Biouptake, toxicity and biotransformation of triclosan in diatom *Cymbella* sp. and the influence of humic acid

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ABSTRACT

Triclosan is one of the most frequently detected emerging contaminants in aquatic environment. In this study, we investigated the biouptake, toxicity and biotransformation of triclosan in freshwater algae *Cymbella* sp. The influence of humic acid, as a representative of dissolved organic matter, was also explored. Results from this study showed that triclosan was toxic to *Cymbella* sp. with 72 h EC50 of 324.9 μg L−1. Humic acid significantly reduced the toxicity and accumulation of triclosan in *Cymbella* sp. SEM analysis showed that *Cymbella* sp. were enormously damaged under 1 mg L−1 triclosan exposure and repaired after the addition of 20 mg L−1 humic acid. Triclosan can be significantly taken up by *Cymbella* sp. The toxicity of triclosan is related to bioaccumulated triclosan as the algal cell numbers decreased when intracellular triclosan increased. A total of 11 metabolites were identified in diatom cells and degradation pathways are proposed. Hydroxylation, methylation, dechlorination, amino acids conjunction and glucuronidation contributed to the transformative reactions of triclosan in *Cymbella* sp., producing biologically active products (e.g., methyl triclosan) and conjugation products (e.g., glucuronide or oxaloacetic acid conjugated triclosan), which may be included in the detoxification mechanism of triclosan.

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1. Introduction

Triclosan (TCS), an antimicrobial agent, is one of the most widely used pharmaceuticals and personal care products and frequently detected emerging contaminants. For example, up to 450 t/year of TCS are consumed in Europe, approximately 96% of which are disposed to wastewater plants (Scientific Committee on Consumer Safety, 2010). Even though the removal rate of TCS during wastewater treatments is >80% (Reiss et al., 2002), TCS is usually detected in surface water with the concentrations in the range of few ng L−1 to several μg L−1 (Halden and Paull, 2005; Young et al., 2008; Brausch and Rand, 2011; Thomaidi et al., 2017). In China, the product of TCS increased from 500 tons in 2003–2900 tons in 2012 and the increasing production of TCS resulted in tremendous discharge and environmental accumulation of TCS (Huang et al., 2014). For example, TCS was frequently detected in the Pearl River Delta, South China with a concentration of up to 1023 ng L−1 (Peng et al., 2008). Previous studies have shown that TCS can pose acute toxicity to aquatic organisms at environmentally concentrations (Cortez et al., 2012), and continuous discharge of TCS into the aquatic environment may pose long-term ecologic effects due to the accumulation and chronic exposure (Halden, 2014; Tato et al., 2018). For instance, TCS had endocrine disrupting effects (e.g., increased vitellogenin mRNA expression and decreased sperm counts) to fish at 1013 μg L−1 (Ishibashi et al., 2004; Raut and Angus, 2010). TCS had EC50 values ranging from 0.53 to 9.2 μg L−1 to green algae, such as *Scenedesmus subspicatus, Pseudokirchneriella*

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subcapitata and Microcystis aeruginosa (Orvos et al., 2002; Yang et al., 2008; Huang et al., 2016). Peng et al. (2017) also reported that TCS posed high risks to algae with risk probability of 83% in the urban rivers of Guangzhou in China due to the discharge of sewage effluents. What’s more, at the base of the trophic food chain, algae such as diatoms represent a source of food for numerous organisms, and these microalgae are particularly relevant or seriously affected by exposure of xenobiotic pollutants in aquatic ecosystems. Diatoms are very sensitive to xenobiotic pollutants in freshwater (Potapova and Charles, 2002; Rimet and Bouchez, 2011). Thus, diatoms were often used to predict the toxicity and the bioavailability of xenobiotics to aquatic environments. Cymbella sp. is one of the most common and occurring diatoms in freshwater (Jasprica and Hafner, 2005). For instance, Wood et al. (2014) reported that atrazine at 500 μg L\(^{-1}\) could cause 54% death of Cymbella sp., indicating that Cymbella sp. was a sensitive diatom species to xenobiotics in natural waters. In addition, transformation of TCS may occur in aquatic organisms after uptake (Balmer et al., 2004; Sun et al., 2017). Incomplete degradation of TCS may produce metabolites with unknown biological activities, leading to unanticipated environmental effects. Dann and Hontela (2010) have speculated that the metabolites of TCS (e.g., methyltriclosan, 2, 4-dichlorophenol, and 2, 8-dichlorodibenzo-p-dioxin) showed higher toxicity to aquatic biota and higher resistance to degradation than the parent compound TCS in waters. Methyltriclosan with a bioaccumulation factor (BAF) of 1200 was detected to be higher than that of TCS (BAF = 500) in snails collected from Pecan Creek, USA (Coogan and La Point, 2008). Thus, the sole consideration of the unaltered TCS could lead to an underestimation in the uptake and risk assessment of TCS. At present, it is yet unknown how TCS is transformed in diatom and whether transformation leads to the formation of incomplete and potentially bioactive products.

