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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.

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; Brodin et al., 2013; Gelsleichter and Szabo, 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-Sörensen 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 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, <1 ng/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 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

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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 STPs 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 (Will 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., 2015).

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 the potential effects of these substances on biota. Furthermore, it is also important to test the sensitivity and responsiveness of distinct test 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 et al., 2015). According to Widdows and Shick (1985) salinity is one of the dominant environmental factors that mostly 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 (*R. 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 (*Ruditapes 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 ± 1 °C, photoperiod of 12 h light:12 h 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