Electrochemical simulation of triclosan metabolism and toxicological evaluation

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HIGHLIGHTS
• Phase I metabolism of triclosan was simulated by electrochemistry-mass spectrometry.
• Ether cleavage, cyclization and hydroxylation of triclosan are the main mechanisms.
• Triclosan and its reaction mixture showed high toxicity on zebrafish embryos.
• Toxicity declined via ether cleavage and toxic products formed via other mechanisms.
• The reaction mixture can activate AhR in zebrafish embryos.

GRAPHICAL ABSTRACT

ABSTRACT

Triclosan (TCS), an antimicrobial agent, is considered as emerging pollutant due to its wide dispersive use in personal care products and high aquatic toxicity. In the present study, phase I metabolism of triclosan was investigated through laboratory electrochemical simulation studies. The products formed in the electrochemical (EC) cell were identified by online and offline coupling with QTRAP and high-resolution FTICR mass spectrometers, respectively. The sequential formation and disappearance of each product, with the continuous increase of voltage from 0 to 3500 mV, was observed to reveal the transformation pathways of TCS. The toxic potential of TCS and the identified products was estimated using Quantitative structure–activity relationship (QSAR) modeling on 16 target proteins. The toxicity change of TCS during simulated metabolism and toxicological effects of reaction mixture were assessed by Fish embryo toxicity (FET) test (Danio rerio) and quantitative real-time polymerase chain reaction (qPCR). Eight metabolites formed during the simulated metabolism of TCS mainly via the mechanisms of hydroxylation, ether-bond cleavage and cyclization. In FET test, the reaction mixture (LC50, 48h = 1.28 mg/L) after electrochemical reactions showed high acute toxicity on zebrafish embryos, which was comparable to that of triclosan (LC50, 48h = 1.34 mg/L). According to the modeling data, less toxic products formed only via ether-bond cleavage of TCS while the products formed through other mechanisms showed high toxicity. AhR-mediated dioxin-like effects on zebrafish embryos, such as developmental retardation in skeleton and malformations in cardiovascular system,
were also observed after exposure to the TCS reaction mixture in FET test. Activation of the AhR by the reaction mixture in zebrafish embryos was further proved in cyp1a gene expression analysis.

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1. Introduction

Pharmaceuticals and personal care products (PPCPs), comprising diverse organic compounds, such as antibiotics, hormones, antimicrobial agents, sunscreen products etc., have raised significant concerns in recent years for their potential threats to ecosystems and human health. Triclosan (5-chloro-2-(2,4-dichlorophenoxy)-phenol, TCS) is a broad-spectrum antimicrobial agent, which is widely used in many consumer products such as toothpastes, antiseptic soaps, detergents, cosmetics, plastic kitchenware, socks, carpets and toys (Bedoux et al., 2012; Reiss et al., 2002; Singer et al., 2002). The global demand for TCS has now exceeded 1500 tons per year in the world (Dann and Hontela, 2011). In Europe, about 350 tons are consumed annually (Pintado-Herrera et al., 2014; Young et al., 2008).

Given the widespread use of personal care products and consumer products, triclosan has been detected in wastewater, surface water, sediment, soil, organisms, and even in human milk (Adolfsson-Erici et al., 2002; Allmyr et al., 2006; Bester, 2005; Singer et al., 2002). The products containing TCS are mostly rinsed down the drain and enter the wastewater treatment plants (WWTPs). In WWTP influent, the concentration of TCS can reach to as high as 86.2 μg/L (Kumar et al., 2010). Although triclosan is biodegradable and can be effectively removed in WWTPs, a small percentage of triclosan and its metabolites are discharged with wastewater effluent into the aquatic environment. TCS was found in wastewater effluent at concentrations ranging from 0.01 to 5.37 μg/L (Bester, 2005; Kumar et al., 2010), in surface water from below the detection limit up to 2.3 μg/L (Kolpin et al., 2002) and in sediments with 0.27 to 2633 μg/kg (Agüera et al., 2003; Zhao et al., 2010).

