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Yes, caffeine, ibuprofen, carbamazepine, novobiocin and tamoxifen have an effect on *Corbicula fluminea* (Müller, 1774)

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ABSTRACT

Reports indicating the presence of pharmaceutical in fresh water environment in the ng L^{-1} to μg L^{-1} range are occurring with increasing frequency. It is also a fact that pharmaceuticals may produce adverse effects on aquatic organisms. Nevertheless, there is still a lack of knowledge regarding how these emergent contaminants may affect aquatic biota. The goal of this research was to evaluate the sublethal responses in *Corbicula fluminea* such as, general stress (lysosomal membrane stability [LMS]), biomarkers of phase I and II (etoxyresorufin O-deethylase [EROD], dibenzylfluorescein dealkylase [DBF], gluthathione-S-transferase [GST]), oxidative stress (gluthathione reductase [GR], gluthathione peroxidase [GPX], lipid peroxidation [LPO]), and biomarkers of effect (DNA damage) after 21 days of exposure to caffeine, ibuprofen, carbamazepine, novobiocin and tamoxifen at 0.1, 1, 5, 10, 15, 50 μg L^{-1}. Environmental concentrations tested in this study caused general stress and produced changes on biomarkers tested. LMS, responses from phase I and II enzymatic activity, oxidative stress, and biomarker of effect represent important ecotoxicological information, and will provide a useful reference for the assessment of selected drugs and the effects which these compounds may have on aquatic invertebrates, using *C. fluminea* as a bioindicator species.

1. Introduction

Water contamination by pharmaceuticals has been an environmental issue since the 1990s (Doerr-MacEwen and Haight, 2006), and there are reports indicating the presence of pharmaceutical compounds and their active metabolites in fresh water environment in ng L^{-1} to μg L^{-1} range, occurring with increasing frequency. Pharmaceuticals enter the aquatic environment through municipal effluents, being detoxification processes in waste water treatment plants (WWTPs) not enough to manage them. The constant discharge, then goes directly to the aquatic environment and gives them a state of pseudo-persistence (Hernando et al., 2006). Moreover, they may cause diverse effects on aquatic biota. The potential toxic effects of these (Aguirre-Martínez et al., 2015; Binelli et al., 2009a; Canesi et al., 2007; Fent et al., 2006; Ferrari et al., 2003; Quinn et al., 2008a, 2008b, 2011; Martín-Díaz et al., 2009a, 2009b). Nevertheless, research is limited to a lesser number of drugs compared to the actual current drugs discharged in the environment, also taking into account the effects that pharmaceuticals may have on aquatic species in general.

A recommended appraisal for the assessment of toxic compounds effects in organisms should include the evaluation of general stress as a toxicity screening tool (Viarengo et al., 2007). In this regard, the evaluation of lysosomal membrane stability (LMS) as biomarker of general stress syndrome has been advised and previously applied in fish, mussels, crabs and clams (Aguirre-Martínez et al., 2013a, 2013b; Buratti et al., 2012; Martínez-Gómez et al., 2008; Moore et al., 2004; Viarengo et al., 2007) and it has been employed as a Tier-1 approach in wide-scale bio-monitoring programmes (Aguirre-Martínez et al., 2013c; Viarengo et al., 2007). Secondly, the use of biomarkers of phase I, phase II, oxidative stress and biomarkers of effect has been proposed by several scientists as early warning tools in environmental risk assessment (Broeg et al., 2005; Depledge and Fossi, 1994; Lam 2009; Lam and Gray, 2001; Van der Oost et al., 2003; Viarengo et al., 2007). An example of those biomarkers includes the activity of biotransformation enzymes (etoxyresorufin O-deethylase [EROD], dibenzylfluorescein dealkylase [DBF], gluthathione-S-transferase [GST]), antioxidant enzymes (gluthathione reductase [GR] and

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gluthathione peroxidase [GPX]); and biomarkers of effect (Lipid peroxidation [LPO] and DNA damage). These responses have been extensively applied as parameters that indicate the presence, availability and even the toxicity of compounds (Walker 1995; 1998). In addition, these biomarkers have been used to study cellular detoxification (Aguirre-Martínez et al., 2013c, 2013d, 2014; Gagné et al., 2007, Martín-Díaz et al., 2004; Morales-Caselles et al., 2008; Ramos-Gómez, et al., 2011a'; Viarengo et al., 2007).

