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Effects of common pharmaceutical drugs (paracetamol and acetylsalicylic acid) short term exposure on biomarkers of the mussel *Mytilus* spp.

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Highlights

- No clear oxidative effects resulted from exposure of *Mytilus* to both drugs
- Metabolic changes were elicited by bot drugs, namely in the glycogen content
- Absence of oxidative effects may be consequence of short term exposure
- Mussels are promising bioindicators but only after prolonged exposures

Abstract

Pharmaceutical drugs in the wild may pose significant risks to non-target exposed organisms. This situation is even more troublesome for coastal marine or estuarine environments, located in the vicinity of large human conglomerates, for which the putative number of pollutants is extremely high, and the regime by which wild organisms are exposed is continuous. In addition, the number of studies addressing this issue is still scarce, despite evidences that show the potential contamination profiles and adverse biological effects in organisms from such areas. In this study, the ecotoxicity of common pharmaceutical drugs (namely paracetamol and acetylsalicylic acid) was assessed, by studying the susceptibility of the mussel species *Mytilus spp* to oxidative stress after being exposed for 96h to increasing but ecologically relevant concentrations of the two mentioned pharmaceuticals (paracetamol: 0, 0.5, 5, 50, and 500µg/L; acetylsalicylic acid: 0, 0.1, 1, 10, and 100µg/L). The oxidative status in exposed organisms was analyzed by measuring oxidative stress biomarkers, namely catalase (CAT), glutathione-S-transferases (GSTs), and lipoperoxidation (LPO) levels, whose alteration was indicative of chemical exposure, in both digestive gland and gills of the organisms. In addition, the food uptake and the nutritional reserve status of exposed organisms were also assessed, by measuring the consumption of ingested food, and levels of tissue reserves of glycogen in gills and digestive gland. No significant alterations were observed in the assessed oxidative stress parameters so it was possible to hypothesize that the studied drugs may have probably exerted a limited alteration of antioxidant defenses and damage, which was

reverted by the activation of defensive adaptive mechanisms. This set of data evidenced that the pro-oxidative metabolism that was already described for both drugs in other animal models, was not fully established in the exposed mussels. On the contrary, glycogen reserves were substantially changed after exposure to both toxicants, being possible to observe opposite responses caused by both drugs. Food uptake was not altered following exposure to the drugs. Further evaluations are thus required to conclude about both drugs ecotoxicity and other parameters, namely seasonality, which should be considered when performing ecotoxicology tests, especially with the selected species.

Keywords: drugs, mussel, oxidative stress, marine and estuarine toxicity, biomarkers, metabolism

Introduction

Pharmaceutical drugs are bioactive compounds used worldwide in diagnosis, treatment, alteration or prevention of disease, health conditions, and also to alter normal physiological functions of the organism (Gómez-Oliván et al., 2014), both human and animal. Even animal-related industries (livestock, poultry, aquaculture) rely on the use of pharmaceutical drugs, namely antibiotics and hormones, to favor both growth and disease control (Kaleniecka and Zarzycki, 2015). The use of modern pharmaceutical drugs dates to a few decades ago and its consumption has increased along time due to the ever increasing human population and its inherent access to healthcare service and medicines, and also due to the widespread use of drugs in veterinary practices (Correia et al., 2016). During the last decades, pharmaceutical residues and their transformation products have been detected in significant concentrations in aquatic systems (Winker et al., 2008), mostly due to a combination of indiscriminate usage and low removal efficiency in

wastewater treatment systems (Brodin et al., 2014), which are not prepared to eliminate such compounds (Nunes et al., 2006). Consequently, drugs are considered environmentally stable, and recalcitrant, and their main source in the wild results from contamination by treated wastewater effluents (Polar, 2007). Also, improper disposal of expired medication in the water, and the release of drug residues from manufacturing activities are important sources of these contaminants (Zuccato et al., 2000). The occurrence of pharmaceuticals and their toxic metabolites in rivers, lakes, subterranean water and oceans may be related also to incidental events, including industrial spills; in addition, animal husbandry operations (including the use of manure as fertilizer, discharges from aquaculture operations and the administration of drugs to poultry, swine and cattle, as therapeutic agents but also as growth promoters) also contribute for the presence of such substances in the aquatic environment (Heberer, 2002). However, pharmaceuticals may undergo various physical and chemical changes as they interact with each other and with the environment (Nunes, 2006) and their concentrations can be attenuated by processes, such as dilution, adsorption to solids, biodegradation, photolysis, or other abiotic transformation. (Snyder et al., 2009).

Because of their resistance to biodegradation and pharmacological efficiency, pharmaceuticals have the ability to persist in the environment and may have biological activity in non-target organisms (Nunes et al., 2006). Drugs are designed to act almost instantly with minimal side effects, and tend to keep this activity even when in the wild (Nunes et al., 2006). Drug exposure may result in generic alterations of the physiology of aquatic organisms, including changes in the way organisms obtain their food after being exposed to pharmaceuticals, by disturbing filtration rates (Nkoom et al., 2019), and in the management and mobilization mechanisms of energetic reserves, such as glycogen (Nunes et al., 2017; Teixeira et al., 2017a). When exposed to organic toxic compounds

(even via environment), organisms trigger defensive mechanisms to prevent potential toxic effects (Valavanidis et al., 2006); such mechanisms involve the biotransformation (and ultimate excretion) of toxicants. Biotransformation is a metabolic process that aims to increase the hydrophilic profile of xenobiotics (Minier and Galgani, 1995), occurring mainly in the liver of vertebrates, and in the digestive gland of aquatic invertebrates, such as bivalves. Among all mechanisms involved in this process, an important role is attributed to enzymes such as glutathione S-transferases (Stanley, 2017), that catalyze the conjugation of glutathione with electrophilic compounds in aquatic organisms (Gonçalves-Soares et al, 2012). The metabolism of most xenobiotics results in the production of residual amounts of reactive oxygen species (ROS), which are unstable chemical entities with one or more unpaired electrons, with potential to damage cells and tissues. In order to attenuate the deleterious effects of ROS, living organisms under pro-oxidative conditions increase the expression of enzymes that play a crucial defense role, including superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GRed) and glutathione peroxidase (GPx) (José et al., 2009). The increased activity of such defensive mechanisms can allow inferring about the putative exposure of an organisms to a xenobiotic whose metabolism favors the production of ROS. Lipid peroxidation (LPO) is an indication of cellular damage following an oxidative insult, and results from free radical attack to polyunsaturated fatty acids (PUFA) from the biological membranes (Machlin & Bendich, 1987). Increased levels of LPO are thus indicative of oxidative stress.

Two of the most important pharmaceutical drugs found worldwide (especially in the aquatic environment) are paracetamol (acetaminophen) (Lourenção et al., 2009; Yang et al., 2008; Solé et al., 2010) and acetylsalicylic acid. These two drugs are mostly used as

antipyretic, analgesic and, in the case of acetylsalicylic acid, anti-inflammatory, being produced and used in the range of several kilotons per year (Cleuvers, 2004).

Paracetamol is a p-aminophenol derivative with analgesic and antipyretic activities, that was first described by Von Mering in 1893 (Wang et al., 2009). It has been considered safe for human use, when administered in common therapeutic dosages (Xu et al., 2008). Given its wide use, it has been found in concentrations up to 65 μg/L (namely in the River Tyne, United Kingdom; Roberts & Thomas, 2006). The mechanism of toxic activity of paracetamol seems to be well evolutionary conserved. The oxidation of paracetamol by the cytochrome p450 enzymes of a large set of species produces a toxic metabolic intermediate, N-acetyl-p-benzoquinoemine (NAPQI), (Xu et al., 2008) which may be conjugated with intracellular glutathione (Prescott, 1980; Patel et al., 1992; Xu et al., 2008). However, if the paracetamol dosage is extremely high, the intracellular pool of glutathione may be depleted, and NAPQI accumulates, leading to multiple toxic effects (covalent biding and arylation of critical cell proteins). Based on ecotoxicological data from the literature (for bacteria, *Daphnia* spp., and fish embryos), Henschel et al., (1997) concluded that paracetamol may be harmful to aquatic organisms. Toxic oxidative effects by paracetamol were again reported by Oliveira et al. (2015) after exposing the crustacean Daphnia magna to this compound. Similar results were obtained by Brandão et al. (2014) and by Solé et al (2010), for the freshwater bivalve *Corbicula fluminea*, and for the marine mussel *Mytilus galloprovincialis*, respectively. In addition, paracetamol was also toxic towards the marine bivalves *Ruditapes decussata* and *R. philippinarum*, with the involvement of antioxidative detoxification pathways (Antunes et al., 2013). Given the involvement of paracetamol in a considerable set of biochemical pathways of aquatic organisms, it is not surprising that glycogen depletion is another feature that has been associated to the exposure to this drug, as shown by Nunes et al. (2017).