A long term and low-level exposure to TCS and their metabolites can cause ecotoxicological effects on aquatic and terrestrial organisms. TCS has been reported to have high toxicity to freshwater aquatic organisms (Di Paolo et al., 2016b; Orvos et al., 2002). TCS can exert growth and production inhibition on green algae, invertebrates and fish, while genotoxicity and teratogenicity were also found in specific aquatic species (Dann and Hontela, 2011; Bedoux et al., 2012). TCS can also induce the antimicrobial resistance in the aquatic environment (Dhillon et al., 2015). In laboratory studies, the TCS-resistance bacteria were found after increasing exposure to TCS and the consequent development of resistant colonies (Russell, 2003). Additionally, TCS has shown weakly estrogenic and androgenic activity at environmental relevant concentrations (Dann and Hontela, 2011; Di Paolo et al., 2016a), suggesting TCS is a suspected endocrine disturbing compound.

Biodegradation of triclosan in the environment and wastewater has recently gained more attention. A number of studies have speculated some metabolites may be more toxic on aquatic organism and human beings than TCS itself (Dann and Hontela, 2011). However, the metabolites of TCS are so far not yet fully understood and only very limited information is available in the literature on the toxicological effects of its metabolites. Therefore, an understanding of TCS metabolism and toxicological effects of its metabolites is urgently demanded.

Traditional methods of studying metabolites are to conduct in field or incubate in different scales. However, they always involve expensive and time-consuming sample treatment processes to extract metabolites. To overcome these drawbacks, Electrochemistry-mass spectrometry (EC-MS), has been developed to simulate phase I metabolism reactions catalyzed by the enzymes of cytochrome P450 (CYP450) (Karst, 2004), which is the first step of the biotransformation (Meyer, 1996). Although electrochemical simulation is not able to perfectly predict actual phase I metabolisms, EC-based approach has the potential to mimic the majority of oxidative metabolism reactions (Karst, 2004). Many studies have reported that electrochemical simulation were in good agreement with results in vivo and in vitro studies (Baumann et al., 2010; Johansson et al., 2007; Lohmann and Karst, 2008; Zhu et al., 2015).

The aim of the present study is to investigate phase I metabolism of TCS using electrochemical simulation method and to evaluate the toxicological effects of TCS and its metabolites. The metabolites of TCS formed in the EC cell were identified using a QTRAP and Fourier Transform Ion Cyclotron Resonance (FTICR) mass spectrometers. The transformation pathways were proposed taking into consideration on the structural information and transformation trends of the products. Toxicity potential of TCS and each transformation product was estimated using quantitative structure–activity relationship (QSAR) modeling on 16 target proteins. The toxicity change during simulated TCS metabolism was evaluated by fish embryo toxicity (FET) test. Aryl hydrocarbon receptor (AhR) mediated toxicity of TCS and its reaction mixture was investigated by quantitative real-time polymerase chain reaction (qPCR) to determine induction of cyp1a gene expression.

2. Materials and methods

2.1. Chemicals and regents

All solvents (chromatographic grade) and chemicals (analytical grade) were used as received from the commercial suppliers. Triclosan (CAS 3380–34–5, 99%) was purchased from Sigma-Aldrich. Ammonium acetate (p.a.), methanol (LiChrosolv purity), and dimethyl sulfoxide (DMSO) were purchased from Merck KGaA (Darmstadt, Germany). Formic acid was obtained from ROMIL (Cambridge, UK). High purity water (18.2 MΩ cm) was produced by a MilliQ plus 185 (Millipore, Molsheim, France).

2.2. Online EC-MS

A commercial EC reactor (Antec Leyden, The Netherlands) with a built-in platinum counter electrode and Roxy potentiostat was set up as reported in previous investigations (Chen et al., 2012; Hoffmann et al., 2011). Boron-doped diamond electrode was chosen as the working electrode in the present study.

The reaction solution was composed of 50 μM of TCS with a 5 mM ammonium acetate buffer, in a mixture of methanol and water (1/v 2:3). The reaction solution was pumped through the EC cell at a constant flow rate of 10 μL/min. The residence time of the solution at the working electrode was approximately 3 s. A potential ramp at a scan rate of 10 mV/s was applied to record the dynamic transformation processes of target ions. The mass spectra of TCS reacted at different reaction voltages were recorded by applying constant voltages to the EC cell. All reactions were conducted at a constant temperature (25 °C) and repeated in triplicate to ensure the stability of the system and minimize bias and random errors.

