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Fate and effects of triclosan in subtropical river biofilms

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Abstract

Triclosan (TCS, 5-chloro-2-(2,4-dichlorophenoxy) phenol) is a broad-spectrum antimicrobial compound. Owing to its wide use, TCS has been frequently detected in river systems, especially in the (sub-)tropics. However, little information on its interaction with river biofilm in the (sub)tropics is currently available. In the present study, subtropical river biofilms were chronically exposed to TCS for 14 d at concentrations of 0.1-100 μg/L in artificial river water, which was followed by a 7 d recovery period. The results show that 100 μg/L TCS inhibited the growth of river biofilms and the no-observed-effect concentration (NOEC) of TCS on river biofilms was 10 μg/L. The affected biofilms did not completely recover within the 7 d of recovery period due to the adsorbed TCS which was not removed together with dissolved TCS. Exposure to TCS caused significant changes in prokaryotic species composition of river biofilms but no significant effects on eukaryotic species composition. In particular, the relative abundance of several TCS-tolerant bacterial species (e.g., *Pseudoxanthomonas mexicana*, *Sphingopyxis alaskensis* and *Sphingomonas wittichii*) in river biofilms increased following exposure to 10 and 100 μg/L TCS. River biofilm efficiently removed TCS from the liquid phase and the pH values of the aquatic system significantly affected the removal efficiency of TCS (from 36% at pH 6.5 to 60% at pH 8.5). No degradation products were detected in the liquid phase after 5 days of exposure, possibly due to strong adsorption of the hydrophobic degradation products to river biofilms and through biodegradation by bacteria utilizing TCS and its degradation products as source of carbon and energy for growth, such as *Methyloversalitis universalis* and *Methylobacterium*

aquaticum.

Keywords: Triclosan; River biofims; Sub-tropics; Growth inhibition; Community composition; Microorganisms

1 Introduction

River biofilms are assemblies of bacteria, algae, and fungi embedded in extracellular polymeric substances (Hall-Stoodley et al., 2004; Branda et al., 2005). They are important constituents of river ecosystems to maintain their function in terms of nutrient retention, producing organic substrates, feeding aquatic animals and organic matter re-mineralization (Huerta et al. 2016; Bechtold et al., 2012; Proia et al., 2012). In rivers and streams, biofilms consist of diverse species, have abundant biomass, are distributed ubiquitously, and are thereby exposed to and interact with various stressors.

Triclosan (TCS, 5-chloro-2-(2,4-dichlorophenoxy)phenol) is a broad-spectrum antimicrobial compound used in a wide range of consumer products i.e. antimicrobial soaps and body washes, toothpastes, cosmetics, clothing, kitchenware, furniture, and toys. As a typical emerging contaminant, TCS has been detected worldwide in rivers and lakes with concentrations up to 8.72 µg/L (Zhao et al., 2010; Ramaswamy et al., 2011; Cuderman and Heath, 2007; Perez et al., 2013; Zhang et al., 2015; Peng et al., 2017; Lehutso et al., 2017). Meanwhile, numerous studies have indicated the toxicological effects of TCS on aquatic communities. For instance, TCS possibly affects multiple target sites in different microalgal species which showed varying sensitivities to TCS (Franz et al., 2008). 7.9 mg/kg TCS in sediment increased the relative abundance of cyanobacteria and resulted in a dramatic die-off of algae within artificial streams (Drury et al., 2013). Besides, exposure to TCS at the concentration of 10 µg/L caused changes in bacterial community composition in river biofilms (Lawrence et al., 2009). In addition, Nietch et al. (2013) observed that stream periphytic biofilms were stimulated at low doses of TCS (0.1, 0.5 and 1 µg/L) but inhibited at high doses (5 and 10 µg/L). The effects of TCS on river biofilm communities remained, even after a recovery period (Proia et al., 2011; Lawrence et al., 2015). However, most of the toxicological studies are limited to temperate regions, although the highest concentrations of TCS were detected in subtropical ecosystems (Zhang et al., 2015; Peng et al., 2017). A recent study performed in Thailand (Khatikarn et al., 2018) reported 96-h LC50 values for five invertebrate species ranging from 72 to 962 µg/L and concluded no significant difference between the sensitivity of aquatic species from tropical and temperate regions. Since algae proved to be more sensitive but hardly any (sub-)tropical data is available (Khatikarn et al., 2018), it is necessary to perform more studies using algal communities to provide a comprehensive understanding on the mechanism of TCS risks in (sub-)tropical freshwater systems.