Acetylsalicylic acid (ASA) is the most commonly used salicylate. After administration, ASA is easily hydrolyzed in most organisms to salicylic acid by nonspecific esterases and then conjugated with diverse biomolecules to salicyluric acid, 2,5-dihydrobenzoic acid (2,5-DHBA) (gentisic acid). However, the metabolism of salicylates often results in oxidative stress, considering the oxidative pathway of its metabolism, with the intervention of CYP 2E1 and CYP 3A4, forming 2,3-DHBA (Coudray & Favier, 2000). Doi et al. (1998) showed that salicylic acid induced LPO in rat livers microsomes, a process known to be related to CYP mediated oxidative metabolism, Similarly, and despite the absence of the biochemical metabolic route in aquatic organisms, salicylates may also exert oxidative effects, as shown by Gómez-Oliván et al., (2014), after determining that ASA exposure induced an increment in LPO in *D. magna*. Similar effects were also found for juveniles of the freshwater fish *Cyprinus carpio* and *Danio rerio*, after being exposed to salicylic acid, as demonstrated by Zivna et al. (2015), and Zivna et al. (2016), respectively. The now existent literature data about the ecotoxicity of salicylates (namely acetylsalicylic acid) is extremely scarce, and no indications about the putative effects of these compounds were found for most aquatic organisms, namely marine mussels.

Individuals of the mussel species *Mytillus* spp. are sessile filter-feeders that collect food particles from the water column, more specifically phytoplankton, dinoflagellates, small diatoms, zoospores, flagellates, other protozoans, various unicellular algae, and detritus (Myers et al., 2013). Mussels have shown to be a robust tool to detect and characterize the dynamics of a large number of contaminants in water systems (Reichwaldt & Ghadouani, 2016), since being filter feeding organisms, they tend to accumulate waterborne pollutants (Beyer et al., 2017). Mussels are also a standard choice for ecotoxicological testing because they are widely distributed, resistant to many

contaminants (Teixeira et al., 2017b), they have long life-cycle, which allows monitoring the bioaccumulation process of many compounds through their life stages (Teixeira et al., 2017b) and also due to the fact that they are easy to capture and maintain under laboratory-controlled conditions. They live in large populations, forming what is called "mussel beds" permitting repeated sampling in the same area. (Cristina & Gomes, 2012).

To assess the environmental risks posed by the two previously mentioned pharmaceuticals, mussels of the species *Mytilus* spp. were exposed for 96h to these chemicals. Estuarine environments are often highly contaminated due to the anthropogenic pressure (Justicia, 2016). As shown by Martínez et al. (2007) over 41% of world global population lives within coastal limits which greatly increases the likelihood of contamination in these areas, including sporadic pharmaceutical releases via municipal effluent discharges (Gaw et al, 2014). Hereupon, the aim of this work was to characterize the short-term toxic response of mussels from the species *Mytilus* spp. to paracetamol and acetylsalicylic acid exposures, using biomarkers of biotransformation and oxidative stress defense mechanism and oxidative damage, such as CAT, GST and lipid peroxidation. In addition, generic markers reflecting the nutritional status (food uptake and glycogen levels) were also measured in tissues of the mentioned species.

Materials and methods

Test organisms

During the month of May 2018, 150 adult mussels (*Mytilus* spp.) with an average shell size of 4.5 ± 0.5 cm, were collected during the low tide period, from Barra, Ria de Aveiro, Portugal (40°38'34.5"N 8°44'07.7"W), a costal lagoon sheltered from wave action. This location, Barra, corresponds to the initial part of the Mira channel close to the Ria de

Aveiro lagoon entrance, and is mainly subjected to naval traffic (Oliveira et al., 2009), not being considerably affected by untreated domestic sewage (the main source of pharmaceutical drugs in the wild). Ria de Aveiro is a large mussel hybrid zone where both *M. edulis* and *M. galloprovincialis* coexist. At this site, a mixture of pure and hybrid individuals occurs (Coghlan & Gosling, 2007). As there is no single morphological character that can be reliably used to separate this mixed population, we were not able to classify which exact species was used in this study.

After collection, the organisms were immediately transported to the laboratory for acclimation, where they were kept in 60L aquaria for three weeks under controlled conditions: artificial seawater, salinity of 34 (the same salinity as the sampling site), continuous aeration, mechanical filtration, temperature 20±1 °C, and a photoperiod of 16 h L:8 h D. During this period, mussels were fed every two days with a *Chlorella vulgaris* suspension ($1x10^5$ cells/mL) (Caetano, 2014). The medium was renewed every week and dead organisms were immediately discarded. Organisms were considered dead when their shell gaped and did not shut when stimulated.

Test solutions and exposure

Stock solutions of the two different drugs were prepared: a stock solution of paracetamol and another of acetylsalicylic acid, both with a final concentration of 10mg/L. The pharmaceuticals were dissolved in artificial seawater (salinity 34) so that the stock solution did not interfere with the final salinity of the exposure solutions. Paracetamol and acetylsalicylic acid are stable when under conditions similar to those found in the wild, as demonstrated by previous studies (paracetamol: Kim, et al., 2007; Wu et al., 2012; ASA: Paíga et al., 2015). All the test solutions were prepared from the two stock

solutions; for paracetamol short term exposure, 5 concentrations were selected (0-control, 0.5, 5, 50, and 500 µg/L); for acetylsalicylic acid short term exposure the concentrations were 0-control, 0.1, 1, 10 and 100 µg/L.

Paracetamol concentrations were based on ecological relevance criteria; the two first concentrations were close to those already reported for aquatic ecosystems: 6–65 µg/L; (these levels were already found to occur in the estuary of the River Tyne, UK; Roberts & Thomas, 2006) and acetylsalicylic acid has been found in levels up to 0.34 µg/L (in water from rivers and streams; Ternes, 1998) but with an estimated predicted environmental concentration (PEC) of 80.4 µg/L in European Union waters (Stuer-Lauridsen, et al., 2000).

For the determination of biomarkers, 8 mussels per concentration were individually exposed (8 replicates for each condition/concentration of drugs), in 1.5L plastic bottles (previously used for human consumption of water), in a final volume of 200mL of artificial seawater per bottle. The mussels were not fed during the exposure to both drugs. The exposure periods lasted for 96h, and 48h after the onset of this period, media were renewed in order to discard excretion products from mussels, and also to maintain the levels of the drugs in the water. All the remaining abiotic conditions were kept similarly to those adopted for the quarantine period. Tests were performed following an adapted version of the Ecological Effects Test Guidelines: OPPTS 850.1710. Oyster BCF (Environmental Protection Agency EPA, 1996).

After the exposures, mussels were sacrificed on ice-cold water (4°C), and tissues of interest (digestive glands and gills) were dissected, isolated and stored at -80ºC in Eppendorf microtubes until the performance of enzymatic assays.

Feeding assay

A feeding assay was conducted with a distinct batch of mussels. 8 mussels per concentration (including the control) were individually exposed to the same levels of both drugs, in similar 1.5 L water bottles. All other experimental conditions were kept. Immediately after the onset of the exposure, a total of $1.5x10^5$ cells of the microalgae *Chlorella vulgaris* were given to each mussel, in a final volume of 200 mL (initial cell density of $7.5x10^5$ cells/L). Aliquots from each replicate were withdrawn with a micropipette 1 h after the onset of the exposure, to be further analyzed and the cells counted, with a haematocytometer. The ingested number of cells during this one hour period, per animal, was calculated subtracting the final measured density of *C. vulgaris* from the initially defined cell density of $7.5x10^5$ cells/L.

Tissue processing for the determination of biomarkers

Tissue samples (8 replicates per concentration) were homogenized on ice (4°C) in a volume of 1 mL of 50 mM phosphate buffer pH = 7.0 with Triton X-100 0.1% (homogenization buffer), using a Branson Sonifier 250 (constant cycle ultrasounds for about 30 seconds). After this step, homogenates of all 8 replicates/concentration were centrifuged at 15 000 g for 10 min at 4°C. Supernatants were recovered and stored (at -80°C) for the determination of all oxidative stress biomarkers.