Dissolved organic matter (DOM) is a typical component in aquatic environment and is usually involved in processes (e.g., redox reaction, fate and transport) of contaminants and nutrient cycling in environmental systems (Huangfu et al., 2013; Coble et al., 2016; Prak et al., 2017). DOM can interact with chemicals by binding and sorption, such as ion exchange, hydrogen bonding, charge transfer, covalent binding, hydrophobic adsorption and partitioning, which have been shown to enhance the distribution of pollutants in water, alter their bioconcentration and toxicity (Zhang et al., 2014; Chang and Bouchard, 2016; He et al., 2016). Approximately 50–75% of DOM was constituted by humic acid (HA) in natural waters (Mamba et al., 2009) and thus HA was usually selected as the model DOM. Kim et al. (2016) found that HA could significantly reduce bioavailability of pharmaceuticals (e.g. tetracycline, 4-octylphenol) and their toxicity to algae. However, the role of HA in biouptake, toxicity, and biotransformation of TCS to diatom is still unclear.

The objective of this study was to explore the biouptake, toxicity, and biotransformation of TCS in a typical freshwater diatom Cymbella sp. Given that DOM commonly exist in actual waters and the presence of DOM will have an influence on fate and effects of pollutants on Cymbella sp., the influence of HA as a representative on the biouptake, toxicity, and biotransformation of TCS was paid special attention. The degradation pathways of TCS in Cymbella sp. were elucidated with an emphasis on the identification of transformation products. This information will be valuable for risk assessment of TCS in natural waters.

2. Materials and methods

2.1. Chemicals

Triclosan (chemical purity: 97%) was purchased from Sigma-Aldrich (China). HPLC-grade methanol and acetonitrile were obtained from Fisher Scientific (China) and EMD Millipore Corporation (USA), respectively.

A stock solution of TCS was prepared by mixing the TCS in methanol at 100 mg L\(^{-1}\). Humic acid (HA, Aladdin, Shanghai, China) stock solution was prepared by dissolving HA in a 7 mM NaOH solution and then adjusted to pH 7.0 ± 0.2 with hydrochloric acid. This solution was then sonicated for 30 min, filtered three times through a 0.45 μm cellulose ester membrane and stored at 4 °C prior to use. The total organic carbon (TOC) content of HA was measured with a TOC analyzer (Multi N/C HT1300, Analytik Jena AG, Jena, Germany) and the HA concentrations were described as milligrams of organic carbon per liter water. All chemicals used in this study were of analytical grade.

2.2. Diatom cultures

The diatom Cymbella sp. was obtained from the Center of Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB-Collection, Wuhan, China). Diatoms were inoculated into 500 mL sterile D1 medium. The constituents in D1 medium was listed in our previous study (Ding et al., 2017). The cultures were incubated at 23 ± 1 °C in an incubator under a controlled lighting regime. Fluorescent lamps were used as the light source with an automated light/dark cycle of 12 h/12 h. The illuminance was maintained at 4000 Lux.

2.3. Measurement of cell growth, total chlorophyll and carotenoid contents

The toxicity of TCS on diatom was determined by monitoring the cell growth, total chlorophyll and carotenoid contents of Cymbella sp. Algal bioassays were conducted for 72 h in the culture containing at 0, 10, 50, 100, 200, 500 and 1000 μg L\(^{-1}\) TCS, respectively. The methanol concentrations in algal cultures were below 1%, which was found to show no effect on the growth of Cymbella sp. in our preliminary experiment. The effect of HA on TCS toxicity to diatom Cymbella sp. was investigated by adding varying HA concentrations (0, 10, 20, 30, 40, 50 mg L\(^{-1}\)) to the medium containing a constant TCS concentration (72 h EC50 TCS). Batch experiments were conducted in 100 mL Erlenmeyer flasks containing 50 mL D1 medium. The algal density was determined by optical densities of the algal suspensions at 680 nm in a UV-2550 spectrophotometer (Shimadzu, Japan). The cellular growth rates (d\(^{-1}\)) were calculated by fitting the cell numbers to an exponential function as reported in our previous study (Ding et al., 2017). All treatments were performed in triplicates.