Biofilms play a vital role in water purification (Chenier et al., 2003; Tien and Chen, 2013) and use certain minerals and organic pollutants, resulting in a decrease in dissolved concentration of such pollutants in the water (Podda et al. 2014). Several studies have investigated the adsorption and degradation processes of TCS in freshwater environments. For example, the stable TCS concentrations in algae compared to the decreasing TCS concentration in the water phase implied a maximum adsorption ability of TCS to algae (Coogan et al., 2007). Higher sorption capacity of the sediment might reduce TCS bioavailability and its degradation rate in sediment (Huang et al., 2015). As to biodegradation, TCS can be degraded by both ammonia-oxidizing bacteria and heterotrophic microorganisms in activated sludge (Roh et al., 2009). Bacterial strains identified as *Pseudomonas* sp. are tolerant to TCS and can degrade TCS under aerobic, anoxic, and anaerobic conditions (Gangadharan Puthiya Veetil et al., 2012). However, little is known on how river biofilms can remove TCS from the overlying water phase, although 14C–triclosan studies have shown that only four to seven percent of the radioactive TCS was recovered sorbed to organic material or retained by the biofilm (Lawrence et al., 2015)..

To fill in the lack of data on the fate and effects of TCS on (sub-)tropical river biofilms, we exposed river biofilms to TCS using a range of concentrations (0.1-100 μg/L) including the concentrations of TCS occurring in natural sub-tropical river systems (Zhang et al., 2015; Peng et al., 2017; Lehutso et al., 2017). We evaluated the shifts in community structure and function using high-throughput sequencing and related statistical analyses. Additionally, we investigated the degradation of dissolved TCS by river biofilms under different pH conditions in terms of degradation rates and degradation products.

2 Materials and methods

2.1 Culture of biofilm

The chemical composition of artificial river water was adopted from Ylla et al. (2009) (12 mg/L Na_2SO_4, 20 mg/L Na_2SiO_3, 30 mg/L $CaCl_2$, 1 mg/L KCl, 2 mg/L $MgSO_4$ and 20 mg/L $NaHCO_3$) and was modified with the addition of 8 mg/L NH_4Cl and 1.6 mg/L Na_3PO_4 to simulate the eutrophication status of the sub-tropical river (Heng River, Boluo, Huizhou, China). The tap water which was used as solvent met the Standards for Drinking Water Quality in China (GB 5749-2006) and was aerated for more than 96 h before the artificial river water preparation.

The inoculum was collected from the submerged surface of about 10 rocks in Heng River and resuspended in the artificial river water of ca. 15 L. Subsequently, 80 pieces of sand-blasted glass slides (2 cm × 10 cm) were placed into the water for 5 weeks for biofilm colonization. The water flow was driven by a mini-pump and kept under a controlled condition at 25 °C and a light intensity of 4000 lux with a dark/light cycle of 12 h: 12 h. Half

of the artificial river water was renewed every week until the surface of each slide was covered by green biofilm after 5 weeks.