Biomarker assays

Considering that the expression of metabolic/antioxidant enzymes is higher in the digestive gland (Livingstone et al., 1992; Cajaraville et al., 1993), both GSTs and catalase activities were determined in this specific tissue.

GSTs activity was measured in the digestive glands of the mussels, by monitoring the formation of the thioether resulting from the conjugation reaction between 2,4-dinitrochlorobenzene (CDNB) and reduced glutathione (GSH) by following the increase in absorbance at 340nm. To maximize the effectiveness of this test, the supernatant from the homogenization of the mussel was diluted 1:100 with phosphate buffer 0.1 M, pH = 6.5. Results were expressed as nanomoles of thioether produced per minute, per mg protein.

Catalase activity was also measured in digestive glands of the mussels and was determined by monitoring hydrogen peroxide (H_2O_2) decomposition at 240nm. To maximize the effectiveness of this test, the supernatant resulting from the homogenization of the mussel was diluted 1:100 with phosphate buffer 0.1 M, pH = 6.5. The results were expressed based on the total soluble protein present in the samples (nmol min^{-1} mg^{-1} protein) (Aebi, 1984). Changes in sample absorbance were spectrophotometrically measured at 240 nm, and activities were expressed as µmol H_2O_2 consumed per min per mg protein.

Lipid peroxidation is a common outcome in mussel (including *M. galloprovincialis*) gills, as demonstrated by Fernández et al. (2010). Consequently, this parameter was measured in the gills of the mussels, and was determined by measuring the absorbance of the complex resulting from the chemical reaction of products of oxidative free radical attack to membrane lipids (thiobarbituric acid reactive substances, especially malondialdehyde) with thiobarbituric acid. This reaction, which occurs at 100ºC, produces a colored complex whose absorbance can be quantified at 535nm (Buege and Aust, 1978).

Protein concentration was determined for every sample dilution (1:50) according to the protocol by Bradford (1976), in order to express enzymatic activities taking into account the protein content of the analyzed tissues. Bradford method involves binding of the Bradford reagent (Coomassie brilliant blue G-250) to the soluble protein present in the samples. This binding gives rise to a colored stable complex that can be quantified at 595nm. Protein standards were prepared using ɣ-globulin (1 mg/mL) so that we could estimate the total protein content of the samples by the resulting calibration curves.

The determination of glycogen was performed in both gills and digestive gland, according to Lo et al. (1970). Both tissues were shown to be adequate for the determination of energetic reserves in bivalves, as demonstrated by Thuy et al. (2011) and Chandurvelan et al. (2013). This method was based on the basic digestion of the samples, precipitation of the dissolved glycogen by ethanol, followed by a reaction with sulfuric acid. In order to optimize the method, samples were diluted 1:5 in ultrapure water. The resulting glucose was quantified spectrophotometrically at 490 nm. Results were expressed in micrograms of glycogen per milligram of fresh weight (μg mg FW^{-1}).

Statistical Analysis

Data were tested for normality using the Shapiro-Wilk test, and to test for homogeneity of variances, a Levene median test was used. If tested data fulfilled the ANOVA assumptions, they were statistically analyzed with one-way ANOVA, followed by a Dunnett's multicomparison test to discriminate significant differences between toxicant concentrations and the control treatment. The level of significance adopted was 0.05. The statistical software that was used was SPSS 24.

Results

Paracetamol short term exposure

Food (algal cells) intake was significantly reduced by paracetamol exposure ($F_{(4,30)}$ =5.261; p<0.001; Fig. 1), along a dose-dependent pattern.

GSTs activity response measured in digestive gland of *Mytilus spp*. was not significantly different among treatments (One Way ANOVA: $F_{(4, 29)}$=0.935; p=0.457; Fig.2)

CAT activity response measured in digestive gland of *Mytilus spp*. was not significantly different among treatments (One Way ANOVA: $F_{(4,24)}$=0.963; p=0.446; Fig.3).

Lipid peroxidation levels were not significantly changed in gills of *Mytilus spp*. after acute exposure to paracetamol (One Way ANOVA: $F_{(4, 33)}$=1.733; p=0.166; Fig. 4).

Glycogen levels in gills were significantly increased after paracetamol exposure (One Way ANOVA: $F_{(4,30)}$ = 8,884; p<0.001; Fig. 5). There were statistical differences between the control group and the concentrations 0.5 µg/L, 50 µg/L, and 500 µg/L. In the digestive gland, no significant statistical differences were found between control organisms and those exposed to the tested concentrations (One Way ANOVA: $F_{(4,27)}$=1.64; p=0.193; Fig. 5).

Acetylsalicylic acid short term exposure

Individuals of *Mytilus* sp. did not change their filtration activity of algal cells after being exposed to ASA ($F_{(4,25)}$=0.694; p=0.604; Fig. 6).

GSTs activity response measured in digestive gland of *Mytilus spp*. was not significantly different among treatments (One Way ANOVA: $F_{(4, 32)}$ =1.422; p =0.249; Fig. 7)

Catalase activity response measured in digestive gland of *Mytilus spp*. was not significantly different among treatments (One Way ANOVA: $F_{(4,34)}$=0.421; p=0.793; Fig. 8).

Lipid peroxidation levels were not significantly changed in gills of *Mytilus spp*. after short term exposure to acetylsalicylic acid (One Way ANOVA: $F_{[4,35)}$ = 0.716; p = 0.587; Fig. 9).

Glycogen levels in gills were significantly altered (One Way ANOVA: $F_{(4,27)}$ =11.039; p< 0.001; Fig. 10), since aninals exposed to the three highest concentrations (1 µg/L, 10 µg/L, and 100 µg/L of ASA) had significantly higher amounts of glycogen in their gills, when compared to control organisms. In the digestive gland no significant statistical differences were found among treatments (One Way ANOVA: $F_{(4,28)}$ =0.946; p=0.452; Fig. 10).

Discussion

The toxic effects of drugs in the wild is not limited to the deleterious alterations of specific pathways. In fact, generic physiological trends, such as energy metabolism and allocation, may also be disturbed by exposure to drugs in the wild. Such effects have been already documented for several antibiotics (Aderemi et al., 2018), carbamazepine and cetirizine (Almeida et al., 2018), caffeine (Cruz et al., 2016), and atorvastatin (Falfushynska et al., 2019), among others. Considering that energetic homoeostasis changes may also be related to modifications in the patterns of food ingestion, the simultaneous assessment of both parameters seems to be an adequate strategy to study deleterious shifts in energetic reserves and management in exposed organisms. The ecological importance of such parameters is obvious, since significant alterations in these features may decisively alter the fitness of exposed organisms. The obtained results demonstrate that both drugs

elicited different responses; paracetamol exposure resulted in a significant short term increase in the ingestion of food by exposed mussels, strongly modulating the energy uptake from the external media. On the contrary, ASA exposure did not result in any significant alteration in the ingestion of algal particles by exposed organisms. These results are extremely interesting if combined with the results concerning the glycogen content determined both in gill and digestive glands of exposed organisms. In fact, the strong increase in food ingestion in paracetamol-exposed mussels was followed by a significant reduction in their gills glycogen levels, while the content of this same parameter was not significantly altered in the digestive gland, albeit the occurrence of a mild reduction. It is possible to hypothesize that the toxic response elicited by paracetamol is energetically demanding, leading not only to an increase in nutrients from the external media, but also to the depletion of cellular energetic reserves present in metabolically active tissues, such as gills. Increased feeding rate after exposure to paracetamol has been already reported by Solé et al. (2010). On the other hand, the influence of paracetamol on glycogen metabolism, in rodents, has been already established by Jepson et al. (1987), showing that different hepatic locations could exhibit distinct response to this drug in terms of glycogen mobilization. According to this study, periportal hepatocytes were enriched in glycogen, while perivenous hepatocytes had their glycogen levels strongly reduced. The work by Neyrinck et al. (1999) showed the involvement of the Küpffer cells in hepatic metabolism of paracetamol in rats, which could be reflected in their hepatic glycogen levels. Similarly, an exhaustion of hepatic glycogen was already documented to occur in the fish *Phaloceros harpagos*, by Matus et al. (2018), after being acutely exposed to paracetamol. On the contrary, exposure to the marine bivalve species *Ruditapes philippinarum* to this same drug caused a metabolic depression reflected by an increase in glycogen content of its tissues, as demonstrated by

Nunes et al. (2017). These data from the literature, when compared to the here obtained results, suggest that the metabolism of paracetamol has clear consequences on energetic reserves and food consumption of exposed organisms. Depending on the species, and on the analyzed tissue, the ultimate response (glycogen increase or decrease) seems to be highly species-specific.

