atlantic Ocean. This lagoon exhibits a spatial gradient of salinity from about 0 in the upper reaches of the estuary to about 36 at the bar entrance (Lopes et al., 2007). Bivalves are commonly used as test animals for sediment toxicity, using various endpoints (Shin et al., 2002). These organisms have been used for the assessment of several endpoints with toxicological importance, from mortality (Cheung and Wong, 1993; Naimo et al., 2000), to bioaccumulation of contaminants (Thompson et al., 2000; Shin et al., 2002). The clam *R. philippinarum*, native of the Indo-Pacific region, was introduced in the European Atlantic and Mediterranean coastal waters for commercial exploitation, and is surely one of the most well adapted and widespread species (Pranovi et al., 2006). Having displaced its autochthonous congeneric species (*R. decussatus*) in several coastal areas, *R. philippinarum* now represents the most important species for commercial clam landings in Europe (Milan et al., 2011). This clam species is adaptable to be used as test organism (Cheung and Wong, 1993), making it an ideal organism for biological testing. This species is able to survive in a large range of salinities (from 14 to 42), but presented high sensitivity to low salinities (0 and 7) (Carregosa et al., 2014a, b).

2. Materials and methods

The clams (approximately 150 specimens) were collected in October 2013 to avoid the reproduction period of this species (Meneghetti et al., 2004; Drummond et al., 2006). In order to minimize the effect of body size on biochemical responses, organisms with similar size (47.3-52.7 mm) were used in the laboratory experiments. Immediately after collection, organisms were subjected to depuration/quarantine/acclimation for 2 weeks. During this period, conditions were kept stable: temperature $18\pm1^{\circ}\text{C}$, photoperiod of 12h light: 12h dark, artificial seawater with mean salinity of 28 ± 1 , and continuous aeration (Freitas et al., 2012). During this period, animals were kept in 2 containers, each with circa 75 organisms; organisms were fed with Algamac Protein Plus (150 000 cells/L/animal) every 2-3 days and, when identified, dead organisms were immediately discarded. After this period, organisms were exposed to different paracetamol concentrations (0.00 mg/L, control; 0.05; 0.5 and 5 mg/L), combined with distinct salinity levels (14, 28 and 35). Prior to the onset of exposure, for 5 days, salinity was gradually decreased and increased to reach the salinities other than the control, 14 and 35 respectively. The salinities tested were selected taking into account the salinity

range found at the sampling site (25 to 35 g/L), in the Ria de Aveiro, where clams were harvested, and salinity changes predicted to occur due to strong rainy events and longer drought periods. In summer and winter periods these values can reach salinities of 38 and 10 g/L, respectively (Santos et al., 2007). The three distinct paracetamol concentrations were obtained by diluting an initial solution, whose concentration was confirmed through colorimetric quantification following an adapted version of the procedure no. 430 by Sigma Diagnostics (Brandão et al. 2014). Test concentrations nominated hereinafter are actual concentrations estimated on the basis of the paracetamol quantification made on the stock solution. Salinity was set up by the addition of artificial sea salt to deionized water. Paracetamol concentrations were based on ecological relevance criteria; the first concentration was close to those already reported for freshwater systems (6 – 65 µg/L; Ternes, 1998; Roberts and Thomas, 2006) and in the same order of magnitude of the levels found in the marine ecosystem (3 µg/L; Nödler et al., 2014). The second selected concentration was 1 order of magnitude above the observed levels in the wild; the highest concentration was used to test the responsiveness of clams to the oxidant effect of paracetamol (Antunes et al., 2013).

For this experiment, a total of 84 organisms were used, divided by plastic vessels with capacity up to 1 liter of water (1 clam per vessel). A total of 7 clams were used per test condition (1 organism/replicate), in 300 mL of artificial seawater. The remaining abiotic conditions were kept similar to those adapted during quarantine: continuous aeration, temperature of $18\pm1^{\circ}\text{C}$, and 12h light: 12h dark. The aeration and mortality were monitored every 24h. Exposures were conducted following an adapted version of the Ecological Effects Test Guidelines: OPPTS 850.1710. Oyster BCF (EPA 1996a; b). Exposure period was of 96h, and the water was renewed 48h after the onset of exposure, to maintain paracetamol levels and salinity during the experiment. At the end of the exposure, clams were frozen at -80°C for further processing.

