Physiological and biochemical effects of triclocarban stress on freshwater algae

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Abstract

Triclocarban (TCC) is considered as a contaminant of emerging concern (CEC) and ranked in the top 10 CEC occurrence. TCC is a high-production-volume synthetic chemical used extensively in various personal care products. The heavy use and emission of TCC pose a serious threat to the environment. To evaluate its ecotoxicity, three freshwater algae were exposed to TCC at concentrations ranging from 0.500 to 16,000 mg/L to determine its growth inhibitory effects. Chlorophyll, malondialdehyde (MDA), antioxidant enzyme activities, and other indicators were used to analyze the mechanism underlying the toxic effect of TCC. With the 96-h median effective concentrations (EC_{50}) of TCC ranging from 5.445 to 10.220 mg/L, the three types of algae exhibited obvious toxic responses, of which *Chlorella vulgaris* was the most sensitive. The calculated 96-h EC_{50} values of TCC were 8.474 mg/L, 9.100 mg/L, and 8.760 mg/L for *C. vulgaris*, *Scenedesmus obliquus*, and *Chlorella pyrenoidosa*, respectively. TCC decreased the chlorophyll content, increased the MDA content, decreased the peroxidase activity, and decreased superoxide dismutase activity in the three algae, indicating that the chemical can inhibit photosynthesis and destroy the activities of antioxidant enzymes in algae. Most of the indicators showed a positive correlation with the concentration of TCC.

Keywords Triclocarban · Green algae · Chlorophyll · Peroxidase · Superoxide dismutase

1 Introduction

The antimicrobial agents triclocarban (3,4,4'-trichlorocarbanilide; TCC) is high-production-volume chemicals that are widely used in a variety of personal care products (PCPs) [1]. Its mass production and daily application have continued for more than 30 years, and a conservative estimate indicates that the world's largest annual usage is over 750×10^3 t [2]. Currently, TCC is among the top 10 most commonly detected organic wastewater compounds for frequency and concentration in the whole world [3]. It has been detected in wastewater effluent in quantities up to micromolar concentrations ($\mu\text{g/L}$) in Asia, Europe, and North America, indicating widespread environmental contamination. This substance is highly lipophilic, can accumulate in various organisms, has been used for a long

time, and poses potential threats to the environment and organisms. Although the detection of the instrument can quantify TCC concentration, it cannot measure directly the influences on test organisms. Due to the persistence and toxicity of TCC, its aquatic ecotoxicity has attracted wide attention [4]. In recent years, its toxic effects, such as inhibitory effects on growth and development, endocrine disruption, and reproductive toxicity, have caused great concerns [5–11]. Several studies provided evidence that TCC interferes with biological receptors in vitro, including a stably transfected aryl hydrocarbon receptor (AhR)-, androgen receptor (AR)-, or estrogen receptor (ER)-responsive firefly luciferase reporter gene that responds to chemicals that can bind to and/or activate the respective receptor [12]. Moreover, TCC could also be a significant

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concern when it coexists with TCS, whose hazard quotient was reported to be over 10 by Brausch and Rand [13].

Algae are at the bottom of the food chain in aquatic ecosystems. These organisms are sensitive to environmental pollution and are also an important segment in the accumulation of toxic substances, which can advance to consumers through the food chain. They are routinely employed as test organisms for ecotoxicological studies. Normally, the molecular, cellular, and physiological levels of algae change dramatically when they are under contamination stress. The examination of 139 water sources in 30 US states by the US Geological Survey [14] and that of the Zhujiang River, Yangtze River, Yellow River, and other water systems in China indicated that TCC was a highly accumulated pollutant. The maximum detected concentration in water is 338 ng/L that in the sediment reaches 2.723 µg/g, and the concentrations in water samples from wastewater treatment plants are as high as 0.4–50 µg/L [14–16]. TCC can also be detected in organisms, at levels ranging from 50 to 400 µg/kg in *Cladophora*, 50–300 µg/kg in freshwater snail, 14,000–80,000 µg/kg in bream bile, 0.75–10 µg/kg in pelagic fish plasma, 9.0 µg/kg in killer whale plasma, 0.4–38.0 ng/g in the plasma of lactating mothers, and 0.022–0.95 ng/g in the breast milk [17, 18]. Therefore, the ecological toxicity of TCC requires attention.

This study aimed to treat three typical freshwater algae with different concentrations of TCC and then analyze the growth inhibition, photosynthesis, and antioxidant capacity of algae to evaluate the possible toxic effect of TCC on algae and its underlying mechanism to lay a foundation for the control of environmental pollution by TCC and for biological toxicology research.

