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Triclosan-induced transcriptional and biochemical alterations in the freshwater green algae *Chlamydomonas reinhardtii*

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ABSTRACT

Triclosan (TCS) is an antibacterial and antifungal agent widely used in personal care products (PCPs). We investigated the effects of TCS (20 μg/L, 100 μg/L and 500 μg/L) on *Chlamydomonas reinhardtii* by measuring the algal growth, chlorophyll content, lipid peroxidation, and transcription of the antioxidant-related genes (superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), glutathione S-transferase (GST), plastid terminal oxidase 2 (PTOX) and thioredoxin (TRX)) as well as biochemical alterations. The results showed significant dose-related effects of TCS on the algal species in terms of growth and chlorophyll content. Malondialdehyde (MDA) increased with increasing TCS concentrations and showed significant difference between the treatment of 405.3 μg/L TCS and control group. Transcription analysis revealed that the expression of SOD mRNA was most sensitive to TCS among the selected genes. In addition, Fourier-transform infrared spectroscopy showed time- and concentration-specific biochemical responses in *C. reinhardtii* when exposed to TCS. The biochemical alterations associated with different doses of TCS were mainly attributed to structural changes associated with lipid, protein, nucleic acid and carbohydrate. The findings from this study reveal that TCS in the aquatic environment may affect algal growth, chlorophyll synthesis, oxidative stress responses and cause biochemical alterations. This study provided important information to achieve a better understanding of the toxic mechanism of triclosan on algae *Chlamydomonas reinhardtii*.

1. Introduction

Triclosan (TCS) as a broad-spectrum antibacterial agent is widely used in many personal care products (Dann and Hontela, 2011), including soaps, deodorants, household cleaners, dental care products, skin care creams and textiles. The concentration of TCS in these products is between 0.1% and 0.3% by weight (Miller et al., 2008; Sabaliunas et al., 2003; Singer et al., 2002). Owing to its wide use and incomplete removal during wastewater treatment process, TCS could be easily found in wastewater treatment plant effluents and receiving environments (Baalbaki et al., 2016; Halden and Paull, 2005; Ying and Kookana, 2007). TCS has been frequently reported in surface water (Chen et al., 2014; Peng et al., 2008; Ramaswamy et al., 2011), sediments (Liu et al., 2015; Peng et al., 2017) and biota samples (Bennett et al., 2009; Foltz et al., 2014; Riva et al., 2012). TCS was found in the range of 3800–16600 ng/L, and 200–2700 ng/L in wastewater influents and effluents, respectively (McAvoy et al., 2002). It has also been detected at concentrations up to 1023 ng/L in surface water (Peng et al., 2008), and up to 403 ng/g dry weight (dw) in river sediment of the Pearl River, South China (Chen et al., 2014). Furthermore, TCS was also reported in human milk at concentrations of 0.07–300 ng/g lipid weight (Adolfsson-Erici et al., 2002).

In aquatic ecosystems, TCS with its $\log K_{ow}$ value of 4.8 has the potential to bioaccumulate via food chain and to cause adverse effects on aquatic non-target organisms (Balmer et al., 2004; Dann and Hontela, 2011). TCS has showed reproductive and developmental adverse effects to fish (Dann and Hontela, 2011; Ishibashi et al., 2004; Schnitzler et al., 2016). It has been demonstrated that TCS has potential weak androgenic effects (Foran et al., 2000), genotoxic and cytotoxic effects on Zebra mussel hemocytes (Binelli et al., 2009a, 2009b). TCS is

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also highly toxic to the asexual reproduction of algae *Closterium ehrenbergii* with an EC_{50} of 0.62 mg/L, and even causes genotoxic effects on *C. ehrenbergii* (Ciniglia et al., 2005). In addition, freshwater microalga *Pseudokirchneriella subcapitata* was rather sensitive to TCS with a no-observed-effect concentration (NOEC) value of 200 ng/L and lowest observed effect concentration (LOEC) value of 400 ng/L (Yang et al., 2008). However, information on the toxicological mechanism of TCS on aquatic organisms like algae is still lacking.

Algae are the primary producer of aquatic ecosystem, playing an important role in maintaining the structure and function of the aquatic ecosystem. Thus, algae have been widely used as ecotoxicological assessment species to indicate aquatic environmental quality (Delahaye et al., 2005; Fabricius et al., 2012; Yokoyama and Ishihi, 2010). The green freshwater alga *Chlamydomonas reinhardtii* is a motile and unicellular green algae commonly found in fresh water environment. It is sensitive to environmental perturbations and ubiquitously distributed (Jamers et al., 2013). Additionally, the entire genome of this organism has been sequenced as part of the Chlamydomonas Genome Project (http://www.chlamy.org/). This microalga is also highly sensitive to a wide range of chemicals such as metals, nanomaterials and organic chemicals with quantifiable responses at the genomic and cellular levels (Jamers et al., 2013; Sanchez et al., 2015; Wang et al., 2008). Previous studies revealed that TCS could pose potential toxic effects to aquatic and terrestrial biota (Hanioka et al., 1996; Schnitzler et al., 2016; Wang et al., 2014; Yang et al., 2008). However, the potential toxic effects to the green alga *C. reinhardtii* are mainly focused on traditional toxicity, such as growth inhibition and enzyme responses. To the best of our knowledge, no investigation has been conducted at the molecular level to evaluate its effects on *C. reinhardtii* in terms of transcription of oxidative-related genes and biochemical alterations.