2.1 Biomarker analysis

The selected biomarkers were the activities of antioxidant enzymes (namely, superoxide dismutase, SOD; catalase, CAT; glutathione reductase, GRed), the activity of the phase II metabolism/antioxidant isoenzymes glutathione-S-transferases (GSTs), the redox balance in terms of ratio of the levels of reduced and oxidized glutathione (GSH/GSSG), and the extent of lipoperoxidative damage (levels of thiobarbituric acid

reactive substances, TBARS, expressed as malondialdehyde). The biochemical parameters were determined as described by Carregosa et al. (2014a; b). Frozen organisms were homogenized in liquid nitrogen, using a tissue homogenizer Ystral mod.X10/20, and immediately divided in separate aliquots of 0.5 g for the determination of biochemical parameters. Previously obtained homogenates were resuspended in phosphate buffer (50 mM, pH = 7.0, with 0.1% Triton X-100) for the determination of Superoxide dismutase (SOD), Catalase (CAT), Glutathione reductase (GRed) and Glutathione S-transferases (GSTs) activities; the homogenates for reduced Glutathione (GSH) and Lipid peroxidation (LPO) were resuspended in TCA (trichloroacetic acid) 20% and were centrifuged at 10.000g for 20 min at 4°C. Supernatants were used immediately or stored at -80°C.

SOD activity was determined by the conversion of nitroblue tetrazolium (NBT) to NBT diformazan (formazan dye) via superoxide radical, whose formation was followed at a wavelength of 560 nm, as described by Beauchamp and Fridovich (1971), and activities were expressed in nanomoles of NBT diformazan per minute, per g of fresh weight (FW).

CAT activity was measured by the reaction of the enzyme with methanol in the presence of H₂O₂ (Johansson and Borg, 1988). The formaldehyde formation was spectrophotometrically measured with Purpald, which forms a colored compound that absorbs radiation at a wavelength of 540 nm. Enzymatic activity was expressed in nmoles of formaldehyde produced per min, per mg of FW.

Reduced GSH quantification was described by Moron et al. (1979), with some modifications. Extraction was performed with TCA 20% and neutralized with NaOH and potassium phosphate buffer. During the oxidation of glutathione by 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), 2-nitro-5 thiobenzoic acid is formed, which can be spectrophotometrically detected by a change of absorption at 412 nm. Results were expressed as micromoles of 2-nitro-5-thiobenzoic acid formed per g of FW. Total GSH concentration (GSH + GSSG) is measured by a colorimetric reaction using DTNB, which reacts with thiolic compounds. GSH reacts with DTNB to generate GSSG and 2-nitro-5-thiobenzoic acid (Akerboom and Sies 1981), whose amount is proportional to total GSH content. The formation of 2-nitro-5-thiobenzoic acid is monitored at a wavelength of 412 nm. Results were expressed as micromoles of 2-nitro-5-thiobenzoic acid formed per g of FW.

GRed activity was determined according to Carlberg and Mannervik (1985). The quantification of enzymatic activity was performed by monitoring the oxidation of NADPH at an absorbance of 340 nm. Enzymatic activity was expressed as the quantity of the enzyme that catalyzes the conversion of 1 μmol of substrate per min per mg of protein (U/mg of protein).

GSTs catalyze the conjugation of the substrate 1-chloro-2,4- dinitrobenzene (CDNB) with glutathione, forming a thioether, whose formation can be spectrophotometrically followed by the increment of absorbance at a wavelength of 340 nm, according to Habig et al. (1974). GSTs activity was expressed by the quantity of the enzyme that catalyzes the conversion of 1 μmol of substrate per min (U/mg of protein).

According to the protocol described by Buege and Aust (1978), the extent of lipid peroxidation was measured by the quantification of TBARSs. This methodology is based on the reaction of LPO by-products, such as malondialdehyde (MDA), with 2-thiobarbituric acid. The amount of TBARS was spectrophotometrically measured at a wavelength of 535 nm ($=156 \text{ mM}^{-1} \text{ cm}^{-1}$), and results were expressed as nmol of MDA equivalents per g of FW.

Protein concentration was determined according to the spectrophotometric (wavelength of 595 nm) method of Bradford (1976) adapted to microplate, using bovine serum albumin (BSA) as standard.

2.2 Statistical analysis

For each descriptor significant differences among paracetamol concentrations and among salinities were assessed. The biochemical descriptors (LPO, CAT, SOD, GSTs, GSH, GSH/GSSG) were analyzed following a one-way hierarchical design, with paracetamol concentration or salinity level as the main fixed factors. The null hypothesis tested were: i) for each parameter and for a given salinity level, no significant differences existed among concentrations; ii) for each parameter and for each paracetamol concentration, no significant differences existed among salinities. Significance levels ($p \leq 0.05$) between conditions were presented with different letters. The pseudo-F values in the PERMANOVA main tests were evaluated in terms of significance among different conditions. When the main test revealed statistical significant differences ($p \leq 0.05$), pairwise comparisons between conditions were performed. Significance levels ($p \leq 0.05$) among concentrations, for each salinity level,

were presented with different letters in Figures and also in Table 1. Significant levels ($p \leq 0.05$) among salinities for each concentration are presented in Table 1.