The chlorophyll content of Cymbella sp. was analyzed by hot methanol extraction as described in our previous study (Ding et al., 2017). Briefly, a 10 mL diatom suspension was collected and centrifuged at 5000 rpm for 10 min. The pellet was re-suspended with 10 mL of methanol: H\(_2\)O (9:1, v/v) and incubated at 60 °C in a water bath for 15 min. The mixture was again centrifuged at 5000 rpm for 10 min. The optical density of the supernatant was measured at 665, 652 and 470 nm wavelengths in a UV-2550 spectrophotometer (Shimadzu, Japan). The chlorophyll-a (C\(_a\)), chlorophyll-c (C\(_c\)) and carotenoid concentrations of the extracts were calculated by the equations as reported by Xiong et al. (2016). The dry biomass of algae was calculated according to Xiong et al. (2016) have been reported. Briefly, 10 mL of a diatom suspension was filtered through a 0.45 μm Whatman filter paper and dried at 105 °C for 24 h. The filter papers with diatom cells were weighed again after cooling to room temperature, and then the dry weight of diatom cells was calculated between the filter paper and filter water with diatom cells.
2.4. Microscopic observations

Scanning electron microscopy (SEM) analysis was carried out to obtain the morphology and surface information of the diatom Cymbella sp. under the exposure of TCS and DOM. Samples were harvested in the culture added with 1 mg L\(^{-1}\) TCS (TCS-treatment) and 20 mg L\(^{-1}\) HA + 1 mg L\(^{-1}\) TCS (TCS + HA treatment), respectively, after 72 h of cultivation. The TCS-free and HA-free treatment was set as control. A drop of algal suspensions in the control and TCS-treatments was placed on the silicon chip and air-dried. The silicon chip was then coated with gold for observation on a HITACHI S3400N SEM (Hitachi Co., Japan).

2.5. Uptake of TCS by Cymbella sp.

The uptake of TCS by Cymbella sp. was performed in 100 mL Erlenmeyer flasks containing 50 mL D1 mediums. The Cymbella sp. was exposed to EC\(_{50}\) of TCS with and without HA. At 24, 48, 72 and 144 h after treatment, three replicates of 10 mL solution were transferred to 10 mL centrifuge tubes and centrifuged at 5000 rpm for 10 min. The supernatant was discarded, and the residue was washed and resuspended with 10 mL distilled water followed by centrifugation at 5000 rpm for 10 min. The pellet left was extracted with 3 mL dichloromethane: methanol (1:2, v/v) by sonication for 1 h, followed by centrifugation at 5000 rpm for 10 min. The extracted TCS was analyzed and represented for the accumulation amount of TCS within the microalgal cells. All samples were filtered through 0.22 \(\mu\)m membrane filters before analysis.

2.6. Chromatographic analysis

A 10 \(\mu\)L aliquot of each sample was subjected to a Waters ACQUITY ultra-performance liquid chromatography (UPLC) in tandem with a Micromass triple quadrupole detector (MS/MS) (Xevo-TQD, Waters, Milford, MA). The MS/MS was equipped with an electrospray ionization source (ESI) operating in the negative-ion mode. A multiple reaction monitoring (MRM) method was used, and data were acquired and processed using the Masslynx 4.1 software. Chromatographic separation of TCS and its metabolites was performed at 35°C, using an ACQUITY UPLC BEH C18 column (2.1 mm \(\times\) 50 mm, 1.7 \(\mu\)m). The injection volume and the flow rate were 10 \(\mu\)L and 0.2 mL min\(^{-1}\), respectively. The elution was conducted by mobile phase consisted of methanol/water (90/10, v/v). The capillary voltage was 3.0 kV. The source temperature was set to 625°C, the desolvation temperature to 325°C; and cone gas flow and desolvation gas (nitrogen) flow to 1.1 and 15 L min\(^{-1}\), respectively. A dwell time of 0.02 s per ion pair was used.

2.7. Statistical analysis

One-way ANOVA was used to evaluate the significance of differences in growth rate, chlorophyll and carotenoid content of Cymbella sp. in controls and TCS treatments. A difference was considered statistically significant at a level of 0.05. The EC\(_{50}\) of TCS to Cymbella sp. was estimated by SPSS 16.0.

