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Removal, biotransformation and toxicity variations of climbazole by freshwater algae *Scenedesmus obliquus*☆

Chang-Gui Pan [a, *], Feng-Jiao Peng [b], Guang-Guo Ying [c, **]

[a] School of Marine Sciences, Guangxi University, Nanning, 530004, China
[b] Department of Aquatic Ecology and Water Quality Management, Wageningen University, P.O. Box 47, 6700, AA Wageningen, The Netherlands
[c] The Environmental Research Institute, MOE Key Laboratory of Environmental Theoretical Chemistry, South China Normal University, Guangzhou, 510006, China

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ABSTRACT

Climbazole (CBZ) is an antibacterial and antifungal agent widely used in personal care products. In this study, we investigated the interactions between climbazole (CBZ) and freshwater microalgae *Scenedesmus obliquus* (*S. obliquus*). Dose-effect relationships between CBZ concentrations and growth inhibitions or chlorophyll a content were observed. After 12 days of incubation, the algae density and chlorophyll a content in 2 mg/L treatment group was 56.6% and 15.8% of those in the control group, respectively. Biotransformation was the predominant way to remove CBZ in the culture solution, whereas the contribution of bioaccumulation and bioadsorption were negligible. More than 88% of CBZ was removed by *S. obliquus* across all treatments after 12 days of incubation, and the biotransformation of CBZ followed the first order kinetic model with half-lives of approximately 4.5 days at different treatments. CBZ-alcohol (CBZ-OH) was the only biotransformation product identified in algal solution. Moreover, the toxicity of biotransformation products was much lower than its corresponding precursor compound (CBZ). The results of this study revealed that *S. obliquus* might have a great impact on the environmental fates of CBZ and could be further applied to remove organic pollutants in aquatic environment.

1. Introduction

Climbazole (CBZ) is an imidazole fungicide substance that is widely used in the formulation of personal care products such as shower gel, toothpaste, conditioner, hair tonics and shampoo as an antidandruff and antimycotic preservative (Asrar et al., 2006; Ramachandran et al., 1999; Rother et al., 2001). For example, CBZ is used in cosmetics up to a maximum content of 2.0%, equivalent to approximately 15 g/L in antidandruff shampoos (SCCP, 2009) and the estimated annual consumptions of CBZ in European Union and China were up to 100–1000 tons and 3800 tons, respectively (Gouin et al., 2012; Pérez-Rivera et al., 2009). However, CBZ cannot be efficiently removed in the conventional wastewater treatment plants (WWTPs) with its aqueous removal rates of 34–76% (Chen et al., 2013; Wick et al., 2010). Therefore, the remaining CBZ is eventually released into aquatic environment, resulting in detection of CBZ at different levels in a variety of media, including WWTPs (Liu et al., 2017; Qi et al., 2015), surface water (Liu et al., 2015; Zhang et al., 2015), and sediment (Chen et al., 2014). Recently, CBZ has been found up to 0.6 μg/L, 1.4 μg/L and 1.2 μg/g in treated wastewater, raw wastewater and activated sludge, respectively (Qi et al., 2015; Wick et al., 2010). These indicated the limited dissipation ability or biodegradability of CBZ in conventional WWTPs and natural environment (Chen et al., 2013, 2015; Liu et al., 2015). For instance, the screening tests listed on ECHAs registered substances database for CBZ indicate not readily biodegradable (ECHA, 2016).

Compared with other imidazole chemicals, CBZ has stronger aliphatic and aromatic properties, which was expected to induce hepatic enzyme production (Kobayashi et al., 2012). The toxicity of CBZ in aqueous solutions towards a variety of organisms including algae, crustaceans, fish and plants has been studied (Richter et al., 2013; SCCP, 2009). Overall, algae were found to be the most

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* Corresponding author.
** Corresponding author.
E-mail addresses: panchanggui@gxu.edu.cn, panchanggui@hotmail.com (C.-G. Pan), guang-guo.ying@gig.ac.cn (G.-G. Ying).

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sensitive species (SCCP, 2009). For example, CBZ can inhibit the growth of *Pseudokirchneriella subcapitata* and *Navicula pelliculosa* at low concentrations, with median effective concentration (EC_{50}) of 87 μg/L and 291 μg/L, respectively (SCCP, 2009; Richter et al., 2013). CBZ is therefore considered to be very toxic to aquatic organisms (Richter et al., 2013).

Algae are ubiquitous as the primary producer with abundant biomass in aquatic ecosystems (Cooganet al., 2007). Previous literature demonstrated that algae can remove organic contaminants, such as nonylphenol, progesterone and norgestrel through bioadsorption, bioaccumulation as well as biodegradation process (Gao and Tam, 2011; Peng et al., 2014a, 2014b; Warshawsky et al., 1988; Xiong et al., 2016, 2017; Zhou et al., 2012). Among the above three processes, the biodegradation has been proven to be the most effective way to eliminate organic contaminants from the aqueous phase due to the complex enzyme system that comprised of phase I (e.g., cytochrome P450) and phase II enzyme families (Xiong et al., 2018). Generally, the cytochrome P450 metabolizes hydrophilic compound to more hydrophilic compound by adding or unmasking a hydroxyl group and usually involves hydrolysis, oxidation, or reduction reactions (Omura, 1999). Phase II enzymes catalyze the conjugation reaction between electrophilic compounds and glutathione (Xiong et al., 2018). Algae can also absorb contaminants as a substrate for bioaccumulation and biomagnification in the food web (Hong et al., 2008; Nelson et al., 1998). The eukaryotic green alga *S. obliquus* is widely distributed in freshwater and plays an important role in aquatic ecosystem (Zhou et al., 2013). *S. obliquus* grows fast and can remove organic compounds such as nonylphenol (NP) and octylphenol (OP) (Zhou et al., 2013). However, we do not know whether this alga is able to degrade other contaminants such as CBZ classified to be toxic to aquatic organisms, especially algae. This information is helpful to understand the environmental fate and behavior of CBZ.