3. Results

The significance values associated for each pairwise comparison (among concentrations for each salinity; among salinities for each concentration) are depicted in Table 1.

Table 1 - Significance values associated for pairwise comparisons: among concentrations for each salinity; among salinities for each concentration.

		SOD	CAT	Gred	GSts	GSH/GSSG	LPO
Comparison among concentrations	S14_CTL vs S14_005	0.0035	0.087	0.0921	0.1535	0.0097	0.8534
	S14_CTL vs S14_05	0.0002	0.393	0.2531	0.5647	0.0085	0.1293
	S14_CTL vs S14_5	0.0001	0.0066	0.6893	0.9047	0.0087	0.0589
	S14_005 vs S14_05	0.7681	0.2523	0.4342	0.2453	0.9624	0.6667
	S14_005 vs S14_5	0.045	0.2618	0.2526	0.0093	0.7182	0.5984
	S14_05 vs S14_5	0.046	0.0274	0.5719	0.3324	0.7676	0.7894
	S28_CTL vs S28_005	0.0121	0.0009	0.5257	0.3969	0.0338	0.0911
	S28_CTL vs S28_05	0.0433	0.9738	0.0478	0.0813	0.0138	0.7858
	S28_CTL vs S28_5	0.3511	0.0135	0.0457	0.0276	0.1394	0.2566
	S28_005 vs S28_05	0.1115	0.0014	0.058	0.8627	0.6703	0.0456
	S28_005 vs S28_5	0.0267	0.0001	0.045	0.6761	0.894	0.0039
	S28_05 vs S28_5	0.0484	0.0133	0.9091	0.7216	0.692	0.0064
	S35_CTL vs S35_005	0.0678	0.564	0.9223	0.8672	0.1594	0.2759
	S35_CTL vs S35_05	0.1578	0.0411	0.9397	0.5246	0.5206	0.008
	S35_CTL vs S35_5	0.5403	0.0421	0.0823	0.2335	0.1081	0.0007
	S35_005 vs S35_05	0.5591	0.0452	0.9701	0.4498	0.1482	0.0138
	S35_005 vs S35_5	0.3741	0.0456	0.2467	0.2166	0.7327	0.0036
	S35_05 vs S35_5	0.6722	0.8438	0.1597	0.3574	0.0656	0.0321
Comparison among salinities	S14_CTL vs S28_CTL	0.0009	0.0009	0.0301	0.2557	0.0103	0.8665
	S14_CTL vs S35_CTL	0.0001	0.0001	0.4985	0.65	0.0153	0.6634
	S14_005 vs S28_005	0.0125	0.48	0.0004	0.2234	0.0339	0.4325
	S14_005 vs S35_005	0.0104	0.0002	0.0729	0.0789	0.6898	0.6667
	S14_05 vs S28_05	0.0434	0.0001	0.0272	0.6157	0.1012	0.0897
	S14_05 vs S35_05	0.0164	0.0042	0.1068	0.8339	0.3895	0.0035
	S14_5 vs S28_5	0.1796	0.0001	0.2309	0.7615	0.1268	0.0008
	S14_5 vs S35_5	0.4712	0.0013	0.7542	0.0427	0.7548	0.0002
	S28_CTL vs S35_CTL	0.0468	0.0098	0.0423	0.1936	0.1772	0.6327
	S28_005 vs S35_005	0.1987	0.0002	0.124	0.9449	0.0357	0.4795
	S28_05 vs S35_05	0.0806	0.821	0.623	0.2706	0.2355	0.0094
	S28_5 vs S35_5	0.2153	0.0838	0.0454	0.0509	0.0912	0.0036

Superoxide dismutase activity

The variation of SOD activity for clams exposed to different salinities and paracetamol concentrations is presented in Fig. 1A. For the lowest salinity (14, Fig. 1A) relative to the control, SOD activity significantly increased for all paracetamol concentrations

(0.05, 0.5, 5 mg/L; $p \leq 0.05$); for organisms exposed to a salinity of 28 (Fig. 1A), SOD activity significantly increased for the two intermediate concentrations (0.05 and 0.5; $p \leq 0.05$) and decreased thereafter; organisms exposed to the highest salinity (35, Fig. 1A) did not evidence any significant changes in their SOD activities.