3. Results and discussion

3.1. TCS toxicity to Cymbella sp.

The growth inhibition dose-response curves for diatom Cymbella sp. exposed to TCS was shown in Fig. 1. The addition of TCS showed no inhibition on growth of Cymbella sp. when lower than 100 \(\mu\)g L\(^{-1}\) TCS was added to the culture. However, as the initially spiked level of TCS increased, the growth inhibition rate for Cymbella sp. which was caused by TCS increased. For instance, after 48 h of exposure, 100% of inhibition was obtained when 500 \(\mu\)g L\(^{-1}\) TCS was added (Fig. 1). The 24, 48 and 96 h EC\(_{50}\) of TCS to Cymbella sp. were 625.8, 240.3, and 324.9 \(\mu\)g L\(^{-1}\), respectively, suggesting that Cymbella sp. is very sensitive to TCS or its potential toxic metabolites, especially in the chronic exposure. Such inhibition may be caused by the accumulated TCS in Cymbella sp., which could be

![Fig. 1](image-url) The dose-response curve of Cymbella sp. exposed to TCS with concentrations ranging from 0.1 to 1000 \(\mu\)g L\(^{-1}\). Error bars indicate standard deviations (n = 3).
combined with biomacromolecules, resulting certain disturbance or damage in algae. For instance, González-Pleiter et al. (2017) reported that TCS could alter the homeostasis of intracellular free calcium and induce reactive oxygen species overproduction in green alga *Chlamydomonas reinhardtii*, resulting in oxidative stress, photosynthesis inhibition and mitochondrial membrane depolarization. The inhibition caused by TCS and its potential toxic metabolites to *Cymbella* sp. would be further explored in the accumulation section. Similar results were found by Proia et al. (2011) that the addition of 60 μg L⁻¹ TCS could result in a mortality of up to 41% on diatom *Achnanthidium minutissimum* after 7 days of exposure. Blue-green algae *Scenedesmus vacuolatus* were found to be more vulnerable to TCS with EC₅₀ values of 4.03 μg L⁻¹ (Bandow et al., 2009). The higher tolerance to TCS for diatom may be attributed to a cellular defense mechanism that the frustules of diatom could prevent pollutants entering their cells (Santos et al., 2013; Yung et al., 2015).

The presence of HA significantly accelerated the growth rate of *Cymbella* sp. when HA was added at the concentration of <30 mg L⁻¹ during 72 h exposure, suggesting a reduced toxicity to *Cymbella* sp. (Fig. 2). When the concentration of HA was higher than 40 mg L⁻¹, the growth of *Cymbella* sp. was significantly retarded.

**Fig. 2.** The growth rate (a) and pigments (b) of *Cymbella* sp. as a function of TCS (72 h EC₅₀) with different HA concentrations. Error bars indicate standard deviations (n = 3). Different letters above adjacent bars denote a significant difference (p < 0.05) between the treatments, whereas the same letter indicates no significant difference.
This finding was consistent with trends observed by Zhang et al. (2014) that lower than 20 mg L\(^{-1}\) of HA facilitated to the growth of diatom *Navicula* sp., while it reduced the growth rate significantly at concentrations > 40 mg L\(^{-1}\). Previous studies reported that HA could affect the growth of aquatic organisms like xenobiotic pollutants (Timofeyev et al., 2007; Steinberg et al., 2008), indicating that the decreased algal growth rate observed in the culture added with >40 mg L\(^{-1}\) HA may be attributed to the synergistic effects of HA and TCS on diatom *Cymbella* sp. Both the Chl-a and carotenoid contents increased in the treatments containing >10 mg L\(^{-1}\) of HA, which is likely due to the fact that small molecular HA can pass through the cell membrane of algae, stimulating the Chl-a synthesis (Bahrs and Steinberg, 2012), and complex of HA and TCS was formed, leading to a reduced bioavailability of TCS to *Cymbella* sp. Similarly, Behera et al. (2010) indicated that the amount of HA-complexed TCS gradually increased with an increase of HA concentration. Furthermore, HA could accumulate on the algal surface, enhancing the electrostatic repulsion between the xenobiotics and the algal cells, and thus inhibited the xenobiotics entering into the algal cells (Campbell et al., 1997; Vigneault et al., 2000; Tang et al., 2015). Therefore, the hindering of TCS by HA from the diatom cells may also contribute to the attenuating toxicity of TCS to *Cymbella* sp.