MS experiments were carried out on QTRAP 2000 ESI-MS/MS (ABSciex, Darmstadt, Germany) and high-resolution ESI-FTICR-MS Ultra (ThermoFisher Scientific, San Jose, CA, USA), respectively. The QTRAP ESI-MS/MS system was used in negative mode on analysis of TCS and its products. The spray voltage was −4500 V for negative mode, the declustering potential (DP) varied between −40 and
Offline electrochemical reactions were conducted in a bulk reactor “SynthesisCell” (Antec Leyden, The Netherlands). 5 mg of TCS with 10 mM ammonium acetate was dissolved in a 50 mL mixture of methanol and water (v/v 1:4). The solution was oxidized in the “SynthesisCell” for approximately 2 h. The reaction solution was stirred throughout this period by a magnetic stirrer and at a constant temperature of 25 °C. 100 mL of the reaction solution was collected by repeating this reaction twice. Based on the properties of TCS and its transformation products, a CHROMABOND® HR-X column from Agilent (3 mL/200 mg, Macherey and Nagel, Düren, Germany) was chosen as the solid phase for solid phase extraction (SPE). The SPE column was conditioned with 6 mL ethyl acetate/acetone (v/v: 1:1), 6 mL MeOH and 6 mL H2O. The sample solution was loaded onto the columns at a flow rate 4–5 mL/min. The column was then washed with 3 mL MeOH/H2O (v/v 1:9), and the components of the sample were eluted three times with 2 mL ethyl acetate/acetone (v/v: 1:1). Finally, the eluate was dried using the SpeedVac and then reconstituted into stock solution in DMSO.

The quantification of TCS in the reaction solution was performed with a QTRAP 6500 instrument (ABSciex, Darmstadt, Germany) coupled with an Agilent 1260 HPLC (Agilent Technologies, Waldbronn, Germany). The separation was achieved on a Zorbax Eclipse Plus C18 column from Agilent (100 × 4.6 mm id., 3 μm particle size) kept at 20 °C during analysis. Gradient elution was done with 0.1% formic acid in water (solvent A) and acetonitrile with 0.1% formic acid (solvent B) at a constant flow rate of 800 μL/min. The gradient profile was 5% B for 2 min isocratic, from 2 to 7 min, a linear increase from 5% B to 95% B, at 7 min with an isocratic step until 13 min and a return to 5% B at 13 min and 2 min isocratic for re-equilibration. The injection volume was 10 μL. The quantitation of triclosan after HPLC separation was performed using ESI-MS/MS detection in the MRM mode. MRM transition involving precursor ions [M+H]+ and the most abundant product ion were used for quantification (m/z 286→ m/z 34).

2.4. Toxicological evaluation

2.4.1. Toxic potential prediction

The VirtualToxLab is an in silico tool for predicting the toxic potential of chemicals. It simulates and quantifies their interactions towards a series of proteins using automated, multi-dimensional QSAR modeling (Vedani et al., 2015; Vedani et al., 2012). The VirtualToxLab comprises 16 models of proteins known or suspected to trigger adverse effects: the androgen (AR), aryl hydrocarbon (AhR), estrogen α (ERα), estrogen β (ERβ), glucocorticoid (GR), the potassium ion channel (hERG), liver X (LXR), mineralocorticoid (MR), progesterone (PR), thyroid α (TRα), thyroid β (TRβ) and peroxisome proliferator-activated receptor γ (PRARγ) as well as the enzymes CYP450 1A2, 2C9, 2D6 and 3A4. The affinities of the transfected compounds towards the 16 proteins are weighted into the toxic potential value ranging from 0 (none) to 1.0 (extreme). Toxic potential values can be classified into seven groups (Vedani et al., 2015): ToxPot > 0.8 (***, extremely risk), 0.7 < ToxPot ≤ 0.8 (****, very high risk), 0.6 < ToxPot ≤ 0.7 (**, high risk), 0.5 < ToxPot ≤ 0.6 (*, elevated risk), 0.4 < ToxPot ≤ 0.5 (*, moderate risk), 0.3 < ToxPot ≤ 0.4 (−, low risk) and ToxPot ≤ 0.3 (−, no risk).