In general, the activity of SOD was lower at salinity 14 than at the other two salinities tested (28 and 35, Fig. 1A). Furthermore, when analyzing the SOD activity obtained at different salinities, for each of the paracetamol exposure concentrations, it is possible to observe that: i) organisms exposed to 0 mg/L presented significant differences ($p < 0.001$) among salinities; ii) organisms exposed to 0.05 and 0.5 mg/L of paracetamol presented no significant differences between salinity 28 and the highest salinity tested (35), but significant differences ($p < 0.05$) were obtained when comparing salinities 14 and 28, and 14 and 35; iii) organisms exposed to 5 mg/L of paracetamol did not show significant differences among salinities.

Catalase activity

Fig. 1B illustrates the variation in the activity of CAT. The here obtained results showed that, in comparison with the control, organisms exposed to a salinity of 14 had a higher CAT activity, that generally increased along the range of paracetamol concentrations (Fig. 1B). However, significant differences were only observed for the concentration of 5 mg/L ($p < 0.001$). Organisms exposed to the salinity of 28 (Fig. 1B), showed a dual response with a significant increase of the CAT activity at the lowest paracetamol concentration (0.05 mg/L; $p < 0.001$), and a significant decrease, in comparison with the control treatment, at the highest concentration (5 mg/L; $p < 0.05$). For animals under the highest salinity (35, Fig. 1B) CAT activity significantly increased for the two highest tested concentrations.

At all the paracetamol concentrations tested, the activity of CAT was higher at salinity 14, than at salinities 28 and 35 (Fig. 1B). Analyzing the CAT activity obtained for different salinities, and comparing the data at each paracetamol exposure concentration, the results also revealed that: i) when organisms were under control condition (0 mg/L) significant differences ($p < 0.001$) were found among all salinity levels; ii) organisms exposed to 0.05 mg/L showed no significant differences between salinities 14 and 28;

iii) significant differences ($p < 0.001$) were obtained among salinities 14 and 35 and salinities 28 and 35; iv) when exposed to 0.5 and 5 mg/L, organisms revealed no significant differences between salinity 28 and salinity 35, while significant differences ($p < 0.001$) were found between the lowest (14) and the two other salinities (28 and 35).

Figure 1

Glutathione reductase activity

The activity of glutathione reductase (Fig. 2A) showed a similar pattern along the increasing paracetamol exposure, especially noticed at salinities 14 and 35. For salinity 14, the activity of this enzyme slightly increased, albeit not significantly, for the

concentrations of 0.05 and 0.5 mg/L (Fig. 2A); the activity of GRed in animals subjected to 28 significantly increased at concentrations 0.5 and 5 mg/L (Fig. 2A); organisms exposed to a salinity of 35 showed no significant enzyme activity alterations for all exposure concentrations (Fig. 2A).

The results obtained showed that the enzyme GRed presented lower activity at salinity 28 than at salinities 14 and 35 (Fig. 2A). The results on GRed activity further demonstrated that: i) organisms exposed to 0 mg/L revealed no significant differences ($p < 0.05$) between the lowest (14) and the highest (35) salinities; ii) organisms exposed to 0.05 and 0.5 mg/L showed a significant decrease ($p < 0.01$) between salinities 14 and 28, but no significant differences were found in terms of GRed activity when comparing salinity 14 with the other two salinity levels (28 and 35); iii) organisms subjected to the highest paracetamol concentration (5 mg/L) showed significant differences ($p < 0.05$) between salinities 28 and 35.

Glutathione-S-transferases activity

The activity of GSTs is presented in Fig. 2B. Although with no significant differences being reported among paracetamol concentrations, GSTs activity of organisms exposed to the salinity of 14 decreased at the lowest paracetamol concentration (0.05 mg/L), and returned to the control value for higher levels of paracetamol (0.5 and 5 mg/L, Fig. 2B). GSTs activity of organisms subjected to the salinity of 28 increased along the concentrations, with significant differences only being noticed between the control and the highest paracetamol concentration (5 mg/L, Fig. 2B). Clams exposed to the highest salinity conditions showed no significant variation on GSTs activity among concentrations (Fig. 2B).

When comparing salinity levels, the activity of GSTs did not change along the paracetamol exposure gradient. The activity of GSTs, when organisms were exposed at 0, 0.05, 0.5 mg/L of paracetamol, showed no significant differences among salinities (Fig. 2B). At 5 mg/L organisms showed significant differences ($p < 0.05$) between salinities 14 and 35 (Fig. 2B).