Although a few studies have reported the toxicological effects of CBZ on microalgae species (Richter et al., 2013; SCCP, 2009), the removal mechanisms and biotransformation behavior of CBZ by microalgae is still unclear. Furthermore, the biotransformation products may be more persistent, toxic and bioaccumulative than CBZ. Therefore, it is necessary to know about the biotransformation products and their toxicity, which will help us understand the environmental fate, transportation and the potential risk of CBZ in aquatic environment.

The purpose of this study was to reveal the removal mechanism of CBZ by environmentally ubiquitous freshwater microalgae *S. obliquus* and to investigate the biotransformation products of CBZ. Moreover, we also evaluated the toxicity variation of CBZ during the biotransformation process.

2. Materials and methods

2.1. Chemicals and materials

CBZ (CAS: 38083-17-9) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, German). The stock solution of CBZ was prepared in methanol at a concentration of 1000 mg/L and stored at −18 °C. Mineral salts (analytical grade) were obtained from Tianjin (China) and used for preparation of algal culturing solution. Formic acid (HPLC grade, purity ≥ 98%) was supplied by Tedia Company (Tedia, USA). All the organic solvents (i.e., acetonitrile, methanol, methylene dichloride, hexane and ethyl acetate) used were HPLC grade and purchased from Merck Corporation (Shanghai, China) or CNW Technologies (Dusseldorf, Germany). Ultrapure water was produced by a Milli-Q apparatus from Millipore (Watford, UK). All glassware was hand-washed with tap water, Milli-Q water, rinsed with methanol, and baked at 450 °C for more than 4 h before use.

2.2. Algal strain and culture medium

S. obliquus (FACHB-12) was purchased from Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). BG11 medium was used as the culturing solution for the *S. obliquus*. The chemical components of BG11 can be found in a previous study (Rippka et al., 1979). The algal culturing was performed in 150-mL Erlenmeyer flasks containing 100 mL culturing solution at 25 °C under the constant shake of 150 rpm in a SKY-211BG rocking incubator (Sukun, Shanghai, China). Light was provided by continuous cool white fluorescent lamps at 3000 lux with a dark/light cycle of 12h: 12h. Algal cells used for the experiment were collected by centrifuging the culturing solution at the exponential growth phase, pellets were then harvested, washed three times with Milli-Q water and re-suspended in Milli-Q water.

2.3. Experimental setup

The experiments were conducted under strict sterile conditions. The stock solution of CBZ was spiked into the sterilized culturing solution to obtain different working concentration of 0.25, 0.5, 1.0 and 2.0 mg/L in super-clean bench. Both solvent control and water control were included by replacing CBZ stock solution with the same volume of methanol and Milli-Q water, respectively. The toxicity of methanol to algal cells was checked and the no observed effect concentration (NOEC) of methanol was determined to be 0.50% (v/v) in the preliminary experiment based on growth inhibition. The final concentration of methanol was set as 0.10% (v/v). Algal cells at the exponential growth phase were inoculated into the culturing solution with the initial algal density of approximately 10^5 cells/mL. The experiments ran 12 days with constant shaking at 150 rpm. Three controls were used for each treatment: culturing solution with live algae without CBZ to assess chemical effect on algal growth; culturing solution with CBZ without algae to measure abiotic losses of the CBZ; culturing solution with CBZ and dead algae to measure bioadsorption of the CBZ onto algae. Dead algae were acquired by high temperature sterilization (85 °C, 20 min) (Majidi et al., 1990). All tests were performed in triplicate.

The optical density at 680 nm (OD_{680}), chlorophyll a content, and concentrations of CBZ in the aqueous culturing solutions and algal cells were monitored at different time intervals (0 d, 2 d, 4 d, 6 d, 8 d, 10 d and 12 d).

2.4. Determination of optical density and chlorophyll a content

To determine the algal density at the start of incubation, the cell density was determined using a haemocytometer under a light microscope. To evaluate the effect of CBZ on the algal growth, the optical density was measured at 680 nm (OD_{680}) using a BMG microplate reader (BMG Lab technologies, Offenburg, Germany). A linear relationship was observed between algal density (cells/mL) and OD_{680} and yielded an equation for the OD_{680}:

$$OD_{680} = 7 \times 10^{-8} \text{ cell density} + 0.0103 \ (R^2 = 0.9954)$$

To determine chlorophyll a content, 1.0 mL of algal solution was sampled from each treatment and added into a 2-mL centrifuge tube, centrifuged at 9168 g for 5 min. The separated algae cells were then extracted with 2 mL 95% (v/v) ethanol for 24 h in the dark. Absorbance at 665 nm and 649 nm were measured using a microplate reader, and the chlorophyll a content was calculated according to the following equation (Zhou et al., 2013):

$$\text{Chlorophyll a} = 13.95A_{665}\text{-}6.88A_{649}$$