2.2 Toxicity experiment

2.2.1 Set-up of treatment and recovery

Stock solutions were prepared by dissolving TCS (purity 99.5 ± 0.5%, QMX Laboratories Ltd., Essex, UK) in acetone (HPLC grade, Merck, Shanghai, China) and stored at -20 °C before use. This toxicity experiment was conducted in 1 L beakers with four TCS treatments (0.1 µg/L, 1 µg/L, 10 µg/L and 100 µg/L; n=4 replicates) and one acetone control (n=4). After adjusting the pH to 7.0 ± 0.1 using 1 M HCl and 1 M NaOH, 1 L of the artificial river water was spiked with 200 µL of corresponding stock solution to obtain the initial TCS concentration in each replicate and the control received 158 mg/L acetone. Three colonized glass slides were dipped in each replicate and the artificial river water plus TCS was renewed every 48 h. Following the14-day exposure period, there was a 7-day recovery period when the water was replaced by new artificial river water at pH 7.0 ± 0.1 every 48 h.

2.2.2 TCS extraction and analysis

To determine TCS concentrations, 500 mL solution was sampled from each replicate after being spiked with the stock solution and the same was done before renewal to assess the decrease in TCS concentration. The TCS in the water samples collected from the toxicity experiment was extracted by solid phase extraction (SPE) method (Zhao et al., 2010). Briefly, each sample was filtered through a 0.7-µm glass fibre filter membrane, and extracted using an Oasis HLB cartridge (500 mg, 6 mL) conditioned with methanol and water. The filtered water samples were passed through the cartridges at a flow rate of 5-10 mL/min. Each sample bottle was rinsed twice with two aliquots of 50 mL of 5% (v/v) methanol in Milli-Q water, which also passed through the cartridge. After loading of water samples, the cartridges were dried under vacuum for 2 h, and then eluted each with 7 mL of methanol and 5 mL of dichloromethane in sequence. The eluates were combined and dried under a gentle nitrogen stream, dissolved in 1 mL of methanol, filtered through a 0.22-µm membrane filter into a 2-mL amber glass vial, and kept at -20 °C before the instrumental analysis.

An Agilent 1200 series high performance liquid chromatograph (HPLC) fitted with a diode array detector was used for the analytical verification (Yang et al., 2011). Briefly, a SGE C18 RS column (100 × 4.6 mm, 5 m) with a guard column (C18, 4.6 × 7.5 mm, 5 m) was used for the separation of TCS. Acetonitrile (ACN, HPLC grade, Merck, Shanghai, China) and Milli-Q water (Millipore, Watford) with 0.1% acetic acid were used as the mobile phase, which was programmed at 70% ACN for 6.5 min followed by a post time of 2 min. The injection volume was 100 µL and the flow rate was set at 1 mL/min. The UV wavelength for detection was 205 nm. The retention time for TCS was 5.0 min. The instrumental limit of quantification (LOQ) for TCS was 5 µg/L and the limit of detection

147 (LOD) was 1 μg/L.

148 *2.2.3 Biomass and chlorophyll-a*

149 At the beginning of exposure (Day 0), biofilm samples were collected from four
150 additional replicates which were not exposed to TCS. By the end of exposure (Day 14) and
151 recovery (Day 21) periods, biofilms were scraped from randomly selected glass slides from
152 each replicate. Specifically, a 18 cm^2 of biofilm sample was taken for biomass measurement
153 and a 12 cm^2 for photosynthetic pigment determination.

154 To measure biomass (dry weight, DW), biofilm samples were dried at 80°C for more
155 than 4 h until constant weight was obtained using an electronic balance. To extract the
156 photosynthetic pigment, a previously described method was adopted (Peng et al., 2014).
157 Briefly, biofilm samples were frozen (-20 °C) for 20 min and thawed (25 °C) for 5 min,
158 which was repeated three times and followed by being frozen (-20 °C) overnight until the
159 cell walls were broken. The processed biofilm was suspended in 95% ethyl alcohol, heated at
160 80 °C for 2 min, and then kept static for 6 h at room temperature. After centrifugation for 5
161 min at 5,445*g*, the absorbance values of the supernatant at 665 nm (A_{665}) and 649 nm (A_{649})
162 within the measurement range of the spectrophotometer were detected and chlorophyll-a
163 (C_A) content was calculated according to:

