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Response of detoxification system genes on *Chironomus riparius* aquatic larvae after antibacterial agent triclosan exposures

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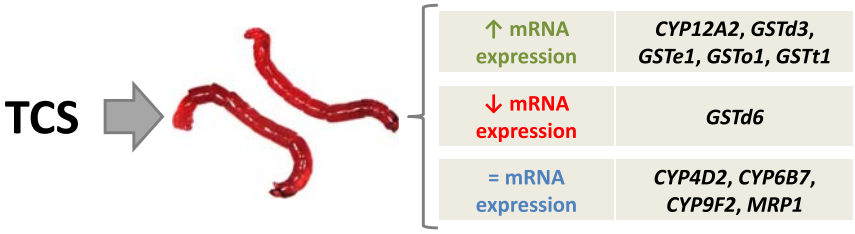
HIGHLIGHTS

- TCS activates phase I and phase II detoxification system genes in *C. riparius*.
- GST enzyme activity is not affected after TCS exposure.
- *MRP1* gene expression, which encodes for an ABC transporter, is not altered by TCS.
- This study gives a putative mode of action of TCS on detoxification system.

GRAPHICAL ABSTRACT



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ABSTRACT

Triclosan (TCS) is an antimicrobial agent used in a range of personal care and consumer products and is commonly detected in aquatic ecosystems. In the present study, the effects of TCS at the molecular level on the detoxification system of *Chironomus riparius* aquatic larvae, a test organism widely used for the assessment of aquatic toxicology, were evaluated. The obtained results show that this xenobiotic was able to induce significant changes in transcripts from different *cytochrome P450s* and *glutathione s-transferases* genes, involved in phase I and phase II of detoxification system, respectively. In contrast, TCS did not affect the glutathione *S*-transferase enzyme activity nor the expression pattern of *multidrug resistance-associated protein 1*, which belongs to phase III of detoxification system. These results provide information about the effects of TCS on the detoxification mechanism of *C. riparius* and offers different biomarker genes that could be useful in ecotoxicological studies, risk assessment and bioremediation.

1. Introduction

One of the most widely used groups of emerging environmental pollutants of pharmaceuticals and personal care products are the antibacterial agents, e.g. Triclosan. Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol or TCS) is a broad spectrum antibacterial agent widely used in many veterinary, industrial, and household products (e.g. deodorant, shampoo, toothpaste, mouthwash, disinfectants, soap, detergent, cosmetics, textiles, and plastic additives). Due to its increasing use over recent decades, TCS is commonly found as a contaminant in aquatic and terrestrial environments (Dann and Hontela, 2011) and has been detected in human urine, plasma, and milk (Wang and Tian, 2015). TCS is one of the top ten most commonly detected organic wastewater compounds (Brausch and Rand, 2011). However, despite the United States Environmental Protection Agency completing its re-registration eligibility decision for the uses of TCS in 2008 (USEPA, 2008), in September 2016 the U.S. Food and Drug Administration banned soaps containing TCS (Federal Register, 2016).

Previous works have reported that TCS invokes disruption of hormonal homeostasis in amphibians (Marlatt et al., 2013; Veldhoen

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et al., 2006) and rats (Kumar et al., 2009; Paul et al., 2010). Moreover, this xenobiotic acts as endocrine disrupting chemical (EDC) in human cell cultures (Ahn et al., 2008; Christen et al., 2010; Gee et al., 2008) and affects the gene expression profile of hormonal receptor genes in frogs (Marlatt et al., 2013; Veldhoen et al., 2006), rats (Feng et al., 2016), and insects (Martínez-Paz et al., 2017). Moreover, TCS induces DNA damage in mollusks (Binelli et al., 2009), insects (Martínez-Paz et al., 2013), annelids (Lin et al., 2010, 2014), protozoans (Gao et al., 2015), crustaceans (Silva et al., 2015), and fish (Capkin et al., 2017).

Despite the increase in recent years in the amount of data available on the environmental toxicity of TCS, information about the potential impact of this xenobiotic on responses of genes and enzymes related to detoxification system in invertebrates, particularly Dipteran, which are important in ecotoxicological studies, is scarce. The larvae of the midge *Chironomus riparius* (Diptera) are widely used as a test organism for the assessment of aquatic toxicology (OECD, 2013; USEPA, 2000). Chironomid larvae are one of the most ubiquitous freshwater benthic invertebrates and are abundant and ecologically relevant to the aquatic food chain (Armitage et al., 2012). Chironomids are being used for toxicity testing using molecular endpoints, such as gene biomarkers for monitoring of both environmental quality and the health of organisms inhabiting polluted ecosystems. In recent years, some genes have been described in *C. riparius* as biomarkers for the evaluation of different aquatic pollutants, among which are those that encode for heat shock proteins (HSPs), ribosomal proteins, cytochrome P450s, and nuclear receptors (Aquilino et al., 2016; Bernabò et al., 2017; Herrero et al., 2015; Martín-Folgar et al., 2015; Martínez-Guitarte et al., 2007; Martínez-Paz et al., 2012, 2014, 2017; Morales et al., 2011, 2013, 2014; Nair and Choi, 2012; Nair et al., 2011a; Ozáez et al., 2013; Park and Kwak, 2008, 2010; Planelló et al., 2008, 2011).

