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Identification of biomarkers responsive to chronic exposure to pharmaceuticals in target tissues of *Carcinus maenas*

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ABSTRACT

A 28-day bioassay was performed with *Carcinus maenas* to evaluate chronic effects caused by exposure to caffeine and ibuprofen (0.1–50 μg L^{-1}) in sea water. Lysosomal membrane stability (LMS) was evaluated in hemolymph applying the neutral red retention assay (NRRA); several biomarkers including ethoxyresorufin O-deethylase (EROD), dibenzylfluorescein dealkylase (DBF), glutathione S-transferase (GST), glutathione peroxidase (GPX), lipid peroxidation (LPO) and DNA damage were studied in gill, hepatopancreas, muscle and gonad tissues. In crabs exposed to environmental concentrations of the drugs, retention time was reduced by 50%. EROD and DBFOD activities were induced by caffeine in muscle and hepatopancreas tissues ($p < 0.05$); GST activity was activated by ibuprofen in gill, hepatopancreas and muscle at the highest concentrations tested ($p < 0.05$). All tissues showed GPX activity and LPO induction ($p < 0.05$). Crabs exposed to caffeine and ibuprofen showed evidence of DNA damage mainly in hepatopancreas tissues ($p < 0.05$). Environmental concentrations of pharmaceuticals induce LMS and the biochemical responses studied in this crab. This methodology is a suitable technique for assessing pharmaceutical toxicity in the marine environment.

1. Introduction

In many countries pharmaceutical products and their metabolites have been reported not only in municipal effluents and sewage treatment plant (STP) effluents but also in rivers, surface water, underground water and sediments (Andreozzi et al., 2002; Zuccato et al., 2000) at concentrations ranging from ng L^{-1} to μg L^{-1} (Ashton et al., 2004; Pomati et al., 2006; Sedlak et al., 2000) (Table 1). Like other compounds, their eventual sink is the sea (Weigel et al., 2002, 2004a). Given their properties (e.g. lipophilicity and persistence), pharmaceuticals can bioaccumulate (Halling-Sørensen et al., 1998) and cause toxic effects in non-target organisms (Grung et al., 2007; Nakada et al., 2006). It has been documented that an organism's ability to function normally in an ecosystem may be impaired at sublethal concentrations of contaminants (Gerhardt et al., 2002). To date, there is scarce chronic toxicity data for the majority of pharmaceuticals in use, thus impeding their adequate risk assessment. It is also of great importance to know the effects these substances pose to aquatic life, so as to allow better risk assessment and environmental protection before the effects are irreversible or costly to remedy (Carlsson et al., 2006; Ringwood et al., 2003). There have been few studies dealing with this topic, and most of those that have been reported are based on acute toxicity. Although acute tests provide valuable information on the toxic effects of drugs (Ferrari et al., 2003), many independent investigations agree that chronic exposure data based on more specific endpoints as biomarkers should be used in risk assessment.

Several published research studies have established chronic effects of pharmaceuticals at environmental concentrations in fresh water environments (Gerhardt et al., 2002; Gagné et al., 2006; Isidori et al., 2006). However, less effort has been applied to the study of chronic toxicity of pharmaceuticals in the marine environment (Aguirre-Martínez et al., 2010, 2012; Buratti et al., 2010; Martín-Díaz et al., 2009a). Various sublethal responses have been measured as indicators of the exposure to pharmaceuticals (Gagné et al., 2006; Laville et al., 2004). Nevertheless, there is a need to identify which pharmaceuticals produce adverse effects and to what extent, in order to characterize and manage the associated environmental risk. Because pharmaceuticals are continuously entering the aquatic environment and being degraded, they will be most likely to have chronic rather than acute toxic effects (Crane et al., 2006).

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Table 1
Measured concentration of caffeine and ibuprofen in sewage treatment plant (STP), creek, river, municipal effluent (ME), waste water treatment plant (WWTP), surface water (Surf. W), sea water (SW) and lake water, worldwide.

Pharmaceutical	($\mu g\ L^{-1}$)	Place of study	Reference
Caffeine	0.01	SW	Weigel et al. (2004a)
	0.07	STP	Weigel et al. (2004a,b)
	0.09	SW	Weigel et al. (2004a)
	0.16	Creek	Yoon et al. (2010)
	0.25	River	Yoon et al. (2010)
	1.9	WWTP	Kosma et al. (2010)
	3.6	ME	Ternes et al. (1998)
	4.42	WWTP	Santos et al. (2005)
	6.0	WWTP	Kolpin et al. (2002)
	10.0	ME	Gagné et al. (2005)
	12.0	STP	Gómez et al. (2007)
	13.9	WWTP	Kosma et al. (2010)
	22.2	WWTP	Gagné et al. (2006)
	293.0	STP	Weigel et al. (2004a)
Ibuprofen	0.01	Lake	Tixier et al. (2003)
	0.01	ME	Thomas and Foster (2004)
	0.01	SW	Weigel et al. (2004a)
	0.03	STP	Weigel et al. (2004b)
	0.05	River	Yoon et al. (2010)
	0.15	Surf. W	Gros et al. (2006)
	0.3	Creek	Yoon et al. (2010)
	0.5	WWTP	Kosma et al. (2010)
	0.7	STP	Weigel et al. (2004b)
	0.8	WWTP	Gros et al. (2006)
	1.0	WWTP	Kolpin et al. (2002)
	1.3	WWTP	Gagné et al. (2006)
	1.3	WWTP	Tixier et al. (2003)
	2.1	STP	Carballa et al. (2004)
	2.3	River	Roberts and Thomas (2006)
	2.6	WWTP	Kosma et al. (2010)
	3.0	WWTP	Roberts and Thomas (2006)
	6.3	WWTP	Santos et al. (2005)
	7.1	STP	Gómez et al. (2007)
	10.1	WWTP	Santos et al. (2005)
	20.0	STP	Weigel et al. (2004b)
	24.6	WWTP	Miège et al. (2009)