A significant increase in gills glycogen content, despite the absence of clear indicators of feeding alterations, was observed in organisms exposed to ASA. Data about the deleterious effects of salicylates on feeding behavior of aquatic organisms are absent from the literature. However, salicylates have been before implicated in the reduction of the hepatic glycogen content of aquatic organisms, namely the fish *Oncorhynchus mykiss* (Gravel and Vijayan, 2007; Gravel et al., 2009). However, results of these studies were obtained after feeding the fish with sodium salicylate, which is not the same form that was used in our work. This is a likely factor that might explain the differences in the response reported to occur in the mentioned study, and the hereby obtained data.

Antioxidant defenses are part of an extremely complex homeostatic system (Regoli et al., 2013) that controls and prevents deleterious modifications to redox status, that may, if not counteracted, result in toxic damage to tissues. This redox status is lessened by the interaction between individual protective factors and prooxidant challenges. Prooxidant mechanisms are of primary importance in modulating toxicological effects of contaminants in marine organisms, exposed via environment. Livingstone (2001) concluded in his studies that there is a potential ROS production stimulated by contaminants in aquatic organisms and that this ROS production can inclusively lead to oxidative damage. The quantification of oxidative stress biomarkers is then a useful tool to detect deleterious effects on the overall organism health condition (Hook et al., 2014).

In our experiment, exposures to the two drugs (paracetamol and acetylsalicylic acid) were performed attempting to provide information about their toxicity and mechanisms of action in the mussel *Mytilus spp*, with special emphasis to oxidative mechanisms and defenses. Results showed that no alterations occurred in the mussel antioxidant defenses in either chemical treatment. This pattern of response is not compatible with previous data, since according to the literature, an oxidative stress scenario should have been accompanied by a response in terms of antioxidant enzymes, including CAT, and GSTs. Increase of CAT activity has been shown to occur after paracetamol exposure, namely in organisms of the freshwater crustacean species *Daphnia magna* (Daniel et al., 2019), and in the sea snail *Gibbula umbilicalis* (Giménez and Nunes, 2019). Previously published studies have indeed demonstrated the effects of paracetamol on aquatic mollusks, with the alteration of GSTs in the freshwater clam *Corbicula fluminea* (Brandão et al., 2014), in the crustacean *D. magna* (Daniel et al., 2019), and in the fish species *Anguilla anguilla* (Nunes et al., 2015a), and *Phalloceros harpagos* (Pereira et al., 2018). The glutathione metabolism, which is critical for paracetamol metabolism, was also affected by this drug in the marine mollusk *Ruditapes philippinarum*, as evidenced by Correia et al. (2016) and Nunes et al. (2017), with the concomitant involvement and activation of the antioxidant enzymatic defense, namely superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities. This set of responses could also have been followed by oxidative damage, with significant increases in lipid peroxidation, a feature that was observed to occur in *A. anguilla* after being exposed to paracetamol (Nunes et al., 2015a).

CAT is an oxidoreductase enzyme present in prokaryotes and eukaryotes (Kashiwagi et al., 1997). It is involved in catalytic conversion of two molecules of hydrogen peroxide into two molecules of water, and one of oxygen (Gonçalves-Soares et al., 2012). By been able to play this role, it prevents cell oxidative stress. CAT activities are often higher in

invertebrates, indicating their important role in the antioxidant protection of these organisms (Livingstone et al., 1992); Regoli et al., 2002). When molecular oxygen is used in aerobic processes or in oxidative stress occasions, catalase may by overexpressed with an inherent activity increase, in response to the excess of formed oxygen peroxide (Oruc et al., 2004). So, this catalase increase might function as a compensatory tool to face oxidative alterations (Oost et al., 2003). In the present study, such response was not registered, since no significant modifications were reported concerning CAT activity, indicating that paracetamol and acetylsalicylic acid exposures, under the selected conditions, were not able to induce any responses in the digestive gland of the exposed mussels. This set of responses contradicts previous toxic patterns found in literature, which obtained significant pro-oxidative responses after exposing other aquatic organisms, such as the zebra mussel *Dreissena polymorpha* (Parolini et al., 2010), the fish *Oreochromis mossambicus* (Kavitha et al., 2011) and the freshwater bivalve *Corbicula fluminea* (Ramos et al., 2014) to paracetamol, and both *Daphnia magna* and *Daphnia longispina* to acetylsalicilic acid (Marques et al., 2004). It is also to mention the study by Parolini & Binelli (2012) where the combined effects of paracetamol with other non-steroidal anti-inflammatory drugs (namely, diclofenac and ibuprofen) were tested on the freshwater bivalve *D. polymorpha*; it was evidenced a significant increase in CAT activity after acute exposure to the mixture. The lack of alterations in CAT activity in both chemical treatments in our study might be related with the fact that mussels manifest other defense mechanisms, namely enzymatic and non-enzymatic forms involved in antioxidant defenses, such as SOD and vitamins, which might have countered the ROS formation (Vlahogianni et al., 2007), but that were not measured in our study. Also, mussels react quite promptly to environmental alterations, manifesting both physiological and behavioral unspecific changes, namely involving their opening of valves and

filtration of water (Kramer et al., 1989). Previous studies have shown that, when exposed to a given toxicant (e.g. chlorine; Kramer et al., 1989), the zebra mussel *Dreissena polymorpha* significantly increased their time closed; a similar response was also reported for the mussel species *Mytilus edulis* when exposed to copper sulphate. This behavior, which is common to mussels exposed to toxic xenobiotics, will probably result in a lower contaminant exposure that may justify the results obtained. However, we cannot discard that despite putatively ecologically relevant, the here adopted experimental design may have led to this absence of results; the exposure period may have not been long enough to elicit a defensive response in mussels, and the here adopted concentrations of both drugs used were not high enough to cause toxicity of the organisms.

Another important enzymatic forms involved in the glutathione phase II metabolism, and antioxidant defense are the glutathione-S-transferases (GSTs) isoenzymes, which catalyze the conjugation of the reduced form of glutathione (GSH) with xenobiotics that can then be detoxified (Blanchette et al., 2007). Glutathione (GSH in its reduced form) and its associated metabolism is a crucial mechanism for cellular protection against oxidative stress. GSH acts as a detoxifying agent at several different levels scavenging free radicals, reducing peroxides and being conjugated with electrophilic compounds thus providing the cell several defenses against ROS and their toxic products (Hayes et al., 1999). In the present study, similarly to catalase, no significant alterations occurred in terms of GSTs, even though digestive gland tissue of mussels exposed to paracetamol showed a slight decrease in their GSTs content. Conjugation with paracetamol is likely to occur via GSTs-catalyzed reactions, but quite paradoxically this parameter did not respond to either exposures. Many evidences have already shown that the patterns of GSTs responses are variable and that they might differ between species and be dose-dependent (Antunes et al., 2013). As shown by the study of Nunes et al. (2015a), gill

tissue of the fish *Angilla angilla* also suffered a decrease in terms of GSTs activity for the concentrations of 125 and 3125 μg/L of paracetamol. Similarly, Correia et al. (2016) tested the oxidative effects of paracetamol on the edible clam *R. philippinarum* under different salinities. Even for the lowest (and possibly more stressful salinity 14), animals that were exposed to paracetamol did not have their GSTs contents affected. Antunes et al. (2013) also tested the biochemical effects of paracetamol in the edible clams *Venerupis decussata* and *Venerupis philippinarum* and a dual response was obtained for *V. decussata*: impairment of GSTs at low concentrations and significant increases in their activity for higher levels, equivalently to what was shown by Brandão et al. (2014), in which GSTs activity in *C. fluminea* exposed to paracetamol decreased for higher concentrations of this drug. This scenario may reflect an oxidative stress response that is only noticeable at high dosages of paracetamol.

