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Effects of 4-MBC and triclosan in embryos of the frog *Pelophylax perezi*

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HIGHLIGHTS

- Effects of two personal care products in Perez's frog early life stages.
- Glutathione S-transferase was inducted by 4-MBC in a bell-shape like pattern.
- Triclosan (TCS) reduced survival, delayed hatching and induced malformations.
- TCS induced detoxification, impaired neurotransmission and anaerobic metabolism.

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ABSTRACT

The widespread and increasing use of personal care products (PCPs) have led to environmental contamination by substances included in these products. These substances have been detected in aquatic compartments and shown to cause adverse effects on non-target aquatic organisms. In this work toxicity of the antimicrobial triclosan (TCS) and of the UV-filter 3-(4-methylbenzylidene) camphor (4-MBC) was assessed in the embryos of Perez' frog *Pelophylax perezi*. Lethal and sub-lethal parameters were evaluated in embryos in Gosner stage 8–9 exposed to 0.00013–1.3 mg/l of 4-MBC and 0.25–2.50 mg/l of TCS during 144 h. Survival, malformations, length and hatching were evaluated as apical endpoints. Biomarkers of neurotransmission, oxidative stress, energy metabolism and estrogenicity were determined at the biochemical level through the activities of cholinesterase (ChE), catalase (CAT), glutathione S-transferase (GST), lactate dehydrogenase (LDH) and levels of lipid peroxidation (LPO) and vitellogenin (Vtg). Embryo exposure to 4-MBC led to few developmental malformations (up to 3%) and a GST induction at 0.013 mg/l. Triclosan exposure reduced survival, delayed hatching (at 72 h) and development and induced malformations. In addiction ChE was inhibited in the highest concentrations tested and GST and LDH were induced at 0.79 mg/l, the LOEC registered for TCS in Perez' frogs. Overall, our study showed that TCS might exert adverse effects on *P. perezi* early life stages, but only at four orders of magnitude above the concentrations found in environment. Furthermore, our results highlight the need to assess PCPs toxicity at different levels of biological organization.

1. Introduction

In recent years, different components of widely used personal care products (PCPs; e.g. shampoos, fragrances, cosmetics, toothpaste, soap) are under scrutiny for their evidences of endocrine disruption (e.g. Witorsch and Thomas, 2010). Under typical use conditions, PCPs are rinsed off and residuals which are not biodegraded or removed in wastewater treatment plants can enter the aquatic environment. Therefore, chemicals used in the composition of PCPs, like bactericides and ultraviolet light filtering compounds (UV-filters) are frequently detected in aquatic ecosystems (Brausch and Rand, 2011). The primary classes of PCPs include antimicrobials, polycyclic musks, phthalates, parabens and UV filters. Many of these compounds are used in large quantities, and recent studies have indicated many are environmentally persistent, bioactive, and have potential for bioaccumulation (Peck, 2006; Mackay and Barnthouse, 2010). Numerous reviews have been published examining PCPs occurrence and toxicity (e. g. Daughton and Ternes, 1999; Jjemba, 2006; Brausch and Rand, 2011).

Among the PCPs, triclosan (5-chloro-2(2,4-dichlorophenoxy) phenol, TCS) is a broad spectrum antimicrobial agent used in a variety of PCPs including soaps, deodorant, and toothpaste (Wang

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et al., 2013). Triclosan is not completely removed in wastewater treatment plants (Dann and Hontela, 2011) and concentrations can reach 2.3 μg/l in natural aquatic environments and 2.7 μg/l in wastewater treatment plants effluents (Chalew and Halden, 2009; Dann and Hontela, 2011), while the mean concentration found in natural waters is 48 ng/l (Brausch and Rand, 2011). Triclosan has an estimated half-life of 60 days in water (Halden and Paull, 2005) and is a relatively stable lipophilic compound (log Kow of 4.8) that is expected to bioaccumulate in aquatic organisms (Halden and Paull, 2005). Several studies report the adverse effects of TCS on aquatic organisms of different trophic levels (Capdevielle et al., 2008), such as algae *Anabaena* (Orvos et al., 2002), crustacean *Daphnia magna* (Orvos et al., 2002), fish *Oryzias latipes* (Ishibashi et al., 2004) and amphibians *Bufo americanus* (Smith and Burgett, 2005) and *Xenopus tropicalis* (Regnault et al., 2016). Another group of chemicals associated with PCPs are UV-filters compounds used in sunscreens and cosmetics (Sparling et al., 2010). UV filters such as 3-(4-methylbenzylidene) camphor (4-MBC) are lipophilic and chemically reasonably stable (Buser et al., 2006). Concentrations of 4-MBC can reach 1.14 μg/l in surface waters in summer and up to 6.5 μg/l in wastewater treatment plants influent and 2.7 μg/l in effluent (Balmer et al., 2005; Rodil et al., 2009). UV-filters enter the aquatic environment either directly via wash-off from skin and cloth, or indirectly via wastewater, which represents a significant source. UV-filters have shown estrogenic and hormonal activity *in vitro* on MCF-7 human breast cancer cells suggesting that they can act as endocrine disruptors (EDC) (Schlumpf et al., 2001). Kunz et al. (2006) reported that some UV-filters are estrogenic in fish, and induce adverse effects on fecundity and reproduction (Fent et al., 2010). Although several studies addressed the impact of 4-MBC and triclosan in aquatic organisms, most are focused on invertebrates leaving a need to assess effects on aquatic vertebrates. In addition, despite some information about the potential neurotoxicity and estrogenicity of TCS as well as on promoting oxidative stress (e.g. Foran et al., 2000; Li et al., 2016), there is a lack of data on initial embryonic development of aquatic vertebrates for these compounds.