Figure 2

Ratio of reduced and oxidized glutathione levels

The ratio GSH/GSSG is presented in Fig. 3A. Control clams exposed to the lowest salinity (14, Fig. 3A) had a much higher ratio, in comparison with animals from all other concentrations. On the contrary, values found for organisms exposed to a salinity of 28 (Fig. 3A) evidenced an opposite pattern, since a significant increase of this ratio was observed with increasing concentrations of paracetamol. Organisms exposed to the highest salinity (35), revealed no significant differences among concentrations (Fig. 3A).

Comparing GSH/GSSG values obtained for organisms exposed to 0 mg/L of paracetamol, no significant differences were found between salinities 28 and 35, while significant differences were obtained between salinities 14 and 28, and salinities 14 and 35. Clams exposed to 0.05 mg/L showed no significant differences between salinities 14 and 35, but significant differences were found between 28 and the other two salinities. When under 0.5 and 5 mg/L clams showed no significant differences among salinity levels.

Lipid peroxidation

The lipid peroxidation levels obtained for organisms exposed to different paracetamol levels under distinct salinities is presented in Fig. 3B. The results obtained showed that at a salinity of 14 no significant differences were found between paracetamol concentrations. At a salinity of 28 (Fig. 3B) organisms showed higher LPO levels at 0.05 mg/L, and lower contents at 5 mg/L of paracetamol. Clams exposed to a salinity of 35 (Fig. 3B), showed that the levels of MDA decreased along the exposure gradient, being only statistically significant for the two highest concentrations (0.5 and 5 mg/L). When analyzing the LPO levels obtained at 0 and 0.05 mg/L, no significant differences were obtained among salinities (Fig. 3B). On the contrary, LPO levels obtained for clams exposed to 0.5 and 5 mg/L revealed a significant decrease ($p < 0.01$) among salinities (Fig. 3B).

Figure 3

4. Discussion

The main objective of this work was to assess the toxic effect of paracetamol on the marine clam species *Ruditapes philippinarum* under different conditions of salinity, thus simulating the impact of a model anthropogenic compound (paracetamol) under a scenario most likely to occur due to climate change. The first observation that can be drawn from the obtained results is the fact that *R. philippinarum* seems to respond differently to contamination, under varied salinity conditions. As Carregosa et al. (2014a,b) showed, the general tolerance of *R. philippinarum* to salinity was confirmed by our results, since mortality was not registered at the salinity levels tested (14, 28 and 35). On the other hand, the sensitivity of this species towards paracetamol demonstrated

the responsiveness of this organism to oxidative stress-based deleterious effects, in line with previously published data from our work group (Antunes et al., 2013).

Several of the tested conditions were able to result in significant increases of CAT and SOD activities, allowing to infer about the most likely occurrence of oxidative stress. In fact, increases in SOD and CAT activities were observed for organisms under salinity of 14; on the contrary, straightforward patterns with the simultaneous involvement of both SOD and CAT were not observable for the other two scenarios. It is possible to conclude that the initial stages of oxidative stress, with overproduction of both radical superoxide and hydrogen peroxide, only took place at a salinity of 14. The other two treatments (28 and 35) were somewhat ambiguous, since significant alterations of these two enzymatic forms were registered, but not their simultaneous co-occurrence. Changes in these two biomarkers seem to be more dependent upon salinity (Carregosa et al, 2014b), but being also modulated by paracetamol levels. It is thus possible to conclude that pro-oxidative adaptive responses caused by paracetamol occur for all salinity conditions, but were more pronounced and effective for the salinity of 14, which is a rather challenging condition for *R. philippinarum* (Carregosa et al, 2014a). The here obtained results are in good agreement with those obtained by Parolini et al. (2010), after exposing individuals of zebra mussel (*Dreissena polymorpha*) to paracetamol. Similarly, these authors found an increase in CAT and SOD activity for the highest concentration (10nM, 1.51µg/L), as observed in this study, evidencing the undisputable pro-oxidative effect of paracetamol on bivalve species. These results were complemented by a more recent study (Parolini et al., 2013), that showed the pro-oxidative, but transient effect of ecologically relevant (1nM, 151.15ng/L, considerably lower than those used in our study) levels of paracetamol to *Dreissena polymorpha*; in fact, in this study authors found an increase of SOD activity only immediately after the onset of the exposure. This finding suggests that the organism had a prompt antioxidant response to paracetamol, preventing it from exerting more damage. It is also possible to infer that a similar response might have occurred in our study, and no alterations were reported in terms of SOD and CAT activities. The crosstalk between salinity and a pro-oxidative chemical such as paracetamol can be well evidenced if one compares data of SOD activity from individuals of *R. philippinarum* kept at distinct values of salinity (but not exposed to paracetamol; Carregosa et al., 2014a) and those from our study. SOD