Lipid peroxidation (LPO) is a measure of the extent of lipid membrane damage consequent to oxidative stress (Alves de Almeida et al., 2007; Regoli & Giuliani, 2014)). In fact, the pro-oxidative effects of paracetamol can result in membrane lipids oxidation and destruction (Fairhurst et al., 1982; Abdul Hamid et al., 2012), which has been shown to occur in several distinct animal species exposed via environment to anthropogenic chemicals, such as mollusks (Solé et al., 2010; Antunes et al., 2013), catfish (Kashiwagi et al., 1997), rainbow trout (Ramos et al., 2014; Rodrigues et al., 2016), and seabream *Sparus aurata* fingerlings (Varó et al., 2007). Even crustaceans, namely *Artemia parthenogenetica* (Nunes et al., 2006) and aquatic plants, are likely to suffer pro-oxidant effects by paracetamol as shown by Gómez-Oliván et al. (2014) and by Nunes et al. (2014) that demonstrated the occurrence of membrane lipid damage in *Hyalella azteca* and *Lemna minor,* respectively. This occurrence was not observed in this study since gill tissue did not show any significant alterations concerning TBARS levels. These results

are somewhat expectable since no alterations in terms of CAT and GSTs activities were reported for any of the short term exposures.

Considering the absence of significant changes in the metabolic antioxidant defensive system (no noteworthy alterations were registered concerning GSTs and CAT activities) and the inexistence of oxidative damage (TBARS levels were roughly unchanged after paracetamol exposure), we cannot conclude about the occurrence of oxidative stress. Despite having being determined in distinct tissues, one could expect the manifestation of oxidative stress with the involvement of any of the tested parameters, measured in any of the analyzed tissues. This species of mussel is known to be extremely resistant to contamination, being able to survive and reproduce in heavily chronically polluted seawater (Minier et al., 1995). It may be hypothesized that the concentrations used in this studied probably exerted a limited oxidative pressure, and are not seriously challenging for the detoxification capacity of this species. Besides these possibilities, mussels may also have a multixenobiotic resistance (MXR) activity related to multidrug resistance, a hypothesis that was put forward by Minier et al., (1995), by identifying the presence of a gene related to MDR genes in *Mytilus edulis.* MDR is associated with the increased formation of transmembrane glycoprotein P170 (Kartner et al., 1983), that acts by enhancing the activities of mechanisms that result in decreases in drug uptake/accumulation, and in its seric contect.. P170 binds to the drug and facilitate its eflux by an energy-dependent process. This enhanced efflux might be determinant for drug accumulation reduction (Morrow and Cowan, 1988) and this decrease would explain, at least partly, why the mussels did not devidence any alterations in their defense mechanisms against paracetamol and acetylsalicylic acid.

Conclusions

In conclusion, despite the occurrence of mild, albeit not significant changes in antioxidant defenses, it was not possible to report a full measure of deleterious modifications in the balance between oxidative damage caused by both drugs, and the activation of antioxidant mechanisms or damage (Regoli et al., 2003). As already mentioned, mussels are widely used as a bioindicator for monitoring of coastal water pollution (Beyer et al., 2017) because of their ecological and biological characteristics, namely their filter-feeding behavior, and their ecological representativity. However, exposures of the mussel *Mytilus* spp. to paracetamol and acetylsalicylic acid was not causative of significant alterations in the assessed toxicological parameters. This implies that the drugs tested did not cause oxidative stress, since no increased hydrolytic capacity was required to cope with excess of ROS, and that the organisms were not responsive to the conditions to which they were submitted. This conclusion is probably related to the short period of exposure/low albeit realistic levels of exposure, which does not reflect actual field conditions, where the mussels are prone to long-term exposures, probably during considerable portions of their life cycles. Besides, the evaluation of a wider range of oxidative stress biomarkers is required to ensure potential toxic effects of these drugs and their targets.

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Figure Captions

Figure 1. Effects of paracetamol acute exposure on the filtration of algal cells by individuals of *Mytilus* spp. Values are the mean of 8 replicates; error bars correspond to standard deviation. * - statistical differences in relation to the control treatment.

Figure 2. Effect of paracetamol acute exposure on GSTs activity measured in the digestive glands of *Mytilus* spp. Values are the mean of 8 replicates; error bars correspond to standard deviation.

Figure 3. Effect of paracetamol acute exposure on CAT activity measured in the digestive glands of *Mytilus* spp. Values are the mean of 8 replicates; error bars correspond to standard deviation.

Figure 4. Effect of paracetamol acute exposure on TBARS concentrations measured in the gills of *Mytilus* spp. Values are the mean of 8 replicates; error bars correspond to standard deviation.

Figure 5. Effects of paracetamol acute exposure on the glycogen levels in tissues of *Mytilus* spp: darker bars - gills; lighter bars – digestive gland. Values are the mean of 8 replicates; error bars correspond to standard deviation. * - statistical differences in relation to the control treatment.

Figure 6. Effects of acetylsalicylic acid acute exposure on the filtration of algal cells by individuals of *Mytilus* spp. Values are the mean of 8 replicates; error bars correspond to standard deviation.

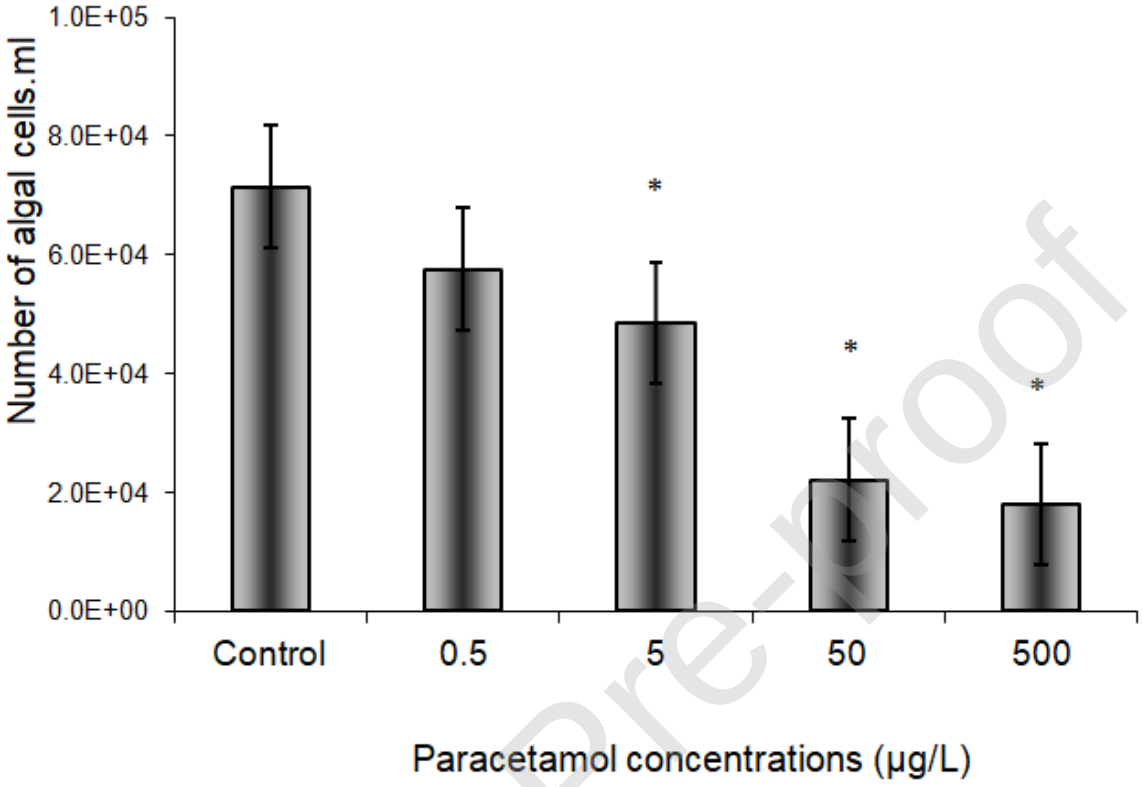
Figure 7. Effect of acetylsalicylic acid acute exposure on GSTs activity measured in the digestive glands of *Mytilus* spp. Values are the mean of 8 replicates; error bars correspond standard deviation.

Figure 8. Effect of acetylsalicylic acid acute exposure on CAT activity measured in the digestive glands of *Mytilus* spp. Values are the mean of 8 replicates; error bars correspond to standard deviation.

Figure 9. Effect of acetylsalicylic acid acute exposure on TBARS concentration measured in the gills of *Mytilus* spp. Values are the mean of 8 replicates; error bars correspond to standard deviation.

Figure 10. Effects of acetylsalicylic acid acute exposure on the glycogen levels in tissues of *Mytilus* spp: darker bars - gills; lighter bars – digestive gland. Values are the mean of 8 replicates; error bars correspond to standard deviation.