Some amphibians have an aquatic life stage followed by a terrestrial adult stage, so they can be exposed to a wide range of contaminants (Dumpert and Zietz, 1984; Lefcort et al., 1998). Along with habitat loss, environmental pollution has been considered responsible for the global decline of amphibian populations registered over recent decades (Blaustein and Wake, 1995; Blaustein et al., 2003). Indeed, amphibians are considered by some authors, more sensitive to aquatic contaminants than other species because they readily absorb chemicals through both their gills and permeable skin (Boyer and Grue, 1995). Amphibian larvae are potentially exposed to several pollutants in their aquatic habitats which may influence their performance (Egea-Serrano et al., 2012). For instance, amphibian larvae are known to reduce their swimming activity in presence of low concentrations of various contaminants (Bridges and Semlitsch, 2005). In this context, the test organism chosen for the present study was early life stages of the Perez's frog *Pelophylax perezi* (Seoane, 1885), a low concern frog endemic of the Iberian Peninsula and Southwest of France (Europe).

The use of biochemical biomarkers in ecotoxicology is becoming a useful routine, and various endpoints have been proposed as valuable tools to assess the effects and mode of action of environmental chemical contamination (Quintaneiro et al., 2006; Monteiro et al., 2007), including in amphibians (Venturino et al., 2003; Venturino and de D'Angelo, 2005). Biochemical markers assessed in this work will include neurotoxicity (cholinesterase, ChE), endocrine disruption (vitellogenin, Vtg), oxidative stress (glutathione S-transferase, GST, and catalase, CAT, lipid peroxidation, LPO) and energy metabolism (lactate dehydrogenase, LDH) biomarkers.

The main objective of this study was to improve the knowledge of the toxicological effects of PCPs. Within them, TCS and 4-MBC were selected based on their occurrence in the environment and on reported effects or gaps about their ecotoxicological effects. Triclosan and 4-MBC effects were assessed in early life stages of the amphibian *Pelophylax perezi* at different levels of biological organization: i) at the biochemical level the impairment of energy production and potential neurotoxicity, oxidative stress induction and estrogenicity were assessed; ii) at the organism level, mortality, hatching rate, growth and embryo malformations and development impairment were evaluated.

2. Material and methods

2.1. Chemicals

The chemicals used in these experiments, like TCS, 4-MBC, acetone and the chemicals used for FETAX medium and biochemical analysis were obtained from Sigma-Aldrich (Germany), except the Bradford reagent which was purchased from Bio-Rad (Germany).

2.2. Sampling of Pelophylax perezi eggs

Egg masses of *P. perezi* in stages 10–12 described by Gosner (1960) were collected in a channel at Quinta da Boavista (40°35′48.8″N 8°41′43.4″W) near Aveiro (Portugal), and were transported to the laboratory in local water. At the laboratory the egg masses were gently cleaned with a Pasteur pipette and eggs were separated carefully to not damage the chorion.

2.3. Experimental design

Stock solutions of TCS and 4-MBC were prepared using acetone as a solvent and FETAX (Frog Embryo Teratogenesis Assay - Xenopus) medium was used for the necessary dilutions. Triclosan and 4-MBC sub-lethal concentrations were tested in the range of 0.25–2.50 mg/l (0.86–8.6 mM) and 0.00013–1.3 mg/l (0.52–5.2 mM), respectively, plus a negative control (FETAX medium) and a solvent control with acetone at 1% (v/v) as recommended by the guideline (ASTM, 2012). The sub-lethal concentrations chosen for each chemical were based on the LC_{50} levels and/or survival obtained for other related species on triclosan (e.g. Ishibashi et al., 2004; Oliveira et al., 2009; Palenske et al., 2010) and 4-MBC (e.g. Torres, 2013; Li et al., 2016).

Pelophylax perezi eggs were exposed during 144 h to TCS and 4-MBC test solutions under controlled temperature (20 ± 1 °C) and photoperiod conditions (16 h light: 8 h dark). Tests followed the FETAX guideline (Dawson and Bantle, 1987; ASTM, 2012) with some adaptations. Briefly, six replicates (n = 6) of ten eggs each were used per treatment. Each group of ten eggs of *P. perezi* were placed in each well of 6-well plates containing 10 ml of test solutions per well. Replacement of test solutions was performed at 72 h. Amphibian embryos and larvae were observed daily under the stereoscope, mortality was checked and dead organisms were removed. During embryo stage egg viability, Gosner stage and malformations were evaluated according to Gosner (1960). After hatching, malformations and larval stage were recorded, as well as length and mortality.

At the end of the test surviving organisms were used for biochemical analysis. For subsequent enzyme analysis and determination of LPO levels, 6 pools of 3–4 larvae from each well were assayed per concentration. Similarly, six pools of 5 larvae from each