The objective of this study was to investigate the toxicity of TCS exposure to freshwater microalgae *C. reinhardtii* at the molecular level. The endpoints assessed include algal growth, lipid peroxidation, transcription change of antioxidant-related genes (SOD, GPX, CAT, GST, PTOX and TRX) and biochemical alterations. The emphasis was to get insights into molecular responses by qRT-PCR and into biochemical alterations by Fourier transform infrared (FTIR) spectroscopy. The results of this study can provide the basis for assessing ecological toxicological effects of TCS and its potential ecological risk in the aquatic environment.

2. Materials and methods

2.1. Algal strain and culture medium

The test freshwater green alga *Chlamydomonas reinhardtii* (No. FACHB-479) was purchased from Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). Selenite Enrichment (SE) growth medium was used to culture *C. reinhardtii*. The growth media contained the following chemicals: $NaNO_3$, 0.25 g/L; K_2HPO_4, 0.075 g/L; $MgSO_4{\cdot}7H_2O$, 0.075 g/L; $CaCl_2{\cdot}2H_2O$, 0.025 g/L; KH_2PO_4, 0.175 g/L; NaCl, 0.025 g/L; $FeCl_3{\cdot}6H_2O$, 0.0005 g/L; and EDTA-Fe, 1 mL/L. A5 trace mental solution (1 mL/L) included : H_3BO_3, 2.86 g/L; $MnCl_2{\cdot}4H_2O$, 1.86 g/L; $ZnSO_4{\cdot}7H_2O$, 0.22 g/L; $Na_2MoO_4{\cdot}2H_2O$, 0.39 g/L; $CuSO_4{\cdot}5H_2O$, 0.08 g/L; $Co(NO_3)_2{\cdot}6H_2O$, 0.05 g/L, and soil extraction liquid, 40 mL/L.

2.2. Experimental design

Triclosan was purchased from Dr. Ehrenstorfer GmbH (Augsburg, German). The stock solution was prepared in dimethyl sulphoxide (DMSO) at a concentration of 1000 mg/L and spiked into the growth medium to obtain different working concentrations: 0 μg/L, 20 μg/L, 100 μg/L and 500 μg/L. The toxicity of DMSO to algal cells was checked and no observed effect concentration of DMSO was determined to be 0.20% (v/v) from the preliminary experiment. The final concentration of DMSO in the experiment was set as 0.10% (v/v) for all experimental media. The low concentration of 20 μg/L simulates the concentration of TCS present in raw sewage. If the wastewater lacks treatment, this concentration could potentially be discharged to the aquatic environment and affect the *Chlamydomonas reinhardtii*. Thus, this concentration has a direct environmental relevance. The high concentration (100 μg/L and 500 μg/L) that will most likely never be present in the riverine aquatic environment; these high concentrations were chosen, however, in an attempt to better understand whether *Chlamydomonas reinhardtii* become stressed after exposure to TCS and how the responses differed between different concentrations.

Algal cells at the exponential growth phase were inoculated into the growth medium spiked with different TCS concentrations, with the initial algal densities of *C. reinhardtii* being 2.35×10^5 cells/mL. Culturing was performed in 250-mL Erlenmeyer flasks containing 100 mL culture volume at 150 rpm and 25 ± 1 °C. Light was provided by continuous cool white fluorescent lamps at 3000 lx with a dark/light cycle of 14 h: 10 h. The experiments were conducted for 96 h, and all tests were carried out in triplicate under strict axenic conditions. Controls were performed with TCS spiked growth medium without algae, which were used to measure the abiotic loss of TCS during the exposure period.

2.3. Determination of cell density and chlorophyll content

Optical density (OD) of the algae cultures was measured daily at 680 nm as the cell density indicator using a BMG microplate reader (BMG Lab technologies, Offenburg, Germany). The cell density was determined using a haemocytometer under a light microscope. The linear relationship between algal density (cells/mL) and OD_{680} was shown in the following equation:

$$OD_{680} = 2 \times 10^{-7}\text{cell density} - 00.047 (R^2 = 0.9836)$$

For chlorophyll content, algae samples were placed into a 2-mL centrifuge tube, then centrifuged at 3500*g* to separate the algal cells and extracted with 2 mL 95% (v/v) ethanol for 24 h in darkness at 4 °C. Absorbance values at 665 nm and 650 nm were measured using a microplate reader (BMG Lab technologies, Offenburg, Germany), and the chlorophyll content was obtained according to the following equation (Yoshida et al., 2004):

$$\text{Chlorophyll content (mg/ml)} = 0.0255A_{650} + 0.004A_{665}$$

2.4. Determination of TCS concentrations in growth media

Culture solution (1.5 mL) was sampled daily from each Erlenmeyer flask (0 h, 24 h, 48 h, 72 h, and 96 h) and centrifuged at 9168*g* for 5 min to remove the algal cells. The concentration of TCS in supernatant was analyzed using Agilent 1200 liquid chromatograph equipped with a diode array detector (HPLC-DAD) (Santa Clara, CA, USA). A Zorbax Eclipse XDB-C18 (4.6 ×150 mm, 5 μm, Agilent) was used for the analysis of the target compound, with the oven temperature of 40 °C. The mobile phase was consisted of 70% of acetonitrile and 30% water at a constant flow rate of 1 mL/min. The injection volume was 50 μL and the UV wavelength used for detection was 205.4 nm. The method limit of quantification (MLOQ) was 5 μg/L.

2.5. Lipid peroxidation

Levels of lipid peroxidation products were measured according to the manufacturer's protocol (Nanjing Jiancheng Bioengineering Institute, China). In brief, 10 mL of sample was harvested from each flask, centrifuged and then followed by cell disruption using ultrasonication. Then the homogenate was reacted with thiobarbituric acid (TBA) and then centrifuged, the supernatant was analyzed for malondialdehyde (MDA) at 532 nm using a microplate reader.