This research is focused on two pharmaceuticals: caffeine and ibuprofen. They are active principles widely used and frequently combined in pharmaceutical preparations. Caffeine is a methylated xanthene, and a potent stimulant of the central nervous system; it has been added to ibuprofen in various combinations in order to improve analgesic efficacy. Caffeine is one of the most commonly consumed alkaloids (Fent et al., 2006; Palo and Choudhury, 2006) and is recognized as one of the pharmaceuticals present most widely in the environment (Nikolau et al., 2007; Khoshayand et al., 2008). This drug is soluble in water and presents a slow rate of degradation; caffeine can persist in aquatic environments (Seiler et al., 2005) and has the potential to biomagnify through the food chain and concentrate over time (Gibson et al., 2012). Ibuprofen is one of the most common medicines found in water treatment plants (WTP) (Gagné et al., 2006; Miège et al., 2009; Santos et al., 2005). Ibuprofen is a non-steroidal drug, used as an anti-inflammatory, analgesic and antipyretic in the human treatment of fever and pain (Ciriaco et al., 2009), this drug has relatively high mobility in aquatic environments, but a low persistence compared to other pharmaceuticals (Buser et al., 1999). Its half life in the field has been estimated at approximately 32 days (Tixier et al., 2003). Research studies have demonstrated acute and chronic effects of caffeine and ibuprofen in fresh water organisms (Heckmann et al., 2007; Quinn et al., 2008a,b). However, no conclusive data on the chronic toxicity of ibuprofen and caffeine in marine biota have been reported.

The aim of the present study is to evaluate the bioavailability and possible adverse effects of ibuprofen and caffeine at environmentally relevant concentrations dissolved in marine water, using the European green crab *Carcinus maenas* as bioindicator species (Bamber and Depledge, 1997). For these objectives, biomarkers of exposure and effect were determined in different tissues of this crab. The specific redox reactivity of pharmaceuticals forms the basis for their respective biological (therapeutic) effects, metabolism, elimination, and toxicity. Exposure to these products can alter the oxidation state of cells and thereby increase oxidative stress. Induction of the detoxification metabolism by the superfamily of cytochromes P450 has been described by several authors (e.g. Thibaut and Porte, 2008) and it is well known that this releases oxygen radicals that can lead to oxidative stress. Aquatic biota exposed to these pharmaceutical compounds can be adversely affected, as they are possibly not as efficient as mammals in eliminating lipophilic drugs and oxygen radicals (Gagné et al., 2006).

In this study special attention has been given to lysosomal membrane stability (LMS) and biochemical responses induced by pharmaceuticals, including expression of the cytochrome P450-related activities, oxidative stress (antioxidant enzyme and lipid peroxidation) and genotoxicity, using consolidated biomarkers (Gagné et al., 2006; Viarengo et al., 2007). Lastly the suitability of using this battery of biomarkers to assess the environmental risk of caffeine and ibuprofen in marine ecosystems is discussed.

2. Material and methods

2.1. Laboratory bioassay

Intermoult females of *C. maenas*, carapace width from 47 to 57 mm, were collected in baited traps from a reference site at the Bay of Cádiz, Spain. All individuals used in this study were caught on the same day from a single location. Crabs were acclimated to laboratory conditions prior to experimentation. After the acclimatization period, six crabs were placed in a 20 L glass aquarium, supplied with constant aeration and exposed during 28 days to different treatments of pharmaceuticals, including environmental concentrations (Table 1). In order to expose organisms to the pharmaceutical concentration required (Table 2), a volume of the stock solution of caffeine and ibuprofen was dissolved in DMSO (0.001% v/v). Solvent control assessment was also undertaken in parallel with the experiment as recommended by Eades and Waring (2010) and Quinn et al. (2008a,b). A detailed description of the bioassay parameters is given in Table 2.

Table 2
Experimental design.

Bioassay	Description
Species	*Carcinus maenas*
Acclimatization	1 week (tanks)
Aquarium	26 glass aquarium, 20 L volume
No. of individuals	6 per aquarium
Parameters	pH (7.8–8.2), T (17 ± 1 °C), salinity (33.8 ± 0.3), dissolved oxygen (>5 mg L^{-1}, 60% sat.), constant aeration, natural photoperiod 12 h/12 h dark:light regime.
Pharmaceuticals & concentrations	Caffeine (0.1, 5, 15, 50 $\mu g\ L^{-1}$), ibuprofen (0.1, 5, 10, 50 $\mu g\ L^{-1}$)
Treatment	Control treatment, DMSO-treated control (50 $\mu g\ L^{-1}$) and pharmaceuticals treatment (spiked sea water with selected concentrations). Each treatment was performed in duplicate
Food	Frozen mussels (given every two days)
Cleaning	Water (filtered sea water) changed every 24 h
Duration of test	28 days
Sampling	Day 28