To better understand the interactions of the TCS-HA-algae system, algal cell morphology was examined after 72 h of incubation. The SEM images show that the diatom cells *Cymbella* sp. were enormously damaged under 1 mg L\(^{-1}\) TCS exposure (Fig. 3c and d), as compared to the intact cells in the control (Fig. 3a and b). After addition of 20 mg L\(^{-1}\) HA to the treatment containing 1 mg L\(^{-1}\) TCS, the damage on diatom cells was almost repaired (Fig. 3e and f), indicating that the toxicity of TCS and its potential toxic metabolites on *Cymbella* sp. was effectively reduced by the addition of HA. This result is in agreement with the observations from the kinetics of algal cell growth, chlorophyll a and carotenoid content tested in the present study.

### 3.2. Biouptake of triclosan by diatom *Cymbella* sp.

TCS can be accumulated in diatom *Cymbella* sp., and the intracellular TCS concentration was time-related as it decreased with time under exposure of 324.9 µg L\(^{-1}\) TCS (Fig. 4a). For example, the accumulated TCS in microalgal cells was 24.37 mg g\(^{-1}\) biomass\(^{-1}\) after 24 h of incubation and decreased to 4.02 mg g\(^{-1}\) biomass\(^{-1}\) after exposure for 144 h. The time-related decrease of intracellular TCS may be due to the fact that TCS could release from the algal cell to the medium or undergo transformation in *Cymbella* sp., which is consistent with the results by Escarrone et al. (2016) reported that the increase of intracellular TCS in the tissues of *Poecilia vivipara*.
within 7 days and then the TCS content decreased.

The addition of HA significantly inhibited the accumulation of TCS in *Cymbella* sp. throughout the 144-h incubation (Fig. 4a). For instance, the intracellular TCS concentration (5.99 mg g⁻¹ biomass⁻¹) was significantly lower in the treatment amended with HA than that in treatment without HA (11.37 mg g⁻¹ biomass⁻¹) at 48 h. It is likely that HA may combine or adsorb TCS in the culture rather than the cell, leading to a decreased level of TCS within diatom cells or a reduced bioavailability. A variety of functional groups, such as -OH, -CONH₂, -CONH, alcohol, carboxylic and carbonyl groups, were found to exist on the surface of HA (Zhang et al., 2014), and can interact with hydroxyl groups in TCS molecular. In addition, the zeta potential of diatom *Cymbella* sp. was −27.05 mV, implying that *Cymbella* sp. was negatively charged (data not shown). Tang et al. (2015) found that HA could increase the electrostatic repulsion between the xenobiotic pollutants and the algal cells. Thus, the decreased uptake of TCS in *Cymbella* sp. may also be caused by the enhanced electrostatic repulsion between anion TCS and algal cells in the presence of HA. Furthermore, Campbell et al. (1997) and Vigneault et al. (2000) found that HA

![Fig. 4. Intracellular TCS concentrations in the diatom *Cymbella* sp. in the presence/absence of humic acid. (a) Intracellular TCS content at different incubation time; (b) Relationship between the intracellular TCS concentration and the cell numbers of *Cymbella* sp.](image-url)
<table>
<thead>
<tr>
<th>Compound</th>
<th>Product Mw</th>
<th>Chemical structure</th>
<th>Retention time (min)</th>
<th>ESI(−)MS² (m/z)</th>
</tr>
</thead>
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<tr>
<td>Triclosan</td>
<td>289.5</td>
<td><img src="#" alt="Chemical structure of Triclosan" /></td>
<td>0.90</td>
<td>287 &gt; 35</td>
</tr>
<tr>
<td>3-chlorophenyl 2-oxoacetate</td>
<td>TP 184 184</td>
<td><img src="#" alt="Chemical structure of 3-chlorophenyl 2-oxoacetate" /></td>
<td>0.39</td>
<td>183 &gt; 137 &gt; 107 &gt; 62 &gt; 35</td>
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<tr>
<td>Methyl triclosan</td>
<td>303</td>
<td><img src="#" alt="Chemical structure of Methyl triclosan" /></td>
<td>0.45</td>
<td>302 &gt; 263 &gt; 216 &gt; 130 &gt; 62</td>
</tr>
<tr>
<td>6-(5-chloro-2-(2,4-dichlorophenoxy)-4-hydroxyphenoxy)hexane-1,1,2,3,4,6-hexaol</td>
<td>TP 486 486</td>
<td><img src="#" alt="Chemical structure of 6-(5-chloro-2-(2,4-dichlorophenoxy)-4-hydroxyphenoxy)hexane-1,1,2,3,4,6-hexaol" /></td>
<td>0.53</td>
<td>485 &gt; 470 &gt; 421 &gt; 369 &gt; 280 &gt; 62</td>
</tr>
<tr>
<td>1-(4-chloro-2-(dihydroxymethoxy)phenoxy)ethane-1,2-diol</td>
<td>TP 250 250</td>
<td><img src="#" alt="Chemical structure of 1-(4-chloro-2-(dihydroxymethoxy)phenoxy)ethane-1,2-diol" /></td>
<td>0.65</td>
<td>249 &gt; 233 &gt; 191 &gt; 157</td>
</tr>
<tr>
<td>(4-chloro-2-hydroxyphenoxy)methanediol</td>
<td>TP 190 190</td>
<td><img src="#" alt="Chemical structure of (4-chloro-2-hydroxyphenoxy)methanediol" /></td>
<td>0.68</td>
<td>189 &gt; 157 &gt; 115 &gt; 75</td>
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<tr>
<td>2-(3-chlorophenoxy)ethane-1,1,2-triol</td>
<td>TP 205 205</td>
<td><img src="#" alt="Chemical structure of 2-(3-chlorophenoxy)ethane-1,1,2-triol" /></td>
<td>0.82</td>
<td>205 &gt; 189 &gt; 173 &gt; 76</td>
</tr>
</tbody>
</table>

