Science of the Total Environment 650 (2019) 1752–1758

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Environmental concentrations of triclosan activate cellular defence mechanism and generate cytotoxicity on zebrafish (*Danio rerio*) embryos

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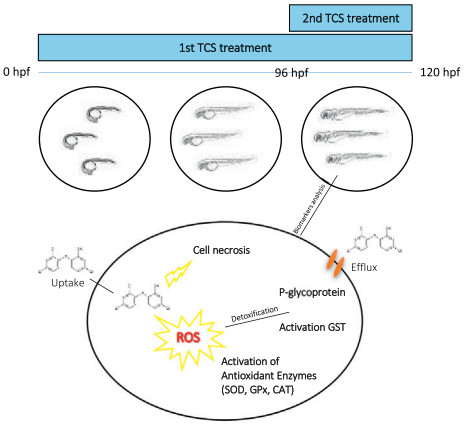
HIGHLIGHTS

- TCS exposure induces P-gp efflux functionality and oxidative stress enzymes.
- Embryos cellular defence system prevents the occurrence of oxidative damage by TCS.
- High levels of cell necrosis underline TCS cytotoxicity potential.
- TCS occurrence in the aquatic environment poses an actual risk for wildlife.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 8 June 2018
Received in revised form 20 September 2018
Accepted 21 September 2018
Available online 26 September 2018

Editor: Damia Barcelo

Keywords:
Triclosan
Danio rerio
Oxidative stress
Biomarkers
Genotoxicity
Cytotoxicity

ABSTRACT

Triclosan (TCS, 5 chloro 2 (2,4 dichlorophenoxy) phenol) is becoming a major surface waters pollutant worldwide at concentrations ranging from ng L^{-1} to μg L^{-1}. Up to now, the adverse effects on aquatic organisms have been investigated at concentrations higher than the environmental ones, and the pathways underlying the observed toxicity are still not completely understood. Therefore, the aim of this study was to investigate the toxic effects of TCS at environmental concentrations on zebrafish embryos up to 120 hours post fertilization (hpf). The experimental design was planned considering both the quantity and the exposure time for the effects on the embryos, exposing them to two different concentrations (0.1 μg L^{-1}, 1 μg L^{-1}) of TCS, for 24 h (from 96 to 120 hpf) and for 120 h (from 0 to 120 hpf). A suite of biomarkers was applied to measure the induction of embryos defence system, the possible increase of oxidative stress and the DNA damage. We measured the activity of glutathione *S* transferase (GST), P glycoprotein efflux and ethoxyresorufin *o* deethylase (EROD), the level of ROS, the oxidative damage through the Protein Carbonyl Content (PCC) and the activity of antioxidant enzymes. The genetic damage was evaluated through DNA Diffusion Assay, Micronucleus test (MN test), and Comet test. The results showed a clear response of embryos defence mechanism, through the induction of P-gp efflux functionality and the activity of detoxifying/antioxidant enzymes, preventing the onset of oxidative damage. Moreover, the significant increase of cell necrosis highlighted a strong cytotoxic potential for TCS. The overall results obtained with environmental concentrations and both exposure time, underline the critical risk associated to the presence of TCS in the aquatic environment.

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https://doi.org/10.1016/j.scitotenv.2018.09.283
0048-9697/

1. Introduction

Triclosan (TCS, 5 chloro 2 (2,4 dichlorophenoxy) phenol) is an emerging contaminant included as antimicrobial agent in several personal care products (PCPs), such as soaps, deodorants, toothpastes, cosmetics and laundry detergents (Dann and Hontela, 2011), with a typical range of 0.1–0.3% of products weight (Montaseri and Forbes, 2016). The ubiquitous use of these products has drawn the attention of the research community, due to the well documented TCS toxicity in mammalians models, including irritation of eyes and skin, allergies, detrimental effects on development and reproduction, weakening of the immune system, inhibition of muscle function and genotoxicity (Barbaud et al., 2005; Binelli et al., 2009; Dann and Hontela, 2011; Cherednichenko et al., 2012; Savage et al., 2012; Halden, 2014). High levels of TCS were recently found in human urine, blood sample, liver, adipose tissue, brain and breast milk (Ruszkiewicz et al., 2017) and its structure is similar to the polychlorinated phenoxyphenols that, under oxidizing conditions, cyclize to other toxic byproducts, which present very harmful effects on human health (Solá-Gutiérrez et al., 2018). Furthermore, a recent proteomic study (Li et al., 2018) revealed a distinct evidence of the potential impact of TCS on human metabolic pathways.

Nowadays, there is growing awareness for TCS environmental implications so that the marketing of over-the-counter antibacterial soaps and body washes containing TCS was banned in the United States since 2016 (FDA, 2016), while the European Union (EU, 2016) has disapproved the use of TCS in human hygiene biocidal products (product-type 1). Nevertheless, the occurrence of TCS in the aquatic ecosystems is a worldwide issue, being commonly detected in surface waters (lake/river/streams with known input of raw wastewaters) with concentrations ranging from 1.4 to 40,000 ng L^{-1}, and in sea water from <0.001 to 100 ng L^{-1} (Dhillon et al., 2015). There is a scientific evidence of TCS emission from Wastewater Treatment Plants (WWTPs) into the aquatic environment, due to their partial inability to remove it, with detected concentrations ranging from 10 to 2,210 ng L^{-1} in European WWTP effluents (Bedoux et al., 2012). TCS and mostly its methylated degradation product (methyl triclosan) are lipophilic and volatile (Wang and Kelly, 2017), causing a high tendency for bioaccumulation in aquatic organisms, which uptake them directly from food or water with bioconcentration factor (BCF) of 2.7–90 (Dhillon et al., 2015). Detected concentrations of TCS are reported in different wild organisms, such as algae and invertebrates, with concentrations up to 400 μg/kg (Coogan and La Point, 2008), and fish, with values up to 300 ng/g wet weight (ww) in muscle tissue (Yao et al., 2018).