2 Materials and methods

2.1 Chemicals

The TCC standard was obtained from J&K Scientific Ltd (Beijing, China) with a purity of 97%. The HPLC-grade reagents including methanol, acetonitrile, ethyl acetate, hexane, dichloromethane, formic acid, and acetic acid were supplied by Merck (Germany), CNW Technologies (China), and Tedia (USA). Glass fiber filters (GF/F, pore size 0.7 µm) were obtained from Whatman (Maidstone, UK). Oasis HLB cartridges (200 mg, 6 mL) were from Waters (Milford, USA). HPLC-grade water was from a Milli-Q water purification system (Millipore, Watford).

2.2 Test organisms

To create a representative subtropical community, we used three species of algae: *Chlorella vulgaris*, *Scenedesmus*

obliquus, and *Chlorella pyrenoidosa*, which were provided by the Institute of Hydrobiology, Chinese Academy of Sciences. The algae were laboratory cultured in BG11 medium at 23 ± 1 °C and a light intensity of 4000 lux (provided by 40 W white fluorescent lamps, Philips F40 T12/DX) with a dark/light cycle of 14 h/10 h. The preparation of BG11 medium can be found in a previous study [19]. The biomass was measured by microscopic counting and optical density (OD) at 690 nm at 24-h intervals during 120 h of culture. Growth curves were plotted to establish the relationship between the cell density and OD₆₉₀ values for the different algae using a linear regression equation.

2.3 Toxic test of TCC on algae

2.3.1 Exposure strategies

In the exposure experiment, the algae at the logarithmic growth phase were collected, washed, and then diluted by freshwater to the initial concentration of 2×10^5 cells mL⁻¹. The three species of algae were cultured with the final concentrations of TCC of 0.5, 1, 2, 4, 8, and 16 mg/L. After 96 h of culture, the biomass, chlorophyll, malondialdehyde (MDA), peroxidase (POD), superoxide dismutase (SOD), and other indicators were measured to evaluate the corresponding toxic effects, with the biomass analyzed once every 24 h [20]. Three replicates were set for each concentration group.

2.3.2 Growth inhibition of algae and calculation of the 96-h median effective concentration (EC₅₀)

OD was measured every 24 h of exposure, and algal growth curves were generated based on OD values. After establishing the linear regression relationship between the inhibition rate (*P*%) and the natural logarithm of the concentration, the concentration value of 50% (EC₅₀) was determined. The inhibition ratio was calculated as follows:

$$P(\%) = (\text{control OD}_{690} - \text{treated group OD}_{690}) / \text{control OD}_{690}.$$

2.3.3 Determination of the chlorophyll content in algae

The algae were collected and filtered through a microporous membrane, and 7 mL of ethanol was added. The contents of the flask were transferred to a centrifuge tube, and the volume was adjusted to 10 mL. The biomass was extracted in the dark at 4 °C for 4 h, and the extract was centrifuged at 3000 rpm for 20 min. The supernatant was transferred to a colorimetric tube, and the volume was adjusted to 10 mL by adding 90% ethanol. OD values of

the supernatants were measured in a 1-cm cuvette, and the chlorophyll-*a* concentration was calculated as follows:

$$\text{Chlorophyll } a(\text{mg/L}) = (11.64 A_{633} + 2.16 A_{645} + 0.10 A_{630}) V_1 / 1000 V_2,$$

where V_1 is the volume of the extract (mL) and V_2 is the volume of the filtrate (L).

2.3.4 Determination of the algal MDA content

The algal fluid was filtered through a membrane and then mixed with 10% tricarboxylic acid. The homogenate was centrifuged at 12,000 rpm for 10 min to collect the supernatant as the MDA extract, of which 2 mL was transferred to a test tube, mixed with 2 mL of 0.6% thiobarbituric acid, and heated in a boiling water bath for 10 min. After centrifugation at 4500 rpm for 10 min, OD of the supernatant was measured, and the MDA concentration was calculated as follows:

$$\text{MDA concentration} = 6.45 (A_{532} - A_{600}) - 0.56 \times A_{450}.$$

2.3.5 Determination of algal SOD and POD activities

SOD activity was assayed at 560 nm by the nitro-blue-tetrazolium (NBT) photoreduction method [21]. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction in NBT as monitored at 560 nm by a spectrophotometer (722-type, Shanghai Instrument Factory, China). Scavenging of O_2^- by SOD prevents the accumulation of intermediates, which is used for the determination of the enzyme activity. POD activity was determined at 470 nm using the guaiacol method [22]. The reaction mixture (3 mL) contained 50 mM PBS (pH 5.5), 20 mM guaiacol, 1 mL 30% (w/v) H_2O_2 , and 0.1 mL enzyme extract.