(continued on next page)
could accumulate on the algal surface and retard the diffusion of solute to the absorb sites on the cell membrane.

A good correlation between the cell numbers of Cymbella sp. and the intracellular TCS concentration in the presence of HA was observed with exponential decay functions ($R^2 = 0.70, p < 0.01$) (Fig. 4b). In other words, the cell numbers of Cymbella sp. increased with the decreased the intracellular TCS concentration, indicating that the toxicity of TCS and its potential toxic metabolites to algae is

<table>
<thead>
<tr>
<th>Compound</th>
<th>Product M&lt;sub&gt;w&lt;/sub&gt;</th>
<th>Chemical structure</th>
<th>Retention time (min)</th>
<th>ESI(-) MS&lt;sup&gt;2&lt;/sup&gt; (m/z)</th>
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<tr>
<td>4-chloro-2-methoxyphenyl 3,4,5,6-tetrahydroxytetrahydro-2H-pyran-2-carboxylate</td>
<td>TP 334 334</td>
<td><img src="image1" alt="Chemical structure" /></td>
<td>1.18</td>
<td>333 &gt; 315 &gt; 275 &gt; 157</td>
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<td>3-chlorophenyl 2-amino-3-methylbutanoate</td>
<td>TP 228 227.5</td>
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<td>1.94</td>
<td>347 &gt; 315 &gt; 189 &gt; 157</td>
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<tr>
<td>5-chloro-2-methoxyphenoxy(pyrrolidin-2-ylidene)methanol</td>
<td>TP 256 255.5</td>
<td><img src="image5" alt="Chemical structure" /></td>
<td>2.31</td>
<td>255 &gt; 231 &gt; 116</td>
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resulted from its accumulation or uptake in algae, leading to the damage on cell organelles. This result is consistent with the decreased algal growth (*Microcystis aeruginosa*) caused by the higher intracellular concentration of TCS (Huang et al., 2016). Recently, Almeida et al. (2017) reported that intracellular TCS could result in the oxidative damage on green algae *Chlamydomonas reinhardtii*, leading to the decreased algal growth.

### 3.3. Transformation products of TCS in the diatom Cymbella sp.

The transformation products of TCS were extracted in *Cymbella* sp. under exposure of 324.9 μg L$^{-1}$ TCS. The extracts were subjected to UPLC-MS/MS analysis to structurally identify the intermediates. A total of 11 metabolites, which are labeled herein as TP 184, methyl TCS, TP 486, TP 250, TP 190, TP 205, TP 334, TP 228, TP 348, TP 408, and TP 256 with the increasing retention times, were found in algal cells during 144 h of incubation (Fig. S1). Because of the lack of commercially available standards for reference, tentative structural identification of the intermediates was based on the analysis of the total ion chromatogram (TIC) and the corresponding mass spectrum, as shown in Figs. S2–S13. The structural and fragmentation information for TCS and its degradation metabolites in algae are shown in Table 1.