164 $$C_A\,(mg/L) = 13.95\,A_{665} - 6.88\,A_{649} \quad (1)$$

165 *2.2.4 Molecular endpoints*

166 For molecular analyses, a 20 cm^2 of biofilm sample was collected from each replicate
167 at 3 time points (Day 0, Day 14 and Day 21) according to the same protocol of paragraph
168 2.2.3 and stored at -80 °C.

169 Total genome DNA of biofilm was extracted using the CTAB/SDS method (Saghai-
170 Maroof et al. 1984; Dellaporta et al. 1983). 16S rRNA/18SrRNA genes of distinct regions
171 (16SV4/16SV3/16SV3-V4/16SV4-V5, 18S V4/18S V9) were amplified using specific
172 primers (e.g. 16S V4: 515F-806R, 18S V4: 528F-706R, 18S V9: 1380F-1510R, et al.) with
173 the barcode. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master
174 Mix (New England Biolabs). The same volume of 1X loading buffer (contained SYB green)
175 was mixed with PCR product in equidensity ratios and electrophoresis was operated on 2%
176 agarose gel for detection. Samples with bright main strip between 400-450 bp were chosen
177 for further experiments. Then, PCR product was purified with Qiagen Gel Extraction Kit
178 (Qiagen, Germany).

179 Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample
180 Preparation Kit (Illumina, USA) following manufacturer's recommendations and index codes
181 were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo
182 Scientific) and Agilent Bioanalyzer 2100 system. Lastly, the library was sequenced on an

Illumina HiSeq2500 platform and 250 bp paired-end reads were generated.

Sequences analyses were performed by Uparse v7.0.1001 (http://drive5.com/uparse/) (Edgar, 2013). Sequences with ≥ 97% similarity were assigned to the same operational taxonomic units (OTUs). Representative sequence for each OTU was screened for further annotation based on the GreenGene Database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) and RDP classifier v2.2 (http://sourceforge.net/projects/rdp-classifier/) (Wang et al., 2007). OTUs abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences.

2.2.5 Statistical analyses on toxicity endpoints

To compare the differences of biofilm response between the TCS treatments and the control in the toxicity experiment, the Williams tests (Williams, 1972) were carried out to determine the no-observed-effect concentrations (NOECs) of TCS based on the untransformed data of dry weight, chlorophyll-a content and their ratio (C_A/DW). The analyses were performed with the Community Analysis computer program version 4.3.05 (Hommen et al., 1994) using a significance level of 0.05.

The community composition of each sample was summarized and compared based on the top 10 relative abundance data at the levels of phylum, class, order, family, genus and species. Meanwhile, the total relative abundance data at the species level were arcsine transformed for principle response curve (PRC) analyses in Canoco 5.1 (Van den Brink et al., 1999; Ter Braak and Smilauer, 2012). The PRC method, based on redundancy analysis (RDA), shows the effects of chemical stress on community response over time compared to control test systems (Miranda et al., 2018). The principal component was plotted against time, yielding a principal response curve of the community for each treatment.

To assess the significance of the effects of TCS concentrations on the biofilm community composition, RDA analyses accompanied with Monte Carlo permutation tests were also performed per sampling date for the relative abundance dataset testing each treatment separately against the control.

2.3 TCS degradation experiment

2.3.1 Set-up of degradation systems

1 L of artificial river water was spiked with 200 µL of TCS stock solution to obtain the final concentration of 100 µg/L in each beaker. Replicated (n=3) treatments containing five pH regimes were established by respectively adjusting their pH values to 6.5, 7.0, 7.5, 8.0, and 8.5, as described in paragraph 2.2. Three colonized glass slides were dipped into each replicate. As a negative control, clean glass slides were employed in sterilized artificial river water at pH 7.0 ± 0.1. The systems were kept at 25 °C and no illumination was provided during this experiment to minimize the photolysis of TCS.