2.4 Statistical methods

All experiments were performed at least in triplicate. The quantitative data were expressed as mean and standard deviation/standard error. If the homoscedasticity assumptions of the data were satisfied, least significant difference (LSD) tests were further conducted to analyze the statistical significance among individual treatments using SPSS v25 (IBM, Armonk, NK, USA). $P < 0.05$ was considered statistically significant, and $P < 0.01$ was considered highly significant.

3 Results

3.1 Establishment of a spectrophotometric method for algal biomass

As shown in Fig. 1, there was a good linear relationship between the biomass and OD values for the three algae. The regression equations established for the three algae were as follows: *C. pyrenoidosa*: $y = 0.0164x - 0.0299$ ($R^2 = 0.9905$); *C. vulgaris*: $y = 0.0172x - 0.04$ ($R^2 = 0.9894$); and *S. obliquus*: $y = 0.0168x - 0.0271$ ($R^2 = 0.9966$).

3.2 Inhibitory effects of TCC on the growth of the three species of algae

The rates of algal growth inhibition by TCC were shown in Fig. 2. Within the test range, the inhibition rates of the three algae by TCC all showed a positive correlation with the TCC concentrations; the calculated 96-h EC_{50} value of TCC for *C. vulgaris* was 8.474 mg/L, that for *S. obliquus* was 9.11 mg/L, and that for *C. pyrenoidosa* was 8.76 mg/L. Among the three species of algae, *C. vulgaris* was the most sensitive to TCC.

Fig. 1 Relationship between the biomass and OD values for the three algae (left: *C. pyrenoidosa*; middle: *C. vulgaris*; right: *S. obliquus*)

Fig. 2 Inhibitory curves of different concentrations of TCC for the three algae (left: *C. pyrenoidosa*; middle: *C. vulgaris*; right: *S. obliquus*)

Fig. 3 Effects of different concentrations of TCC on the chlorophyll content in the three algae

Fig. 4 Effects of different concentrations of TCC on the MDA content in the three algae

3.3 Effects of TCC on the chlorophyll content in the three algae

TCC caused significant decreases in the chlorophyll-*a* content in the three algae (shown in Fig. 3), and the extent of the decrease was positively correlated with the exposure concentration. The decreases in the chlorophyll-*a* content were stronger than those in biomass, indicating that they were caused not only by the decreases in biomass but also by a targeted toxic effect of TCC on the photosynthesis in algae. Among the three algae, *S. obliquus* showed the best TCC tolerance and *C. pyrenoidosa* was the most sensitive.

3.4 Effects of TCC on the MDA content in the three algae

TCC exposure elevated the MDA levels in all three algae, indicating the occurrence of stress (shown in Fig. 4). Except that in *C. vulgaris*, the MDA level was basically positively

correlated with the TCC concentration. The highest effect was caused not by the highest concentration but was observed at 8 mg/L TCC, showing that the stress resistance in algae is complicated.

MDA is the final decomposition product of membrane lipid peroxidation, and its content reflects the degree of stress damage to algae. The TCC concentrations in the range tested in this study caused an increase in the MDA content, indicating that the algae experienced stress, leading to cell membrane damage and increased cell membrane permeability. Using the TCC effect on *S. obliquus* as an example, the MDA content in the control group was 0.068 µmol/g, and that in the treated groups was 0.060, 0.093, 0.105, 0.125, 0.154, and 0.141 µmol/g, which was up to 2.35 times of that in the control group.

Fig. 5 Effects of different concentrations of TCC on the POD activity in the three algae

Fig. 6 Effects of different concentrations of TCC on the SOD activity in the three algae

3.5 Effects of TCC on the POD activity in the three algae

As shown in Fig. 5, TCC caused significant decreases in the POD activity in the three algae, and the degree of inhibition was positively correlated with the TCC concentration. The differences among the three algae were not significant, and their sensitivities were relatively similar.

3.6 Effects of TCC on the SOD activity in the three algae

TCC caused significant decreases in the SOD activity in the three algae (shown in Fig. 6), and the degree of inhibition was positively correlated with the TCC concentration. The maximum TCC concentration reduced the SOD activity to

approximately 1/4 of the normal level, indicating significant damage to SOD activity by TCC.