The metabolite TP 184 was tentatively identified as 3-chlorophenyl 2-oxoacetate, a glyoxal acid conjugated transformation product. The fragment ion m/z 137 represented the successive losses of a chloride and a hydroxyl group. The m/z 107 corresponded to the losses of a hydroxyl and methyl group. The fragment ion at m/z 62 may correspond to the ethane-1, 1-diol (Fig. S2). The mass difference of 14 between methyl TCS and its parent compound TCS is suggestive a methylation of TCS. The fragment ion at m/z 263 may be attributed to the loss of a methyl group and the cleavage of benzene. The successive dechlorination and demethylation lead to the fragment ion at m/z 216 (Fig. S3). TP 486 was tentatively identified as 6- (5-chloro-2- (2, 4-dichlorophenoxy)-4-hydroxyphenoxy)hexane-1, 1, 2, 3, 4, 6-hexaoxl, which was a glucuronide conjugated compound of TCS. The molecular ion of m/z 485 suggested a molecular weight of 486 and fragments detected from MS$^2$ revealed the formation of several fragments: 470 (-OH), 421 (-OH, -OH and -CH$_3$), 369 (-Cl and -OH), and 280 (-Cl and -CH$_2$CHCH$_2$) (Fig. S4). The fragment ion at m/z 233 for TP 250 corresponded to the loss of a hydroxyl group. The fragment ions at m/z 191 and 157 were indicative of the loss of CH-CHOH and subsequently losses of two hydroxyl groups, respectively (Fig. S5). TP 250 was tentatively identified as 1- (4-chloro-2- (dihydroxyphenoxy)phenoxy)ethane-1, 2-diol. TP 190 was tentatively identified as (4-chloro-2-hydroxyphenoxy)methanediol, which was probably formed by the esterification of 4-chlorobenzene-1, 2-diol, which was generated from the cleavage of ether bond in TCS. The fragment ion at m/z 157 corresponded to the consecutive losses of two hydroxyl groups. The fragment ion at m/z 115 may be attributed to the loss of methoxyl group and the cleavage of benzene (Fig. S6). TP 205 was an oxalic acid-conjugated metabolite, which may be tentatively identified as 2- (3-chlorophenoxy)ethane-1, 2-triol. The fragment ions at m/z 189 and 173 for TP 205 was mostly attributed to the successive losses of hydroxyl groups, respectively (Fig. S7). TP 334 is also a glucuronide-conjugated metabolite. The fragment ions at m/z 315, 275, and 157 were indicative of the consecutive losses of H$_2$O, an ethylene-ethanol molecular, and the glucuronide, respectively. Thus, analysis of the fragment ions provided the tentative identity of TP 334 as 4-chloro-2-methoxyphenol 3, 4, 5, 6-tetrahydroxytetrahydro-2H-pyran-2-carboxylate (Fig. S8). TP 228 had a retention time of 1.22 min and was a valine-conjugated metabolite, which was determined to be 2-amino-1-(3-chlorophenoxy)-3-methylbut-1-en-1-ol. Fragmentation experiments (MS$^2$) conducted in negative ion mode revealed the formation of fragments m/z 209 (H$_2$O), 152 (-CH(CH$_3$)$_2$-NH$_2$) and 43 (-C$_6$H$_5$Cl) (Fig. S9). The oxaloacetic acid is usually generated in the Krebs cycle in algae and the combination of TCS with oxaloacetic acid could lead to the formation of TP 408. The fragment ions at m/z 391 corresponded to the loss of -OH. The daughter ion at m/z 348 was likely generated from the subsequent loss of an acetaldehyde molecular. The m/z at 280 may be attributed to the loss of -Cl and the subsequent loss of two hydroxyl groups (Fig. S10). TP 408 was then determined to be 5-chloro-2-(2, 4-dichlorophenoxy)phenyl 2,4,4-trihydroxybutanote. The metabolite TP 348 was tentatively identified as 5-chloro-2-methoxyphenoxy (pyrrolidin-2-ylidine) methanol. The fragment ions at m/z 237 and 116 may be attributed to the loss of H$_2$O and proline, respectively (Fig. S12). The metabolites found in *Cymbella* sp. without HA were same to that in the presence of HA, indicating that the metabolism of TCS in *Cymbella* sp. was not influenced by HA.

The kinetics of metabolites of TCS in the algae are shown in Fig. S14. The metabolites including methyl TCS, TP 113, TP 334, TP 408, TP 486, and TP 250 showed a decrease trend during 72 h of exposure, whereas the opposite trends were observed for TP 190 and TP 348 (Fig. S13). After the addition of HA, the kinetics of several metabolites (e.g., TP 486, TP 350, TP 334, and TP 408) showed different trends (Fig. S14), while the peak areas of TP 190 and TP 348 basically kept unchanged (data not shown). TP 486 was higher in the treatment with the addition of HA than that in the HA-free treatment at 24 h (Fig. S14a), whereas the similar trend of TP 250 was observed after 24 h exposure (Fig. S14b). TP 250 may be a transformation product for TP 486, and was combined with the carboxyl group of carbonate in diatom. Additionally, TP 334 and TP 408 were detected to be significantly higher in the treatments with HA during the whole exposure (Figs. S14c and d). These results suggested that the kinetics of these metabolites was significantly affected by HA.