2.4.2. Fish embryo toxicity test (Danio rerio)

To determine the acute toxicity of TCS and the reaction mixture, the FET test was carried out according to OECD (2013). Zebrafish (D. rerio) maintenance and egg production can be found in Hollert et al. (2003), Peddinghaus et al. (2012) and Lammer et al. (2009).

The embryos were exposed to the controls (Negative control: artificial water, Solvent control: 0.2% DMSO, Positive control: 3.7 mg/L 3,4-DCA), TCS and reaction mixture in 96-well plates, respectively. The exposure concentrations of TCS and the reaction mixture ranged from 10 to 0.313 mg/L with 1:2 dilution series. Each egg was incubated individually in 200 μL exposure medium. Each chemical/sample was tested in at least three independent replicates with ten embryos per test concentration and controls. Coagulation of embryos, irregularities in somite formation, non-detachment of the tail as well as lack of heart-beat was recorded as lethal endpoints (OECD, 2013). The sub-lethal endpoints were recorded as follows: weak heartbeat, weak or no blood circulation, edema, weak or no pigmentation, malformed or underdeveloped embryos, missing eye primordial or eye pigmentation, deformed spine and no or malformed fins. Tests were regarded to be valid if the mortality rate of negative control and DMSO control did not exceed 10%, and the positive control induced effects in > 20% fish embryos.

The mortality rates from experimental triplicates were plotted using the software (Prism 6.0, GraphPad, La Jolla, USA). The median lethal concentration (LC₅₀) was computed from the concentration–response curves using log (agonist) vs. response with variable slope, where the top and bottom of the curve was respectively set to 0% (minimum of negative control) and 100% (maximum of positive control).

2.4.3. Quantitative real-time polymerase chain reaction (qPCR)

According to Schiwy et al. (2015), fertilized fish eggs exposed to the lowest effect concentrations (312.5 μg/L) of triclosan and the reaction mixture in previous FET test were selected visually using a binocular microscope (SMZ 1500, Nikon Gmbh, Düsseldorf, Germany). Only normally developed fish eggs at least in the eight-cell stage were chosen for further testing. The embryos were also exposed to corresponding solvent control with <0.1% DMSO. A pool of 30 eggs was exposed under static conditions in glass dishes, covered with Parafilm® (Pralfilm, Menasha, WI, USA) and incubated at 26 ± 1 °C for exposure time of 48 h. At least three independent replicates were conducted for each sample. Only tests with a survival rate > 90% were used.

qPCR was conducted according to Braunig et al. (2015). The exposure was terminated after 48 h. Overlaying test solution was removed. The embryos were anesthetized with saturated benzocaine solution, transferred to 2 mL tubes, washed with phosphate-buffered saline. The samples were shock-frozen in liquid nitrogen to stop metabolism and stored at −80 °C until further testing. Total mRNA was extracted using NucleoSpin® RNA isolation kit (Macherey and Nagel) following the instructions of user’s manual. Subsequently, cDNA was generated using a random primer mix (hexamers and anchored-dT primers), and M-MuLV reverse transcriptase (all purchased from New England Biolabs) according to manufacturer’s protocol. Gene specific primers for eff1α and cyp1α were designed by Primer® software (Table S1).

qPCR was performed on 96-wellplates (Peqlab) with SYBR® Green Real-Time PCR Master mix (Thermo Fisher Scientific Inc.). Expression of target gene was quantified using comparative quantification cycle method with adjusted PCR efficiency according to the methods reported by Pfaffl (2001). The expression of the target gene cyp1α was normalized the expression of reference gene eff1α. Levels of gene expression were expressed relative to the average value of the solvent controls as a fold change. The statistical differences between the exposed samples and the control were calculated by a un-paired, two-tailed t-test (p < 0.05) using SigmaPlot 13.0 (Systat Software Inc.).