POD is the enzyme responsible for the decomposition of peroxides, while SOD is the enzyme responsible for scavenging oxygen free radicals, and both are key enzymes allowing algae to resist oxidative damage and remove metabolic wastes, which is very important for the growth and metabolism of algae. TCC was shown to significantly damage both activities. Taking the effect of TCC on the POD activity in *C. pyrenoidosa* as an example, the activity in the control group was 32 mg/g/min and that in the exposed groups was 29, 25, 20, 16, 13, and 9 mg/g/min. The lowest activity in the exposed groups was 28.13% of that in the control group. Taking the effect of TCC on the SOD activity in *C. pyrenoidosa* as an example, the activity in the control group was 178 U/g-fresh weight (Fw) and that in the exposed groups was 160, 143, 118, 88, 56, and 27 U/g Fw. The lowest activity in the exposed groups was 15.17% of that in the control group.

4 Discussion

The effects of TCC differed among the organisms that were introduced to the microcosms. In these microcosms, both phytoplanktonic and periphytonic chlorophyll-*a* content decreased as the concentrations of TCC in the sediment increased. Algae are known to be particularly sensitive to TCC, with growth inhibition typically being recorded at EC_{50} values of 0.53 $\mu\text{g/L}$ –430 $\mu\text{g/L}$, depending on the test conditions and species [23, 24]. In this study, TCC caused a significant decrease in the chlorophyll content in the algae. The 96-h EC_{50} values of TCC for *C. pyrenoidosa*, *C. vulgaris*, and *S. obliquus* were between 5.445 and 10.22 mg/L. Although the TCC content in most of the current water systems has not yet reached the 96-h EC_{50} values for the algae, the lowest-concentration groups of the algae (0.5 mg/L) also showed some toxicity effects, indicating that the monitoring and control of TCC should be strengthened.

Among the indexes analyzed in this study, chlorophyll was the executor of photosynthesis, and its content directly reflects the growth and reproduction ability of algae. Taking the effect of TCC on *C. vulgaris* as an example, the concentration of chlorophyll-*a* in the control group was 0.764 $\mu\text{g/L}$, while that in each group was 0.623, 0.496, 0.391, 0.295, 0.227, and 0.130 $\mu\text{g/L}$, respectively. The lowest group was only 20.86% of that in the control group, the effect was very obvious, and the experiment of other algae showed similar characteristics.

TCC and its transformation products (4,4'-dichlorocarbide (DCC), 1-(3-chlorophenyl)-3-phenylurea (MCC) and carbanilide (NCC), 2'-OH-TCC, 3'-OH-TCC) were detected in

the environmental matrices. TCC is an antimicrobial agent and also emerging endocrine disruptor that can cause immune dysfunction and affect human reproductive outcomes. Furthermore, TCC alters the expression of proteins related to binding and metabolism, skeletal muscle development and function, and nervous system development and immune response. TCC has potential health risks in wildlife and humans. Several animal studies illustrate that it can cause various adverse effects, which can be monitored by antioxidant biomarkers (CAT, GST, and LPO) [25]. The first mechanism is a possible increase in intracellular oxidative stress, which could arise from a pathological increase in the production of oxyradicals (mainly H_2O_2 , which is produced in mitochondria by dismutation of O_2^-) through the cellular mitochondrial enzymatic complex chain [26]. Alternatively, TCC may act together as a DNA adduct and/or DNA intercalant to directly exert genotoxic effects [27].

Numerous uncertainties remain including the evaluation of antioxidant enzymes, the possible interference with hormone metabolism, and the role played by the metabolites of TCC. It is necessary to fully understand the biological toxic effect of TCC on this sentinel organism. As these questions continue to be addressed, more definitive and comprehensive aquatic risk assessments should be developed to raise greater public awareness about the potential risk of TCC released into aquatic environments.

5 Conclusions

TCC showed a significant acute toxicity to the freshwater algae, significantly inhibiting their growth, destroying their photosynthetic ability, and impairing their antioxidant function. Because this study was a short-term exposure study, taking into account a long-term cumulative effect of TCC in the environment, it is necessary that TCC should be listed as an important environmental pollutant of great concern, and its content should be tested in each major river system. Furthermore, this paper provides the basic information regarding the physiological and biochemical effects of TCC stress on freshwater alga, and further systematic toxicology research should be carried out on model organisms at all levels to understand toxicity mechanisms.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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