2.3.2 TCS and degradation products

Dissolved TCS concentrations were directly analysed by HPLC following the same method as described in paragraph 2.2.2 by sampling 1 mL of the solution from each replicate at 0 h, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h, 72 h, 96 h, and 120 h.

At the end of degradation experiment (120 h), 100 mL of the solution from each replicate was collected for breakdown product analysis. The potential degradation products of TCS were extracted by liquid-liquid extraction (Yang et al., 2011). Briefly, 100 mL of the solution was collected from each replicate, the pH value was adjusted to about 2 using 2 M HCl, and subsequently NaCl was added until the solution was saturated. Then the solution was mixed with 3 × 10 mL dichloromethane (DCM, HPLC grade, Merck, Shanghai, China) by vigorous shaking. The DCM containing extract was passed through an anhydrous Na_2SO_4 column (1 g, CNW Technologies, Dusseldorf, Germany) to remove water. Then the DCM was evaporated under a gentle nitrogen stream and the extract was re-dissolved in 1 mL of methanol. Each final extract was then filtered through a 0.22 m nylon syringe filter (Shanghai ANPEL, China) into a 2 mL amber glass vial which was kept at -20 °C until analysis.

The degradation products were detected by a gas chromatography-mass spectrometer (GC-MS), which was an Agilent 6890N gas chromatograph (Agilent, USA) connected to an Agilent 5975B MSD mass spectrometer with a DB-5MS capillary column (30 m × 0.25 mm, 0.25 m film thickness) (J&W, USA). The GC conditions were given as follows: a sample volume of 5 µL injected in the splitless mode at 250 °C, the oven temperature programmed from 50 °C (5 min) to 300 °C at 8 °C/min followed by a 5 min hold at 280 °C, and helium used as the carrier gas at a flow rate of 1.0 mL/min. Mass spectrometer was operated under electron ionization mode at 70 eV with a mass scan range of 40-500 amu. The temperatures of the ion source and interface were 250 °C and 300 °C, respectively.

2.3.3 Statistical analyses on degradation efficiencies

The degradation rates of TCS were calculated in SPSS 16.0 based on the first-order dynamic equation:

$$Pr = a * e^{(-kt)} \quad (2)$$

Where *Pr* stands for the proportion of residual TCS to corresponding initial concentration, *t* means the time after starting the removal experiment (h), *k* is degradation rate, and *a* is a constant.

In addition, the half-life ($T_{1/2}$) of TCS was derived out according to the equation:

$$T_{1/2} = (\ln 2)/k \quad (3)$$

The dissolved TCS concentrations were natural logarithmic transformed before

254 performing Williams tests (see paragraph 2.2.5). Since we were interested in the influence of
255 pH on TCS degradation, the lowest pH treatment (pH = 6.5) was used as reference.

256

257 **3 Results**

258 *3.1 Effects of TCS on river biofilms*

259 *3.1.1 Biofilm growth*

260 The biofilms had a total biomass of 64.8 µg DW/cm^2 and a chlorophyll-a content of
261 1.26 µg C_A/cm^2 at the beginning of the exposure (Day 0). During the renewal interval of 48 h
262 in the exposure period, the dissolved TCS concentrations in the treatments decreased from
263 0.1 to not detected, from 1 to 0.068 µg/L, from 10 to 1.26 µg/L, from 100 to 11.6 µg/L,
264 respectively (Table S1). After the 14-day exposure, the biomass and chlorophyll-a of the
265 biofilms increased in all treatments including the control (Figure 1). The Williams test results
266 show that only the 100 µg/L of TCS treatment significantly inhibited biofilm growth
267 compared to the remaining treatments in terms of total biomass (252 µg DW/cm^2 versus 424-
268 578 µg DW/cm^2) and chlorophyll-a contents (3.02 µg/cm^2 versus 5.18-5.42 µg/cm^2). After
269 the 7-day recovery period, biofilms in all treatments increased 1.24-1.99 times in total
270 biomass and 1.75-2.53 times in chlorophyll-a content compared to the levels at the end of the
271 exposure. But a significant effect was still indicated for the highest treatment level, so no
272 complete recovery took place (Table S2). On the contrary, the calculated chlorophyll-a to dry
273 weight ratios showed no significant differences among all treatments compared to the control
274 during the whole experiment period (Figure 1 & Table S2).