3. Results and discussions

3.1. Metabolites and transformation mechanisms

The mass spectra of triclosan reacted at constant voltages of 0, 1500, 2000, and 2500 V were then recorded to identify the possible oxidation product peaks. Eight major products with masses of 143/145, 161/163/165, 177/179/181, 267/269/271, 281/283/285, 303/305/307/309, 317/319/321/323 and 333/335/337/339 Da were detected (Fig. S1). Some products showed a distinct isotope pattern originating from the natural 35Cl/37Cl distribution, which indicates the presence of di-
and tri-chlorinated products. The elemental composition/molecular formulas were created on the basis of the exact masses (error < 1 ppm) with FTICR-MS. The low errors indicated the high grade of confidence in the assignation of the elemental composition. Product 5 was not detected in the FTICR-MS, possibly due to the differences in sensitivity of mass spectrometers. On the basis of the mass spectrometry analysis data of QTRAP and FTICR-MS, we elucidated the chemical structures of possible transformation products (Table 1).

While the reaction voltage increased from 0 to 3500 mV with the rate of 10 mV/s, we observed the transformation trends of transformation products via the 2D mass voltammogram (Fig. 1). Due to the multiple peaks of each product, we only chose one mass of each product to be recorded. The separate mass voltammogram of each individual product were shown in Fig. S2. We found that P1 (m/z 143), P2 (m/z 161), P3 (m/z 179), P4 (m/z 267), P6 (m/z 303) and P7 (m/z 317) were almost formed at the beginning of the reaction. The intensities of P1, P2, P3 and P7 continuously increased until the reaction ended. The findings indicate that the ether-bond and hydroxylation reactions were more likely to occur during biotransformation of triclosan. The intensity of P4 slightly declined at voltages higher than 2500 mV, indicating that P4 can be oxidized to other product at higher oxidation voltages. We found P5 formed at the voltage of 2000 mV and increased until the end of the reaction, which indicate P5 may be transformed from other products. P8 is excluded in the figure due to the low intensity comparing with the other products.

The transformation pathways of TCS during electrochemical simulation were elucidated in Fig. 2. Hydroxylation, ether cleavage and cyclization of TCS were the major transformation mechanisms in the present study.

TCS was attacked by the hydroxyl radicals and formed the metabolites, 4-Chlorocatechol (P1) and 2,4-dichlorophenol (DCP) (P2) via the mechanism of ether bond cleavage. 2,4-DCP (P2) was then oxidized to 3,5-dichlorocatechol (DCC) (P3). Meanwhile, hydroxylation of TCS occurred at the less chlorinated benzene ring to form monohydroxyl-TCS (P6). Subsequently, methylation of P6 led to the formation of P7, which later hydroxylated to P8. Monohydroxyl-TCS (P6) was also transformed to hydroxyl-dichlorodibenzodioxin (DCDD) (P4) via a C—Cl bond breaking and hydroxyl radical induced cyclization (Kanetoshi et al., 1987; Nilsson et al., 1974). Methylation of P4 led to the formation of P5.

We found electrochemical simulation showed good agreement with the biotransformation reactions in the literature. Monohydroxylated TCS (OH-TCS), which has been identified as metabolite in rats (Tulp et al., 1979; Wu et al., 2010) and as well as found to be biotransformation product in biological wastewater treatment (Chen et al., 2015; Lee et al., 2012), were successfully predicted by electrochemical simulation. 4-Chlorocatechol (P1), 2,4-DCP (P2) and 3,5-DCC (P3), which are known to be the major metabolites via ether bond cleavage of TCS during biotransformation (Kim et al., 2011; Mulla et al., 2016; Tulp et al., 1979), were also found in the electrochemically simulated reactions. Besides successful prediction of the known metabolites, two dioxin derivatives P4 and P5 were predicted as potential metabolites of TCS during...