activity in non-exposed organisms, as shown by Carregosa et al (2014b) tends to peak at a salinity circa 14, decreasing to residual values while salinity increases. The role of paracetamol can however be dramatic in the modulation of SOD expression, and our data show that animals exposed to this drug had a tendency to have higher SOD activity values for higher salinities. This is a particularly interesting result since it clearly establishes that pollutants can change profiles and patterns of biological response, including antioxidant homeostasis, depending upon abiotic variables (such as salinity). However, one must bear in mind that not all bivalves are responsive towards paracetamol, as demonstrated by Brandão et al. (2014). This workteam showed that the freshwater bivalve *Corbicula fluminea* was not affected, in terms of its catalase activity, by both acute and chronic exposure to paracetamol. A somewhat similar pattern of response, without alterations in CAT activity, was reported by Solé et al. (2010) after exposing individuals of the marine mussel species *Mytilus galloprovincialis* to paracetamol. It is thus possible to infer that an antioxidant response to paracetamol may not derive strictly from salinity to which organisms are exposed. Among both salt and freshwater organisms, some are the examples of bivalves that respond to paracetamol by promptly activating their first line of antioxidant defense, such as CAT. This pathway seems to be highly species-specific, rather than abiotic conditions-specific.

The maintenance of the redox homeostasis strongly depends upon the metabolism of glutathione: reduced glutathione (GSH), which is the biologically active form of this co-factor, can undergo oxidation to its oxidized form (GSSG) (Tietze, 1969). It is possible to infer that the pool of glutathione, both in the reduced and in the oxidized form, is the main buffer responsible for the non-enzymatic antioxidant mechanisms, of most living beings (Haberhauer-Troyer et al., 2013). In normal conditions, GSSG represents only a small fraction of the total glutathione intracellular pool, but this situation can be reverted under pro-oxidative conditions. GSH decreases with the increase of GSSG, indicating that it is being used as an antioxidant to cope with a chemical insult. In other words, oxidative stress increases GSSG levels, thus reducing the GSH/GSSG ratio (Haberhauer-Troyer et al., 2013). A significant increase of the GSH/GSSG ratio was observed for control organisms exposed to seawater with a salinity of 14, suggesting that salinity *per se* can have a decisive role in the biosynthesis of this specific intracellular antioxidant. In fact, previous studies with marine and

estuarine invertebrate species (namely the crab *Scylla serrata*), show that respiration and oxygen consumption, which are directly connected to the regulation of redox homeostasis, can be strongly modulated by salinity to which organisms are exposed. Exposure to increased salinity (until a maximum of 35) resulted, as described by Paital and Chainy (2012) in increased indicators of oxidative stress (lipid peroxidation, production of hydrogen peroxide, and protein carbonylic content). On the contrary, exposure to low salinity values (around 10) was not causative of any antioxidant adaptation. Our results strongly contradict this general trend, since a strong shift towards the biosynthesis of GSH occurred for control animals exposed to low salinity (around 14). GSH biosynthesis in bivalves is a function of salinity as previously evidenced, and can be up-regulated during times of stress as observed by Tikunov et al (2010). The tolerance towards salinity evidenced by our test species, *R. philipinarum*, shows that this organisms is highly sensitive to low salinity conditions, while it tolerates high salt levels (Carregosa et al., 2014a). The increase in the amount of GSH suggests the occurrence of an adaptive and transient anti-oxidant response, depending only on low salinity levels, with involvement of glutathione as an antioxidant scavenger of ROS. Similar data evidencing the occurrence of oxidative stress in bivalves exposed to low salt levels were obtained by An and Choi, after exposing the bivalve species *Scapharca broughtonii* (ark shell) to hyposaline conditions. Both hyper- and hyposaline conditions were able to significantly alter the antioxidant activity and the oxidative status of exposed organisms, but low salinity values were able to cause more dramatic changes, with involvement of ROS. Similarly, the study by Carregosa et al (2014b) with the same species used in our study, reinforces the concept that low salinity can indeed favor the biosynthesis of glutathione. This alteration seems transient and may constitute an early response to a challenging abiotic condition to which our organisms were able to respond. In fact, and considering that this pattern was only reported for control animals, exposure to a set of more deleterious conditions (such as salinity + paracetamol) caused the reversion of this response, by activation of a more complex group of defenses (eg.: CAT, SOD and GRed) that acted simultaneously to prevent oxidative stress. It is this possible to conclude that low salinity, per se, can elicit an early response though enhancement of GSH biosynthesis in *R. philipinarum*; exposure to a combination of anthropogenic toxicants and to low levels of salinity results in the activation of

complimentary antioxidant mechanisms, and no extreme events of GSH synthesis are required to attain oxidative homeostasis.