### 3.4. Proposed metabolic pathway of TCS in diatom

On the basis of the identified metabolites and their kinetics during incubation, possible degradation pathways of TCS in *Cymbella* sp. are schematically shown in Fig. 5. Methyl TCS was formed by the biological methylation of TCS in *Cymbella* sp. and was found to show higher environmentally persistent and more toxic potentials than the parent compound TCS (Gaume et al., 2012; Macedo et al., 2017), Bester (2003) and Balmer et al. (2004) indicated that methyl TCS was also generated in water treatment process, leading to a higher detection level in WWTP effluent than influent. Coogan and La Point (2008) and Balmer et al. (2004) found that methyl TCS was detected in surface waters and fish with concentration of up to 0.9 ng L$^{-1}$ and 35 ng g$^{-1}$, respectively. Therefore, the formation of methyl TCS in *Cymbella* sp. may contribute to the higher degree of environmental persistence than its parent compound in surface water and other impacted environment. Hydroxylation is considered as a main degradation pathway of contaminants in diatom due to the presence of cytochrome P-450 (CYP 450) (Quintana et al., 2005). In the present study, methyl TCS may be hydroxylated and the metabolite TP 348 was likely formed by the esterification of hydroxylated methyl TCS. Pietrini et al. (2015) reported that glucuronidation of ibuprofen was likely a detoxification process in plant *Lemna gibba* L. Thus, the glucuronidated TCS (TP 486) was
probably included in detoxification processes in Cymbella sp. and the increased toxicity of TCS in diatom with the incubation time may be due to the decrease of TP 486 (Fig. S14). Glucuronidated TCS was previously found in fish and edible plants (James et al., 2012; Macherius et al., 2012). TP 408 was generated by combination of TCS and oxaloacetic acid, which plays an important role in the Krebs cycle in organisms. The dichlorophenol (DCP) was found in the biodegradation of TCS (Sun et al., 2017; Tohidi and Cai, 2017). Dechlorination of DCP may generate the chlorophenol in the present study. Briones et al. (1997) found that oxalic acid was abundant in the blue-green algae. TP 205 may be yielded by the esterification of oxalic acid and chlorophenol (Fig. 5). The esterification of glyoxal acid and chlorophenol lead to the formation of TP 184. The combination of amino acids and chlorophenol could generate a series of TCS metabolites, such as TP 228 (valine-conjugated) and TP 256 (proline-conjugated). TP 190 and TP 250 are carbonate-conjugated metabolites. TP 334 may be formed by glucuronidation of DCP, which may also be an important detoxification mechanism in the diatom Cymbella sp.

4. Conclusions

Results from the present study showed that TCS and its potential toxic metabolites has high toxic effects to Cymbella sp. with 72 h EC50 of TCS of 324.9 µg L\(^{-1}\), and the uptake of TCS in Cymbella sp. was ranged from 24.8% to 69.0%. However, the toxicity of TCS and its potential toxic metabolites to Cymbella sp. obviously decreased when HA added in the algal cultures with the concentration of <30 mg L\(^{-1}\). The SEM analysis confirmed that the diatom cells Cymbella sp. were enormously damaged under 1 mg L\(^{-1}\) TCS exposure but repaired after addition of 20 mg L\(^{-1}\) HA. In addition, HA could significantly inhibit the bioaccumulation of TCS in Cymbella sp. throughout the 144-h incubation. The growth of diatom Cymbella sp. is positively related to bioconcentration capacity of TCS in the presence of HA (R\(^2\) = 0.70, p < 0.01), suggesting that bioconcentration is a prerequisite for TCS toxicity in Cymbella sp. A total of 11 transformation products have been detected and identified, including some biologically active products (e.g., methyl TCS) and conjugation products (e.g., glucuronic or oxaloacetic acid conjugated TCS), which may be included in the detoxification mechanism of TCS. These findings can have important implications for a more general understanding of interaction between TCS and aquatic organism and their biogeochemical cycling in surface waters, providing accurately data for the environmental behavior and risk assessment of TCS in the presence of DOM.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2017.11.051.