275 *3.1.2 Molecular analyses*

276 Before exposure to TCS, the predominant sequences in the detected prokaryotic
277 communities were those representing the phyla Proteobacteria (69%), Planctomycetes
278 (6.3%) and Cyanobacteria (4.4%). Figure 2A shows that exposure to a high dose of TCS for
279 14 days caused an higher increase in the relative abundance of the 10 most abundant families
280 in the two highest treatments (70% in 10 µg/L TCS and 72% in 100 µg/L TCS) compared to
281 the control (63%), resulting in a decreased species diversity. Methylophilaceae and
282 Rhodospirillales became relatively abundant after exposure in all TCS treatments, as well as
283 exposure to 100 µg/L TCS significantly increased the relative abundance of
284 Xanthomonadaceae and Sphingomonadaceae and decreased the relative abundance of
285 Xanthobacteraceae. After the recovery period, the most abundant 10 families still had a
286 larger proportion of the relative abundance in the TCS treatments compared to the control
287 (54-60% versus 48%), although all the biofilm communities had recovered to a certain extent
288 (Figure 2A). In particular, after this period, the relative abundances of Comamonadaceae,
289 Xanthobaceraceae and Methylophilaceae were reduced in all the treatments and the control,

while Planctomycetaceae and Chloroplast increased their relative abundance in all the TCS treatments. It is notable that there was a large increase of Xanthomonadaceae in the 100 μg/L TCS treatment (9.7%) compared to the other treatments and the control (0.36-0.49%).

During the 14-day exposure, the predominant sequences of Chloroplastida and Eukaryota were, to a certain extent, replaced by Fungi and Metazoa, reflected by the reduced relative abundances of Chlorophyceae and Spirotrichea and the increased ones of Nectriacea and Adinetida (Figure 2B). In addition, the relative abundances of Haplotaxida and Eustigmatales increased significantly in all the TCS treatments compared to the control ($P < 0.05$). After the 7-day recovery period, the relative abundance of Chloroplastida increased, while Fungi and Metazoa disappeared in all the treatments including the control (Figure 2B). The relative abundances of Eustigmatales were still significantly higher in the TCS treatments compared to the control after the recovery period ($P < 0.05$).

Using the time series of the control groups as reference, PRC analyses were carried out based on the relative abundances at the species level (Figure 3A). Of all variance, 33% could be attributed to sampling date, which is displayed on the horizontal axis. 30% of all variance could be explained by the exposure levels, 52% of which is displayed in the PRC ($P = 0.04$). The results of Monte Carlo permutation tests show that 14-day exposure to TCS resulted in significant changes of the species composition for all treatment levels, while only the biofilm exposed to TCS at 0.1 μg/L recovered afterwards (Table 1). However, since in the PRC, *Methylotenera* sp. and 'Others' exhibited extremely opposite response compared to the other taxa (Figure 3A), the dataset was reanalysed after excluding *Methylotenera* sp. and 'Others' to optimally show the response of the other taxa as well (Figure 3B). Of all variance, 15% could be attributed to sampling date. 40% of all variance could be explained by the exposure levels, 55% of which is displayed in the PRC ($P = 0.002$). It can be seen that 14-day exposure resulted in a large shift of the community composition, especially in the high TCS treatments (10 and 100 μg/L), as revealed by the high relative abundances for the phyla of Proteobacteria (e.g., alpha_BAC233, *Sphingomonas wittichii*, *Pseudoxanthomonas mexicana* and *Sphingopyxis alaskensis*), Actinobacteria (e.g., *Aciditerrimonas* sp., *Sporichthya* sp. and bacterium_rJ7), and Bacteroidetes (e.g. *Niastella* sp.), as well as the low relative abundances of the phylum of Actinobacteria (e.g. *Gaiella* sp.), Planctomycetes (e.g. *Gemmata* sp. and clone_B55.2011) and some other species of Proteobacteria (e.g. *Methyloversatilis universalis*, *Ralstonia pickettii*, *Caulobacter* sp., *Legionella* sp.). After the 7 d recovery period in uncontaminated water, the differences of bacterial community composition with the control were smaller in river biofilms exposed to 10 and 100 μg/L TCS (Figure 3B).