While organisms under salinity 14 exhibited a marked antioxidant response, probably due to low salinity itself, individuals exposed to the same levels of paracetamol but under salinities 28 and 35 did not respond the same way. In fact, while individuals kept at 35 had their GSH pools unchanged, clams under a salinity of 28 had their ratio increased, suggesting the occurrence of an effective antioxidant response, in which the levels of the biologically active form of glutathione (its reduced form) were enhanced. This suggests a mild aggression, that does not resemble in its severity and consequences the one registered for organisms under 14. The number of studies relating paracetamol exposure and changes in the homeostasis of glutathione is scarce. Despite the absence of bibliographic references supporting this explanation in bivalves, some studies report that the pro-oxidative pathway, in aquatic organisms (namely fish), follow this trend. The work by Kavitha et al. (2011) showed that administration of paracetamol to the freshwater fish *Oreochromis mossambicus* could disrupt the glutathione metabolism, with increased amounts of GSSG (oxidized, non biologically active form of glutathione).

The metabolism of glutathione and its intracellular levels are regulated by the activity of other important enzymatic forms, such as glutathione reductase (GRed). This enzyme reduces the GSSG back into GSH, thus preventing the accumulation of an inactive form of glutathione, and favoring its activity as a scavenger against reactive oxygen species. Data from organisms exposed to a salinity of 14 showed that GRed activity was kept unchanged along the ranges of paracetamol concentrations, which is in agreement with the data obtained for GSH/GSSG. It would be expectable that, under an oxidative chemical insult posed by increasing levels of paracetamol, GRed activity would increase, to augment the conversion rates of GSSG into GSH. This pattern was previously described for other aquatic test organisms, such as the fish *Oreochromis mossambicus* (Kavitha et al., 2011), after being acutely exposed to paracetamol. This trend was not observed in our study, and it is possible to assume that GSH was used as a scavenger to face oxidation, without its prompt conversion to its reduced form. This tendency contradicts the findings by Brandão et al. (2014), who reported a decrease in

GRed activity for high paracetamol concentrations, in the bivalve species *Corbicula fluminea*.

Another important enzymatic form involved in the glutathione metabolism are glutathione-S-transferases (GSTs) isoenzymes, which are able to catalyze the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for ulterior detoxification (Blanchette et al., 2007). Conjugation with paracetamol metabolites, as earlier referred, is likely to occur via GSTs-catalyzed reactions, but quite paradoxically this parameter did not respond to paracetamol exposure, even for organisms under 14. If one considers the data published by Antunes et al. (2013), it was licit to anticipate that *R. philippinarum* exposed to paracetamol, at least for the highest salinity values, could have their GSTs activity significantly increased. It is possible to suggest, however, that this specific group of related enzymes may be the target of ROS to low concentrations of paracetamol. As shown by Brandão et al. (2014), the activity of GSTs in *Corbicula fluminea* exposed to paracetamol decreased for higher concentrations of this drug. Doubts about the responsiveness and/or contradictory trends of response of bivalves to paracetamol exposure can thus be raised; this assumption may be reinforced considering the data obtained by Parolini et al. (2013), that described the lack of response in terms of GSTs activity in *Dreissena polymorpha* exposed to ecologically relevant levels of paracetamol. However, GSTs activity may suffer a dose-dependent induction, but only for higher levels of exposure (Parolini et al., 2010), which may not be attained under the here-proposed conditions.

The results obtained for lipoperoxidation showed that, despite the occurrence of an evident antioxidant response by individuals exposed to paracetamol, especially under 14, oxidative damage did not occur after a 96h exposure assay. The occurrence of oxidative damage is conditioned by the global efficacy of the antioxidant defense mechanisms (including those here depicted), and is likely to occur when the amount of reactive oxidants surpass the scavenging capacity of the antioxidant system. The most prominent effect is peroxidative damage, namely by oxidation of membrane lipids, ending upon in the production of lipidic by-products such as malondihaldehyde, that promptly reacts with thiobarbituric acid, and may be expressed as TBARS levels (Buege and Aust, 1978). However, and considering the lack of effects even for organisms under 14, we can conclude that oxidative damage was not established. This is