The Asian clam *Corbicula fluminea* (Müller 1774) (Bivalvia: Corbiculidae) is a widespread fresh water species which are native to Southern and East Asia. They have spread through most Europe and America (Aguirre and Poss, 1999; Beasley et al., 2003). In particular, this species can be considered as a good model organism due to their wide distribution, abundance, easy of collection, and maintenance under laboratory conditions (Belanger et al., 1986; Cataldo et al. 2001; Doherty, 1990). Therefore, *C. fluminea* has been used in a variety of ecotoxicological studies to evaluate the effects of metals, flame retardants, pesticides and pharmaceuticals (Achard et al., 2004; Baudrimont et al., 1999; Bigot et al., 2010; Brandão et al., 2011; Cooper and Bidwell, 2006; Daughton and Brooks, 2010; Doherty et al., 1987; Geret et al., 2010; La Guardia et al., 2012; Legeay et al., 2005; Peltier et al., 2008, 2009; Shoults-Wilson et al., 2009; Soucek et al., 2001). In the present study five substances from different therapeutic class including caffeine, ibuprofen, carbamazepine, novobiocin and tamoxifen, were chosen to conduct laboratory experiments with *C. fluminea.* Caffeine is a common psychoactive stimulant drug applied worldwide (Fent et al., 2006; Palo and Choudhury, 2006). Ibuprofen is an anti-inflammatory drug used for general pain relief, and it is one of the most abundant anti-inflammatory drugs found in municipal effluents (Miège et al., 2009; Stuer-Lauridsen et al., 2000). Carbamazepine is prescribed for anticonvulsant and mood-stabilizing treatment. Studies of carbamazepine and its degradation products in water indicate high persistence, being frequently found at higher concentrations than other pharmaceuticals in municipal effluents (Cunningham et al., 2010). Novobiocin is a potent antibacterial agent applied worldwide, and tamoxifen is one of the most commonly used chemotherapeutic agents that have been detected in many waste water treatment plant effluents (Bergh, 2003; Osborne, 1998; Powles et al., 1994). Research has demonstrated chronic toxicity of pharmaceuticals at environmental concentrations in fresh water environments (Gagné et al., 2006; Gerhardt et al., 2002; Isidori et al., 2006). In addition, various sublethal responses have been measured as indicators of the exposure to pharmaceuticals (Gagné et al., 2006; Heckmann et al., 2007; Quinn et al., 2008a, 2008b; Laville et al., 2004). Nevertheless these drugs have never been tested in this fresh water species. This research work aimed to evaluate the effects of sublethal doses of caffeine, ibuprofen, carbamazepine, novobiocin and tamoxifen on *C. fluminea* after an experiment lasting 21 days, which estimated the various drugs effects on general stress and also by tracking changes on biomarkers of phases I and II, oxidative stress and effect.

2. Materials and methods

2.1. Acclimation and maintenance conditions

Specimens of *C. fluminea* were collected in the Miño estuary at 22 km from the mouth of the Miño River, in the locality Pontevedresa de Amorín in Galicia, (NW Spain). The Miño estuary has been considered to be at a low chemical contamination level, not rising serious concern (Sousa et al., 2008), and thus it has been used as a reference site in ecotoxicological studies (Cairrão et al., 2004; Elumalai et al. 2007; Monteiro et al., 2007; Moreira et al. 2006). The Minho estuary is included in two natural protected areas of the EU Natura 2000 Galician network, which includes the entire international section of the river (Ferreira and Pombal, 2012; Sousa et al. 2008). This site presents a naturalized population of Asian clams (Araujo et al., 1993; Sousa et al., 2005). All organisms used in this research were of similar size (45 ± 0.5 mm). Once in the laboratory, clams were acclimatized and depurated for one week in 300 L tanks containing tap water which was previously dechlorinated for at least 96 h, and supplied with constant aeration. Water temperature (16 ± 0.2 °C), pH (8 ± 0.3), and dissolved oxygen ($>$ 7.6 mg L^{-1}, 94.5% sat) were strictly controlled in a 12-h light period.

2.2. Selection of pharmaceuticals

Caffeine, ibuprofen, carbamazepine, novobiocin and tamoxifen were purchased from Sigma Aldrich, Spain (Table 1). These compounds were chosen for this research due to their frequent usage, and because they represent different therapeutic groups. Test concentrations of pharmaceuticals were selected based on concentrations detected in municipal effluents (ME), sewage treatment plants effluents (STP), waste water treatment plant (WWTP) effluents, surface water (SW), rivers, creeks and lakes (a detailed list of caffeine, ibuprofen, carbamazepine and novobiocin concentrations detected worldwide can be found in Aguirre-Martínez et al. 2013a, 2013b).

2.3. Bioassay

Based on reported concentrations described above, caffeine was tested at 0.1, 5, 15, 50 μg L^{-1}, ibuprofen at 0.1, 5, 10, 50 μg L^{-1}, novobiocin at 0.1, 1, 10, 50 μg L^{-1}, carbamazepine at 0.1, 1, 10, 50 μg L^{-1} and tamoxifen 0.1, 1, 10, 50 μg L^{-1} during 21 days, in an static renovation system. All concentrations tested in this study were nominal. Stock solutions of these compounds were diluted in dimethyl sulfoxide (DMSO 0.001% v/v). A treatment with DMSO was used as solvent control (tested at 50 μg L^{-1} which corresponds to the highest solvent concentration present in the tests solution at the highest pharmaceutical concentration analyzed) to ensure that there were no adverse effects of the solvent (Eades and Waring, 2010; Quinn et al., 2008a, 2008b). The bioassay was carried out in 44 glass aquaria (20 L). Clams were divided into groups of 80 per treatment (40 per aquarium), and exposed during 21 days to 22 different treatments, (each treatment was performed in duplicate) including; sea water control, solvent control (DMSO) and pharmaceuticals. Clams were fed every two days with Liquifry®. After the feeding process, the water was siphoned out. Waste food, feces, and any other debris were carefully removed and the water was renewed. Then a volume of the stock solution of pharmaceuticals was added to each aquarium, in order to expose organisms to the pharmaceutical concentration required. Physical-chemical parameters during experiment were similar to those applied in acclimation period.

2.4. General stress

Lysosomal membrane stability (LMS) was evaluated *in vivo* in hemolymph from 10 clams per treatment, at the beginning (day 0) and at the end of assay (day 21) using the neutral red retention time assay (NRRA), following the *in vitro* methodology reported in detail by Aguirre-Martínez et al. (2013a). Clam physiological saline (436 M NaCl, 10 mM KCl, 10 mM $CaCl_2$, 53 mM $MgSO_4$ and 20 mM Hepes sodium salt adjusted to pH 7.3 with 1 N NaOH) was prepared following the protocol of Lowe et al. (1995) and Marchi et al. (2004). Briefly, replicates of 40 μL samples were transferred onto microscope slides at room temperature and placed in a lightproof