The chronic and acute toxicity of TCS has been demonstrated in several aquatic models, which include green and blue algae (Orvos et al., 2002) and benthic invertebrates (Dussault et al., 2008), showing effects on biomass and growth rate, and generating oxidative stress and genotoxicity (Binelli et al., 2009; Riva et al., 2012; Martínez-Paz, 2018). In fish, TCS impacts equilibrium, swimming, spinal curvature and quiescence (Orvos et al., 2002; Ishibashi et al., 2004), as well as several studies showed the endocrine disruption effect of TCS, as demonstrated for the Japanese medaka *Oryzias latipes* (Ishibashi et al., 2004; Horie et al., 2018). Some recent studies performed on zebrafish *Danio rerio* embryos highlighted that TCS caused heart edema and slow heartbeat (Zhu et al., 2018), delayed hatching and increased mortality (Falisse et al., 2017), impaired lipid metabolism (Ho et al., 2016), and induced hepatotoxicity (Haggard et al., 2016) at concentrations up to 1.25 mg L^{-1}. Furthermore, Muth-Köhne et al. (2012) demonstrated that exposure to 3 μM TCS (870 μg L^{-1}) may have neurotoxic effects, delaying the development of zebrafish embryos motor neurons. Such results underline the TCS toxic potential for aquatic species, but do not represent a realistic scenario, since exposure concentrations are higher than those detected in aquatic ecosystems.

This study aimed to evaluate the environmental impact of TCS on aquatic ecosystem, assessing its toxic effects at environmentally relevant concentrations, and contribute to a better understanding of the response of the organism detoxification systems to TCS exposure, using zebrafish embryos as experimental model. Embryos were exposed to two environmental concentrations (0.1 and 1 μg L^{-1}) of TCS for two different exposure time, from 0 to 120 hpf and from 96 to 120 hpf, to improve the understanding on cellular detoxification mechanisms and to investigate the temporal variation of chronic toxicity in terms of oxidative stress and cyto-genotoxicity. Actually, we used a biomarker suite based on the measure of reactive oxygen species (ROS) production and the antioxidant enzymes' activity, namely catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GP_x). We also measured the induction of detoxification activities by the glutathione *S* transferase (GST), P glycoprotein and ethoxyresorufin *o* deethylase (EROD), as well as the potential oxidative damage through the Protein Carbonyl Content (PCC) and cyto-genotoxic effects through the DNA Diffusion Assay, Micronucleus test (MN test) and Comet test.

2. Materials and methods

2.1. Preparation of TCS solutions

Triclosan (TCS, CAS 3380-34-5, purity 97%) was purchased from Sigma-Aldrich (Milan, Italy). Firstly, a stock solution of TCS, 1 g L^{-1} in dimethyl sulfoxide (DMSO), was prepared and stored at 4 °C, while two TCS working solutions were prepared successively diluting the stock solution in ultrapure water at 0.1 mg L^{-1} and 1 mg L^{-1}, respectively. The maximum percentage of DMSO was lower than 0.0001%, and not produces any changes on hemocyte viability, DNA damage or enzyme activity (Parolini et al., 2011). These working solutions were then properly diluted to reach the selected exposure concentrations of 0.1 μg L^{-1} and 1 μg L^{-1}, which fall in the range of the current environmental freshwater levels of TCS (Ho et al., 2016; Zhou et al., 2017). These concentrations also fall within the Predicted No Effect Concentration (PNEC) range (70 ng L^{-1} to 1,550 ng L^{-1}) reported by Capdevielle et al. (2007), which made a Species Sensitivity Distribution (SSD) analysis based on pre-existing chronic toxicity data of TCS for 14 different aquatic species.

2.2. Zebrafish maintenance and embryos exposure

Adult zebrafish of the AB strain are raised in the facility of the Department of Biosciences, University of Milan, where they are maintained in tanks into a thermostatic chamber (28 °C) with 14-h light/10-h dark cycle. The facility follows Italian laws, rules and regulations (Legislative Decree No. 116/92), as confirmed by the authorization issued by the municipality of Milan (PG 384983/2013).

Different groups of embryos were collected by means of natural spawning, following the procedure for maximal embryo production described by Westerfield (2007). Control embryos were maintained in zebrafish water (Instant Ocean, 0.1% methylene blue), while others were exposed to TCS (0.1 μg L^{-1} and 1 μg L^{-1}) dissolved in zebrafish water.

We planned two different exposures in order to evaluate the potential temporal variations, one from 0 hpf to 120 hpf (120 h of exposure) and the other one from 96 hpf to 120 hpf (24 h of exposure). Biochemical analysis exposures were performed in 90 × 15 mm petri dish with a maximum of 70 embryos per petri and two replicates per experimental group, adding 25 mL of the appropriate TCS working solution to each treatment group and 25 mL of zebrafish water to control groups (Fig. S1a). ROS level and cyto-genotoxic biomarkers were performed in multi-well plates with 12 and 5 embryos per well, respectively, and three replicates per experimental group, adding 2 mL of the appropriate TCS working solution to each treatment group and 2 mL of zebrafish water to control groups (Fig. S1b). Both exposures (120 h and 24 h) proceeded at 28 °C under semi-static conditions, replacing the exposure