an interesting finding, since it indicates that the antioxidant protection was effective, or the amount of paracetamol absorbed was small, and not sufficient to establish a measurable amount of intracellular MDA-like compounds. If this was the cause of organisms exposed to 14, organisms under the other two set of conditions (28 and 35) even exhibited lower TBARS amounts. This finding suggests that the antioxidant response was stimulated by paracetamol, being consequently effective in preventing the onset of irreversible alterations, such as lipid peroxidation. Bivalves, in general terms, are responsive to the presence of paracetamol, and lipoperoxidation was already described by previous studies. The work by Brandão et al. (2014) showed that the freshwater clam *Corbicula fluminea* had higher levels of MDA after paracetamol exposure. Similarly, exposure of the saltwater mussel *Mytilus galloprovincialis* to paracetamol also resulted in increased lipoperoxidation, as shown by Solé et al. (2010). The absence of results in terms of lipid peroxidation may be related to other defense mechanisms occurring in clams. Coughlan (2009) suggested that the Manila clam may be using organic osmolytes. Organic osmolytes have been extensively reviewed (Yancey et al., 1982; Yancey, 2001; Carregosa et al., 2014a), and found in many species (*Saxidomus giganteus* and *Calymene* spp.) to have cytoprotective properties (Yancey, 2005). It appears that organic osmolytes, such as taurine, betaine and alpha-amino acids, have unique properties such as protecting metabolic reactions and counteracting destabilizing forces on macromolecules, which help prevent cellular damage from occurring (Yancey, 2005).

From the entire set of data, one conclusion seems obvious: extensive oxidative alterations, and the consequent organism's response, were more noticeable in clams kept at a salinity of 14. Low salinity conditions are responsible for the valve closing of animals, a reflex that prevents it from i) absorbing more toxicant from the external media; ii) excrete metabolites of the already absorbed toxicant; iii) allow gas exchanges. Paganini et al. (2010) stated that in marine bivalves reduced environmental salinity can reduce feeding rates, which shows the efficiency of the valve closing reflex. Nakamura et al. (2005) showed that the clam *Ruditapes philippinarum* reduces its clearance rates when exposed to a salinity of 11 g/L, with significant decreases in clearance. Navarro (1988) has shown that the species *Choromytilus chorus*, also an estuarine clam species, closes its valves partially or totally at salinities of 18 and 15. This behavior will induce

hypoxia (Kim et al., 2001), which will have significant effects on cell metabolism (Carregosa et al. 2014). As described by Carregosa et al. (2014b) the metabolites related to anoxic conditions increased especially at low salinities for the same species *R. philippinarum*, a factor that can modulate the antioxidant response. This factor could explain the notorious effects observed for the lowest salinity values, when comparison with responses in organisms under 28 and 35.

A second conclusion points out the dramatic effects caused by salinity in the modulation of toxicity elicited by paracetamol. In fact, toxic profiles were not only different, but were, in some cases, opposite of one another. By conditioning absorption, salinity can enhance (or impair) the capacity of organisms to cope with environmental toxicants. Considering that salinity values are a major driving force, especially in estuaries, their strong variation is an additional challenge that remains almost uncharacterized in toxicological terms, for most aquatic species. The here obtained results showed the importance of studying physiological traits under realistic test environmental conditions, which are likely to vary in great extent as a result of climate change.

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Figure captions

Figure 1 – (A) Superoxide dismutase (SOD) and (B) Catalase (CAT) activity in *V. philippinarum* exposed to the control and paracetamol conditions (CTL, 0.05, 0.5 and 5 mg/L) under the salinities of 14, 28 and 35 (black, dark grey, and light grey bars, respectively). Values are the mean of seven replicates $\pm$ standard error bars. Significant differences between paracetamol concentrations (0.05, 0.5 and 5 mg/L) and the control condition (0 mg/L) are represented with different letters (a-c).

Figure 2 – (A) Glutathione reductase (GRed) and (B) Glutathione S-transferases (GSTs) activity in *V. philippinarum* exposed to the control and paracetamol conditions (CTL, 0.05, 0.5 and 5 mg/L) under the salinities of 14, 28 and 35 (black, dark grey, and light grey bars, respectively). Values are the mean of seven replicates $\pm$ standard error bars. Significant differences between paracetamol concentrations (0.05, 0.5 and 5 mg/L) and the control condition (0 mg/L) are represented with different letters (a-b).

Figure 3 – (A) Reduced (GSH) and oxidized glutathione (GSSG) ratio (GSH/GSSG) and (B) lipid peroxidation (LPO) in *V. philippinarum* exposed to the control and paracetamol conditions (CTL, 0.05, 0.5 and 5 mg/L) under the salinities of 14, 28 and 35 (black, dark grey, and light grey bars, respectively). Values are the mean of seven replicates $\pm$ standard error bars. Significant differences between paracetamol concentrations (0.05, 0.5 and 5 mg/L) and the control condition (0 mg/L) are represented with different letters (a-c).

Figure 1

Figure 2

Figure 3