Fig 1



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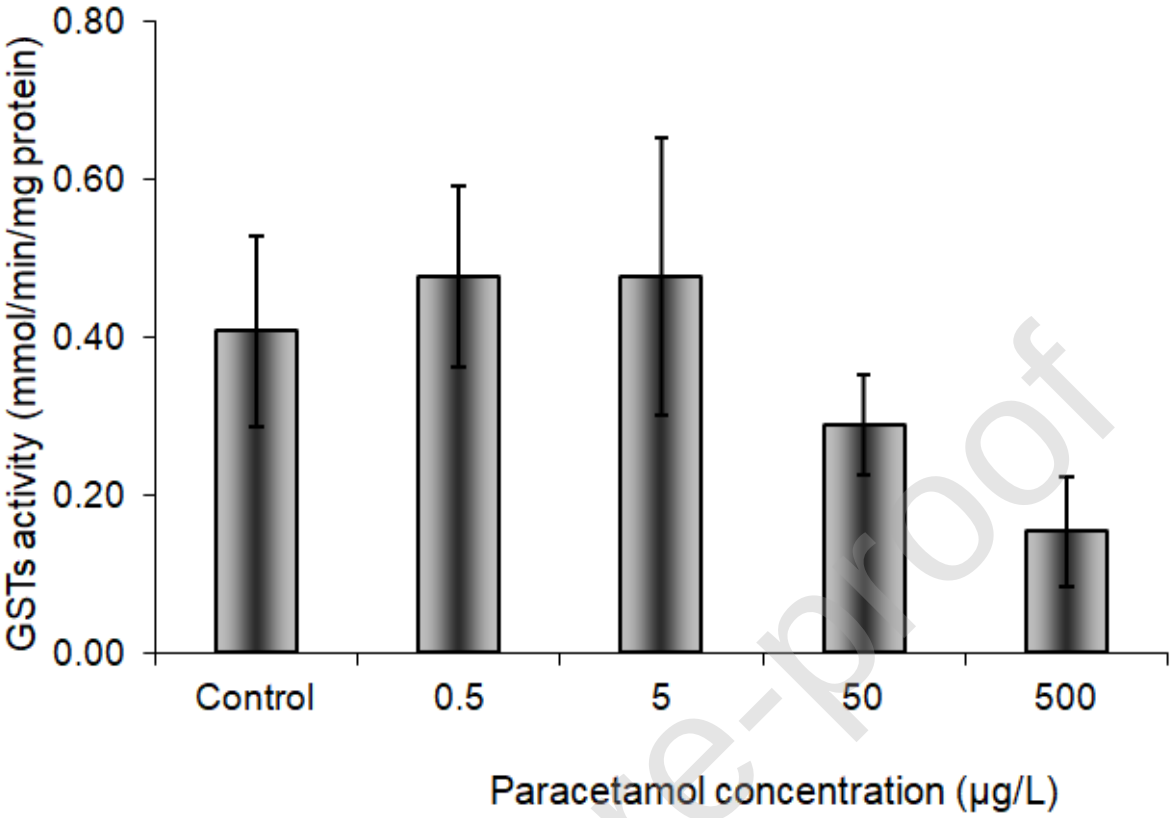

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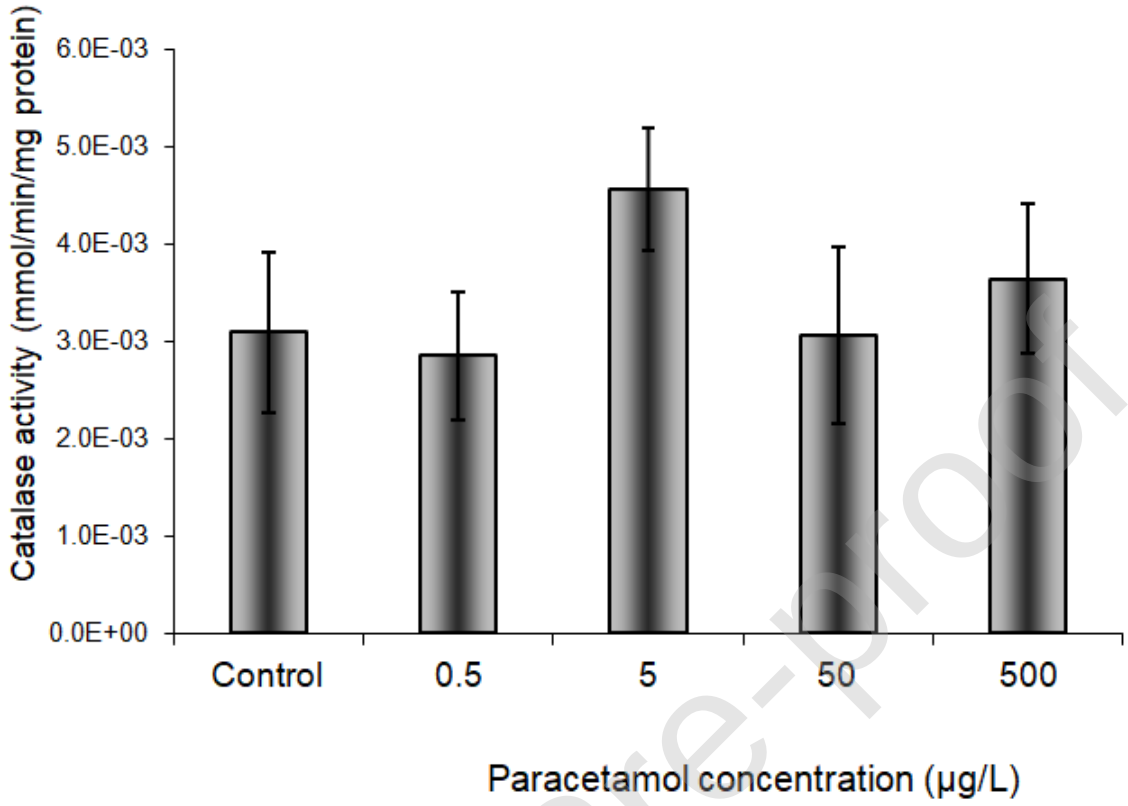

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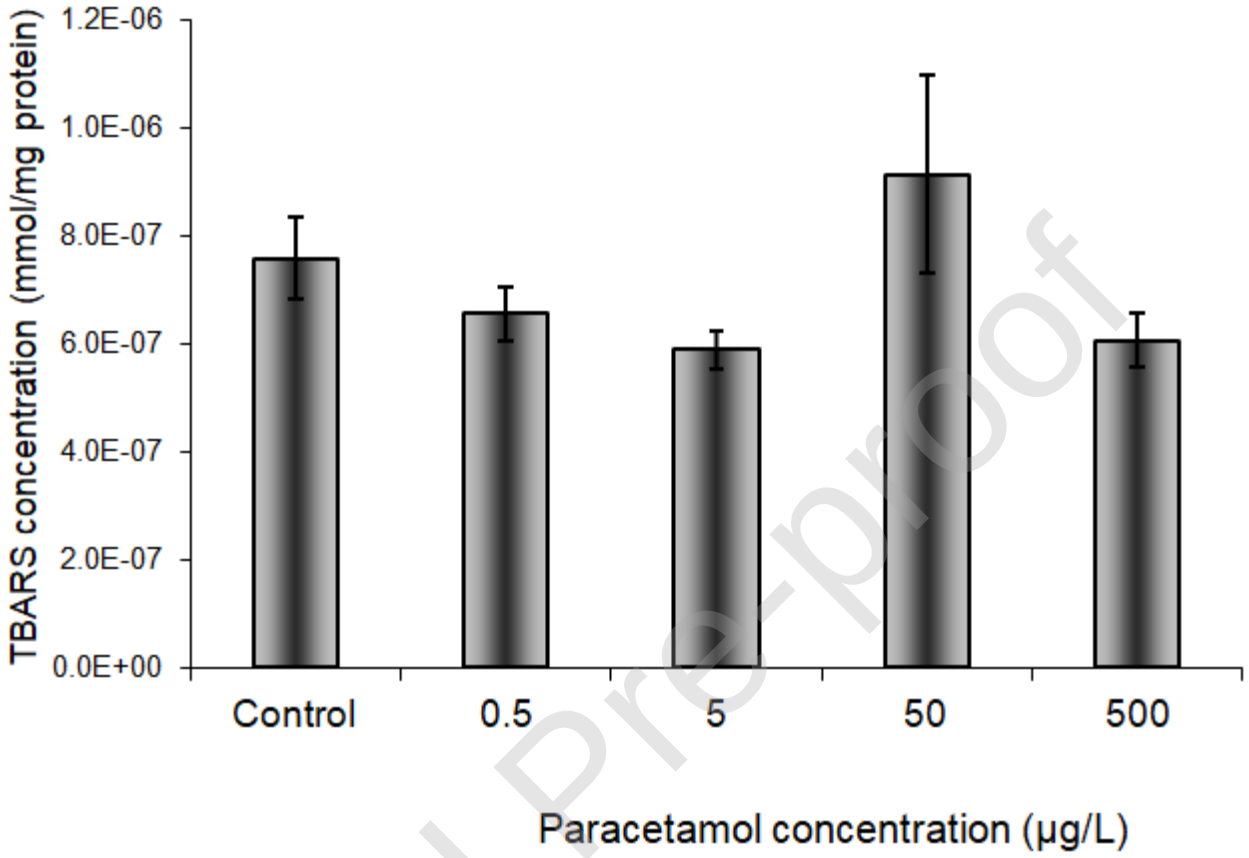