<table>
<thead>
<tr>
<th>Name</th>
<th>Mass [M-H]-</th>
<th>Elemental composition</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triclosan</td>
<td>286.94376</td>
<td>C_{12}H_{7}O_{2}Cl_{3}</td>
<td><img src="image" alt="Structure of Triclosan" /></td>
</tr>
<tr>
<td>P1</td>
<td>142.99054</td>
<td>C_{6}H_{5}O_{2}Cl</td>
<td><img src="image" alt="Structure of P1" /></td>
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<tr>
<td>P2</td>
<td>160.95667</td>
<td>C_{6}H_{4}OCl_{2}</td>
<td><img src="image" alt="Structure of P2" /></td>
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<tr>
<td>P3</td>
<td>176.95158</td>
<td>C_{6}H_{4}OCl_{2}</td>
<td><img src="image" alt="Structure of P3" /></td>
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<tr>
<td>P4</td>
<td>266.96219</td>
<td>C_{12}H_{6}O_{3}Cl_{2}</td>
<td><img src="image" alt="Structure of P4" /></td>
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<tr>
<td>P5*</td>
<td>280.9</td>
<td>C_{13}H_{8}O_{3}Cl_{2}</td>
<td><img src="image" alt="Structure of P5" /></td>
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<tr>
<td>P6</td>
<td>302.93889</td>
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</table>

* The peak was not observed in FTICR-MS.
ToxPot channel, LXR: liver X, MR: mineralocorticoid, PR: progesterone, TR: thyroid groups: 1A2, 2C9, 2D6 and 3A4.


The toxic potential and main target proteins of TCS and its transformation products by Table 2.

Table 2 listed the Toxic Potential values, toxic classification and target proteins of TCS and its metabolites. Only P1 and P2 were suggested to be no risk, in particular no trigger receptor for P1. Triclosan, P3, P4, P5, P6, P7 and P8 were estimated to have the toxic potential and showed binding affinities with most target proteins. Two dioxin derivatives P4 and P5 had weak or moderate binding affinities with 12 of 16 target proteins, indicating P4 and P5 are of great possibility to induce diverse adverse effects in the environment. Additionally, it is notable that TCS, P4, P5 and P7 showed moderate binding to AhR. This finding indicates that the AhR-mediated activities are highly possible to be one of the main adverse effects induced by the transformation products in the environment. Therefore, further toxicological tests were conducted to evaluate the toxicity of TCS and its reaction mixture.

3.2.2. Toxicity change after simulated metabolism

To assess the toxicity change after simulated metabolism, the FET test (D. rerio) was applied to study the developmental toxicity of TCS standard and reaction mixture on zebrafish embryos. The 48 h LC50 value of triclosan is calculated to be 1.34 mg/L (Fig. 3(a)), which well agrees with the toxicity of TCS on zebrafish embryos with LC50,96h of 1.3–1.9 mg/L in our previous study (Di Paolo et al., 2016b). Although our results are higher than LC50,96h for zebrafish embryos exposure in 24-well plates (Oliveira et al., 2009) and other fish species in previous researches (Table S2), this discrepancy could be related to differences in medium volumes and ratin surface area to volume of exposure vessels (Di Paolo et al., 2016b). The 48 h LC50 value of triclosan is calculated to be 1.28 mg/L (Fig. 3(a)). This indicates that the reaction mixture also showed a high developmental toxicity on zebrafish embryos, which was comparable to that of TCS.

The TCS concentrations in the mixture were calculated on the basis of quantification data of TCS in the stock solution obtained from HPLC-MS/MS, which took up 63% (g/g) in the mixture. In order to investigate the contribution of TCS to the toxicity of reaction mixture, the dose-response curve of TCS concentrations in the mixture was compared to the dose-response curve of TCS standards (Fig. 3(b)). The two dose-response curves are close but do not coincide, indicating that TCS is not the only component inducing the toxicity in the mixture.

It is known that some products, such as 2,4-DCP and 3,5-DC, are toxic on fish (Hattula et al., 1981). Dioxin-like products may also induce reproductive and developmental problems on fish (Ding et al., 2015; Latch et al., 2003; Latch et al., 2005; Mezcua et al., 2004; Tohidi and Cal, 2017). To identify the metabolites responsible for the mixture toxicity, we should evaluate the toxicity of each individual product. However, the toxicity data of these products are very limited due to some products are not commercial available. Therefore, we used the ECOSAR (Ecological Structure Activity Relationships) program to predict the LC50 values of individual transformation product on fish (Table S2). The calculated LC50 value of TCS on fish is 0.48 mg/L, which is in good agreement with the available experimental toxicity data. It indicates that the ECOSAR program is a suitable tool to predict acute toxicity of TCS and its products. According to criteria set by European Union, TCS and the five TPs can be classified as very toxic compounds to fish (LC50 < 1 mg/L), while P2 and P3 can be classified as toxic

Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>Toxic Potential</th>
<th>Main target proteins</th>
</tr>
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<tbody>
<tr>
<td>TCS</td>
<td>0.451 °</td>
<td>AR, AhR, ERβγ, GR, LXR, MR, PR, TRα, TRβ</td>
</tr>
<tr>
<td>P1</td>
<td>0.165 °</td>
<td>—</td>
</tr>
<tr>
<td>P2</td>
<td>0.213 °</td>
<td>—</td>
</tr>
<tr>
<td>P3</td>
<td>0.337 °</td>
<td>AR, AhR, ERα, ERβ, PR</td>
</tr>
<tr>
<td>P4</td>
<td>0.389 °</td>
<td>AR, AhR, CYP2C9, CYP2D6, ERα, ERβ, GR, LXR, MR, PR, TRα, TRβ</td>
</tr>
<tr>
<td>P5</td>
<td>0.474 °</td>
<td>AR, AhR, CYP1A2, CYP2D6, ERα, ERβ, GR, LXR, MR, PR, TRα, TRβ</td>
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<tr>
<td>P6</td>
<td>0.454 °</td>
<td>AR, AhR, ERα, ERβ, GR, MR, PR, TRα, TRβ</td>
</tr>
<tr>
<td>P7</td>
<td>0.429 °</td>
<td>AR, AhR, ERβ, GR, LXR, MR, PR, TRα, TRβ</td>
</tr>
<tr>
<td>P8</td>
<td>0.443 °</td>
<td>GR</td>
</tr>
</tbody>
</table>

Toxic potential degrees: °—low risk, °°—moderate risk, °°°—high risk, °°°°—very high risk.

Fig. 2. Transformation pathways of TCS during the simulated metabolism.
compound (1 mg/L < LC₅₀ < 10 mg/L) and P1 as harmful compound (LC₅₀ > 10 mg/L). These findings suggest that most metabolites probably contribute to the toxicity of the reaction mixture together with the parent compound TCS, which is consistent with the results in the FET test.

The evolutions of the acute toxicity through three transformation pathways are shown in Fig. 4 based on the predicted LC₅₀ values of TCS and its metabolites. The three transformation products formed in the Route A were one or two level less toxic than TCS, which indicates the toxicity decreased through breaking ether-bond. The three products (P6, P7 and P8) formed through Route B showed similar toxicity on fish as the parent compound. The results indicate that P6, P7 and P8, which have similar structure as TCS, may retain the toxicological properties with the parent compound. Through Pathway C, the acute toxicity of TCS apparently increased with the formation of highly toxic dioxin-like products P4 (LC₅₀, predicted 0.19 mg/L) and P5 (LC₅₀, predicted 0.11 mg/L). The results suggest the high risk of TCS transformed through cyclization mechanisms. In conclusion, only ether-bond cleavage reactions can detoxify TCS; the other metabolism mechanisms may form similar or higher toxic products.

3.2.3. Lethal and sub-lethal effects of the reaction mixture on fish embryos

The lethal and sub-lethal effects of the reaction mixture after 48 h exposure are shown in Fig. 5 comparing with the solvent control group. Coagulation, developmental retardation, heart edema and slow heartbeats were the main toxicological effects on fish embryo in the present study. Almost all fish embryos coagulated at the exposure concentrations above 2.5 mg/L of the reaction mixture, while mortality rates declined to lower than 10% at concentrations lower than 1.25 mg/L. Abnormal effects, including no pigmentation, missing eye primordial, underdeveloped tail, and malformation in cardiovascular system were mostly observed at relatively low exposure concentrations (≤ 1.25 mg/L). In particular, cardiovascular disorders, such as pericardial edema and slow heartbeats, were found on around 60% of the embryos exposed to 1.25 mg/L and 30% of the embryos exposed to 0.625 mg/L.