It has been reported, in invertebrates, that TCS induces an increase in the activity of the detoxification, antioxidant, and stress defense systems in the clam *Ruditapes philippinarum* (Matozzo et al., 2012), the crustacean *Daphnia magna* (Peng et al., 2013), the terrestrial snail *Achatina fulica* (Wang et al., 2014), the mussels *Unio tumidus* (Falfushynska et al., 2014), the rotifer *Brachionus koreanus* (Han et al., 2016), the freshwater mussel *Elliptio complanata* (Goodchild et al., 2016), and the earthworm *Eisenia fetida* (Ma et al., 2017). In general, the detoxification metabolism system involves the phase I (functionalization), phase II (conjugation) and phase III (excretion). Among the different components of the detoxification system, cytochrome P450 (P450) and glutathione *S*-transferase (GST) enzymes play central roles in defending the cell against toxic substances. P450 enzymes are the most important enzymes in phase I of the detoxification system and mostly, but not necessarily, are primarily responsible for the initiation of the degradation and elimination of endogenous and exogenous compounds. GSTs are the most important phase II enzymes of detoxification system and are capable of conjugating reduced glutathione to the electrophilic centers of toxic compounds. Despite the increase in recent years in the amount of data available on the potential toxic effects of TCS, mainly focused on evaluation of biochemical and enzyme responses, there is a scarcity of information about the response of the detoxification system related genes in aquatic organisms. Thus, the aim of the present work was to analyze the effects of TCS on detoxification system in *C. riparius* aquatic larvae. Therefore, *CYP4D2*, *CYP6B7*, *CYP9F2*, *CYP12A2*, *GSTd3*, *GSTd6*, *GSTe1*, *GSTo1* and *GSTt1* genes, involved on phase I and phase II of detoxification system, have been selected to evaluate its transcription profiles.

On the other hand, multidrug resistance-associated protein 1 (MRP1) is involved in the multi xenobiotic resistance (MXR) mechanisms and phase III detoxification system (Epel et al., 2008; Smital et al., 2004) playing an active role in protecting cells against many kinds of toxics to sublethal levels. This protein belongs to ATP-binding cassette (ABC) transporter proteins superfamily and pump endogenous metabolites and xenobiotic contaminants out of cells. Thus, MRP1 reduce the intracellular concentration of toxic compounds, protecting exposed organisms from potentially toxic effects (Bard, 2000; Litman et al., 2001). In this study, the transcriptional activity of *MRP1* gene has been evaluated with the purpose of analyze the effects of TCS on the MXR mechanisms.

In summary, the aim of present work was to analyze the effect of TCS on the transcriptional activity of genes involved in phase I, and phase II of detoxification system, as well as its effects on the genes that encode to the ABC pump involved in the active efflux of a wide range of both endogenous and xenobiotic substrates. This is one of the first studies that evaluate a suite of detoxification system related genes in invertebrates, with the aim to help to elucidate the mechanisms involved in its metabolic pathway and, additionally, providing a better understanding of the environmental impact of this compound on aquatic ecosystems.

2. Materials and methods

2.1. Chemicals and reagents

Triclosan (TCS) and GST Kit were purchased from Sigma (USA). Stock solution of TCS was prepared in ethanol (VWR, USA) and then diluted in the culture medium to obtain the experimental concentrations of TCS. The commercial kit TRIzol and M-MLV enzyme were obtained from Thermo-Fisher (USA), oligonucleotide dT_{20} primer, phenol:chlorophorm:isoamil alcohol, and gene specific primers were supplied by Sigma (UK), RNase-free DNase and protease inhibitor cocktail were provided from Roche (Germany), $MgCl_2$, dNTPs, and *Taq* DNA polymerase were purchased from BioTools (Spain), EvaGreen dye was acquired from Biotium (USA), and BCA Protein Assay Reagent was supplied by Thermo Scientific (USA).

2.2. Animals

The experimental animals were fourth instar larvae from the midge *Chironomus riparius*. They were originally collected from natural populations in a non-polluted area of Valencia (Spain) and reared under standard laboratory conditions for several generations according to toxicity testing guidelines (USEPA, 2000; OECD, 2011). Larvae were grown from egg masses in glass tanks with culture medium (0.5 mM $CaCl_2$, 1 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $NaHCO_3$, 0.025 mM KH_2PO_4, 0.01 mM $FeCl_3$) supplemented with nettle leaves, commercial fish food (TetraMin), and cellulose tissue. Cultures were maintained under constant aeration at 20 °C and a standard light-dark period of 16:8 h.

2.3. Treatments

Dose selection was chosen based on previous studies in *C. riparius* (Martínez-Paz et al., 2013, 2014, 2017) and in other organisms and cell cultures and concentrations detected in the environment (Dann and Hontela, 2011; Hinther et al., 2011; Perron et al., 2012). For experimental treatments, the fourth instar larvae were exposed to the nominal concentrations of 10 μg/L, 100 μg/L, and 1000 μg/L of TCS diluted in culture medium for 24 h with constant aeration at 20 °C. No food or substrate was provided to the larvae during the TCS treatment. Three independent experiments were performed in each analysis using samples from different egg masses. In each experiment, TCS exposures were performed with a pool of ten larvae for each treatment, three of them were randomly selected for RNA extraction, that was performed from each one separately, and five of them were randomly selected for GST enzyme activity analysis. The control larvae used were exposed to the same concentration of solvent (ethanol 0.033%) as the corresponding treatment and were also assessed in triplicate. Thus, the global numbers of individual larvae obtained from three independent experiments were $n = 9$ for the mRNA expression analysis and $n = 15$ for the GST activity evaluation. Untreated larvae and larvae exposed to TCS and were stored at −80 °C until RNA and protein isolation was carried out.