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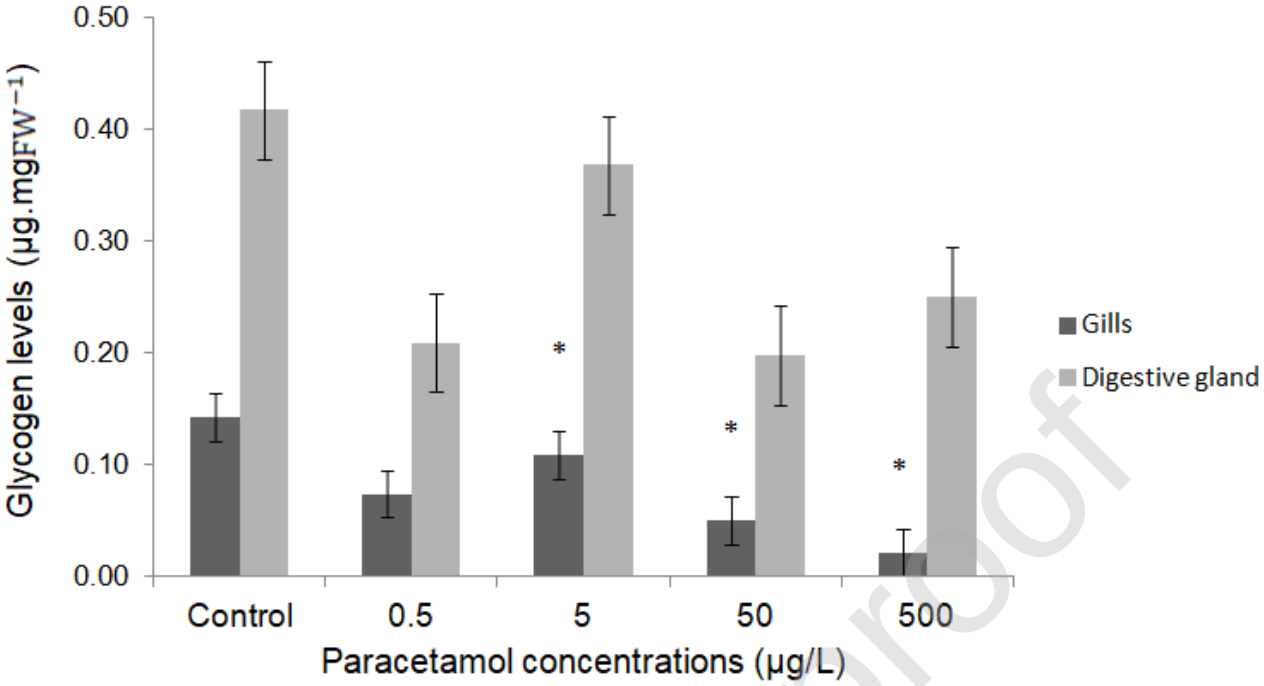

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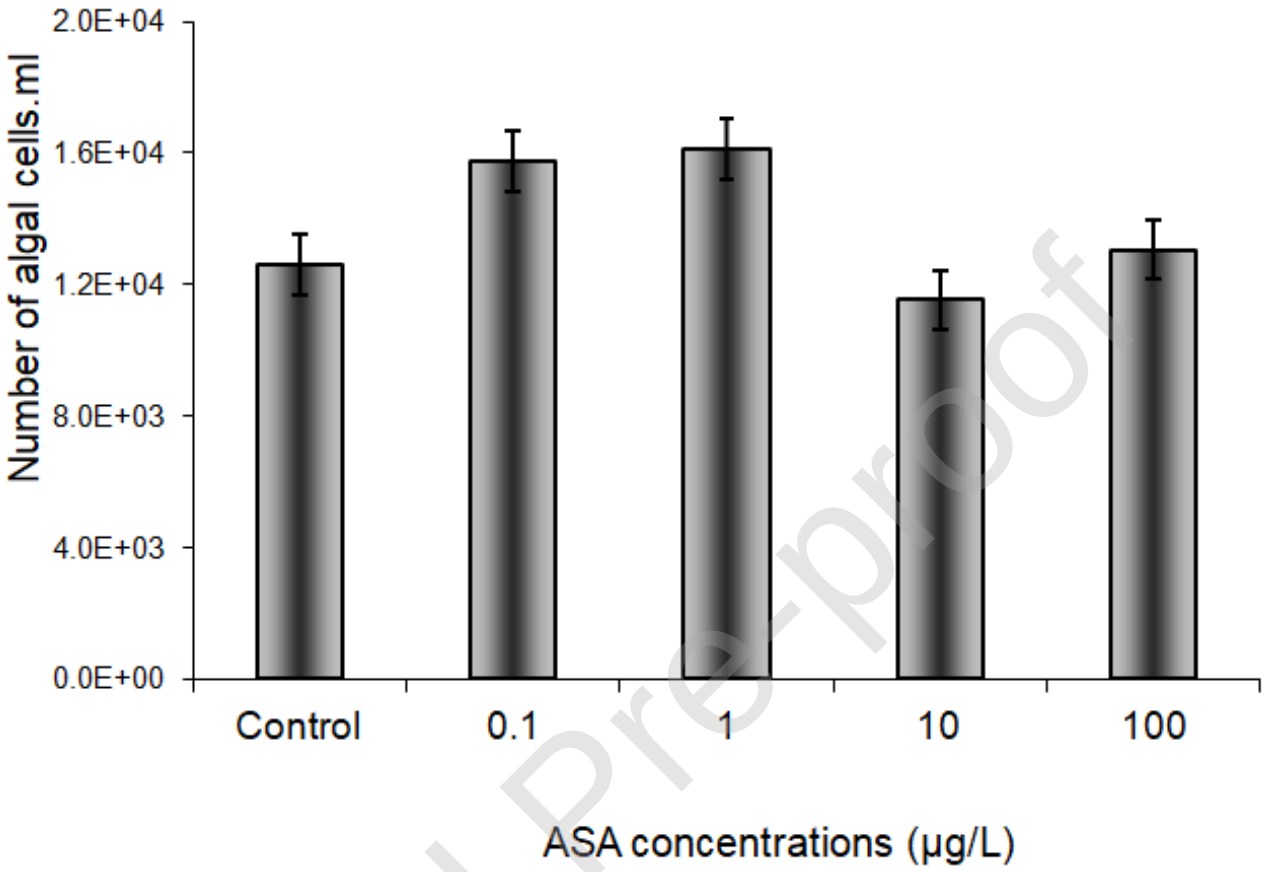