In the study of Oliveira et al. (2009), the toxicological effects of triclosan on zebrafish embryos and adults were well studied. Developmental effects, including delay on otolith formation, delay on eyes and body pigmentation, pericardial edema and spine malformations, were observed at the early-stages of zebrafish. Nassef et al. (2010) found TCS caused eye developmental retardation, hemorrhage, yolk-sac shrinkage, effects on heat-beat rate and spine curvature in medaka fish embryos and larvae. Obviously, the toxicological effects of triclosan reaction mixture on zebrafish embryos observed in our study are consistent with the findings of triclosan in the literature. It indicates the metabolites may induce similar adverse effects on the zebrafish embryos as triclosan, in particular cardiovascular disorders, which are mostly observed.

Furthermore, the malformations in cardiovascular system and skeleton, such as abnormal development of tail, pericardial edema and slow heartbeats, were reported to be typical AhR-mediated effects in zebrafish embryos (Di Paolo et al., 2015; Xiao et al., 2016), which are consistent with the QSAR modeling prediction of AhR activity. In addition, the products with dioxin like structures may induce AhR mediated effects on fish by blocking the activity of enzymes (Peterson et al., 1993). Therefore, AhR mediated effects of triclosan and the reaction mixture on zebrafish embryos should be further evaluated.

3.2.4. AhR mediated activity

To investigate the potential of TCS and its reaction mixture to activate AhR in zebrafish embryos, we took the fishes exposed to the lowest concentration of triclosan and the reaction mixture for 48 h in FET test for cyp1a gene expression analysis. Some organic pollutants can represent ligands for AhR, which mediates transcription of cyp1a gene. Therefore, induction of cyp1a has been intensively used as a biomarker of environmental pollution for AhR mediated toxicity in many previous researches (Fleming and Di Giulio, 2011; Toyoshiba et al., 2004). As shown
in Fig. 6, significantly upregulated cyp1a expression was found in the sample of TCS reaction mixture (2.8 fold), while no significant induction was found after exposure to TCS (1.2 fold) for 48 h. The results indicate that the TCS reaction mixture may activate the AhR, which is consistent with the AhR mediated effects on zebrafish embryos observed in FET test. No significant induction of cyp1a expression in zebrafish embryos under 48 h TCS exposure was found in the present study. Although induction and inhibition of cyp1a gene expression by exposure to triclosan were reported in the literature (Ku et al., 2014; Liang et al., 2013; Szychowski et al., 2016; Zhou et al., 2017), it seems to depend on the exposure concentrations and exposure time. Our findings indicate that the observed AhR mediated activity of the reaction mixture at 48 h exposure in FET test might primarily be related to the transformation products in the mixture. Therefore, further study on cyp1a gene expression of triclosan and the reaction mixture should take different exposure concentrations and time points into consideration.

4. Conclusions

In the present study, we successfully simulated TCS metabolism using laboratory EC-MS, and evaluated the potential risks of TCS and its metabolites using modeling tools and bioassays.

Eight potential metabolites of TCS formed mainly via the mechanisms of hydroxylation, ether cleavage and cyclization in this study. Two dioxin derivatives via cyclization reactions of TCS were for the first time reported as potential metabolites in the present study.

The reaction mixture after electrochemical reactions induced high toxicity on zebrafish embryos, which was comparable to that of TCS in FET test. The evolution of the acute toxicity through three transformation pathways showed that only ether-bond reactions can detoxify TCS while the products formed through the other transformation mechanisms are highly toxic.

Typical AhR mediated effects, such as developmental retardation in skeleton, and malformations in cardiovascular system, were exerted by TCS reaction mixture on zebrafish embryos. Furthermore, a significant induction in cyp1a gene expression was found after 48 h exposure to the TCS reaction mixture while there was no induction or inhibition observed in the sample of TCS, suggesting the metabolites of triclosan may activate AhR in zebrafish embryos even at very low concentrations in the mixture.

This study highlights that TCS and its metabolites may cause serious adverse effects in aquatic system if TCS is continuously used and released into the environment. Therefore, this chemical should be considered on the priority list of emerging contaminants and its utilization in all products should be regulated.

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