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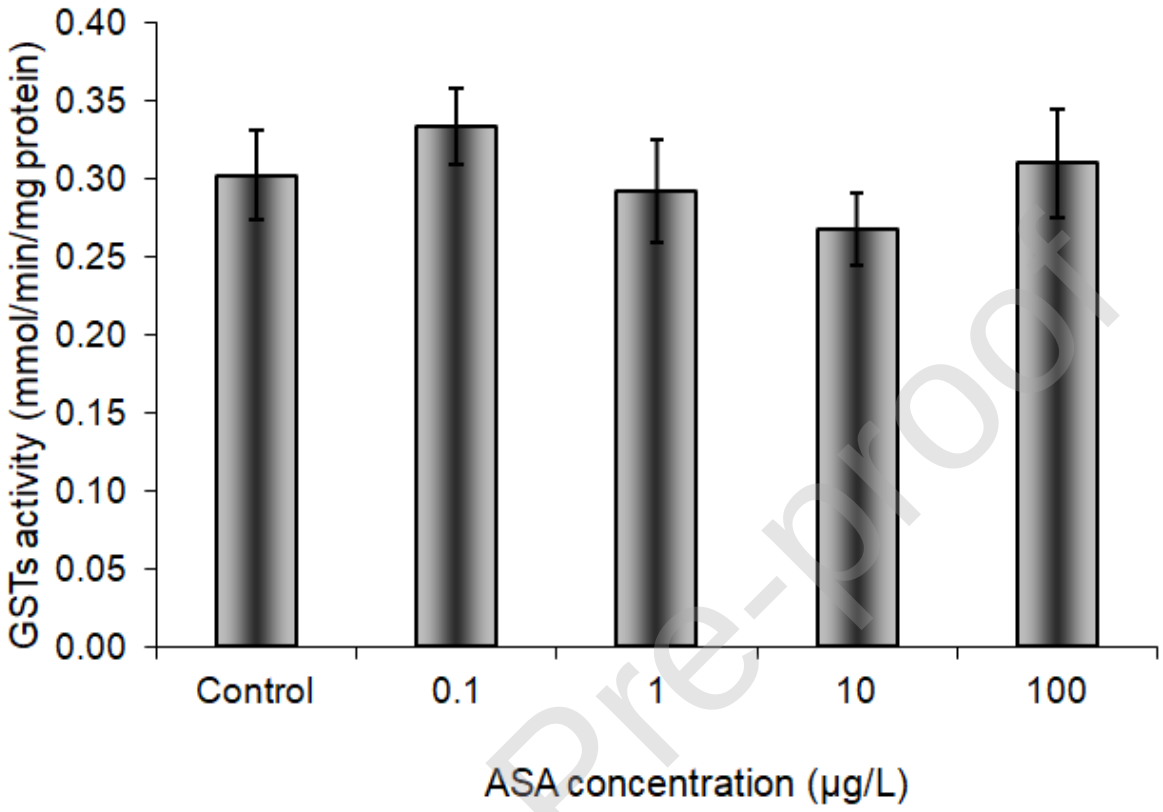

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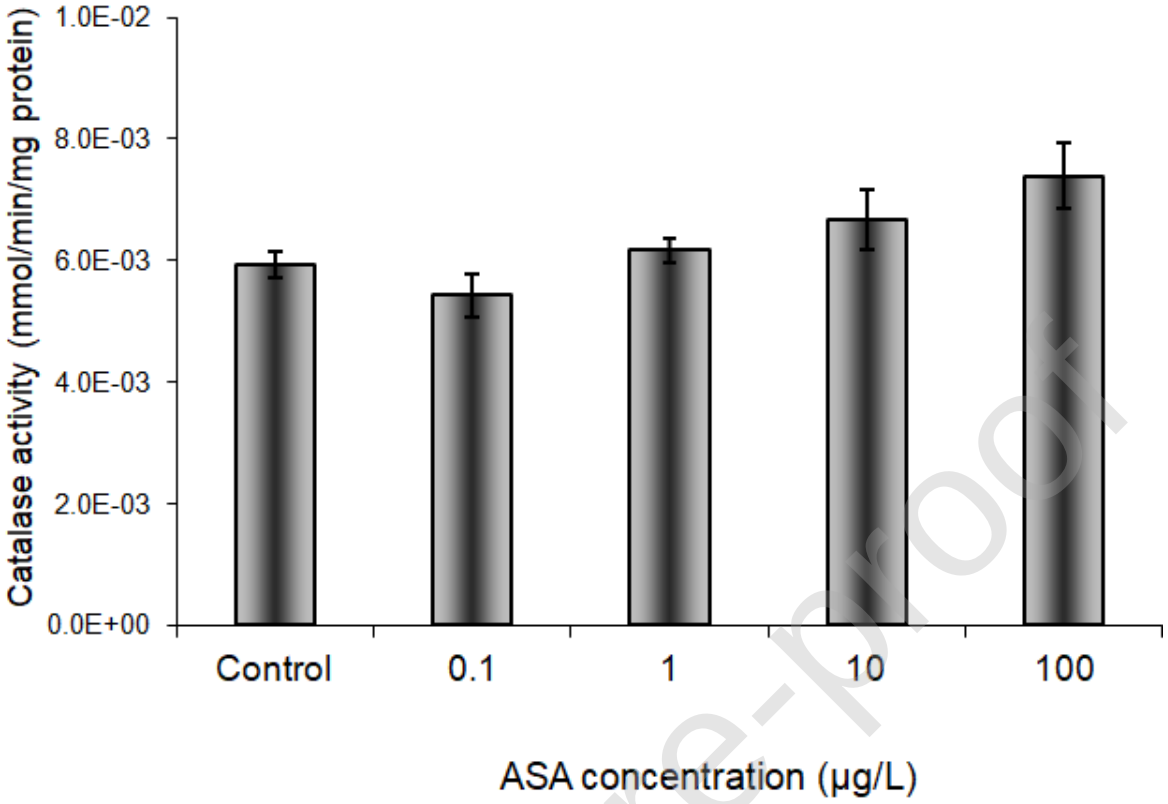

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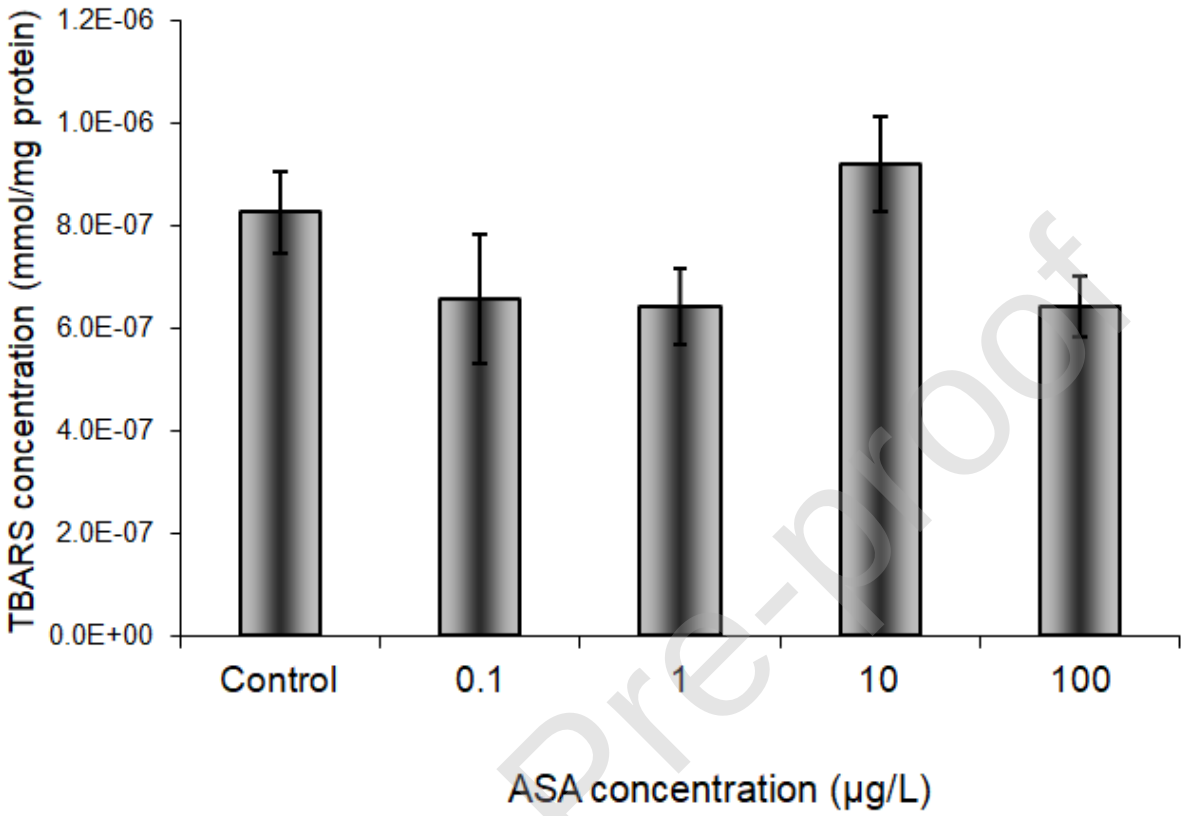

Fig 10