The results of PRC and Monte Carlo permutation tests on eukaryotic data showed that the TCS treatments did not explain a significant part of the variation in eukaryotic community composition ($P > 0.05$).

3.2 Degradation of TCS by river biofilms

The initial TCS concentrations in the treatments were: 100 µg/L (sterilized control), 92 µg/L (pH6.5), 92 µg/L (pH7.0), 94 µg/L (pH7.5), 93 µg/L (pH8.0) and 101 µg/L (pH8.5). Figure 4 shows that the TCS concentration was constant in the sterilized control, implying that no other degradation processes than microbial degradation was of importance during the whole experiment. At 120 h, the residual dissolved TCS in each pH treatments were reduced to 36 to 60%, showing a stronger removal efficiency at higher pH values.

The half-life of TCS at pH8.5 was calculated as 3.7 d (Table 2), reflecting the relatively high degradation or dissipation efficiency of TCS by river biofilm at pH8.5 when compared to those of the other lower pH treatments (5.1-7.8 d). This is confirmed by the Williams test which calculated a NOEC value of pH of 8.0 at the 120 h time point (Table 3).

Degradation products of TCS were not detected in the artificial river water used in the TCS removal experiment.

4 Discussion

4.1 Effects of TCS on river biofilms

TCS blocks bacterial lipid synthesis through specific inhibition of the NADH-dependent enzyme ENR (enoyl-acyl carrier protein reductase) (Adolfsson-Erici et al., 2002), and affects algae primarily by inhibiting fatty acid synthesis and causing protein aggregation (Xin et al. 2017). Furthermore, Escalada et al. (2005) summarized that low TCS concentrations (20-500 µg/L) affected the growth of several bacteria, while several microalgae were reported with sensitivities (EC50) between 0.7 and 19.1 µg/L, making them the most susceptible group compared to activated-sludge microorganisms, invertebrates and fish, based on single species studies (Orvos, 2002; Tatarazako, 2004). This was confirmed by Lawrence et al. (2015) who showed that exposure to 1.8 µg/L TCS resulted in significant reductions in algal and cyanobacterial biomass but no significant effects were observed on bacterial biomass in river biofilms cultivated in rotating annular reactors. At 0.1 to 0.5 µg/L, TCS even stimulated the stream biofilm with increased bacteria cell densities, but higher doses (5 µg/L and 10 µg/L) significantly decreased bacterial cell densities and cyanobacteria abundance (Nietch et al. 2013). Compared to reductions in algal biomass observed in continuous exposure to TCS, the absence of significant effects of TCS at low levels (≤10 µg/L) in the present study (Figure 1) might be explained by the reduced actual concentrations of TCS resulting from the periodic dosing and degradation process of TCS between renewal intervals. Table S1 shows that after 2 days of exposure only 5-14% of the initial dose is left in the water phase, making the time weighted average concentrations lower that the nominal concentrations, but our characterisation of the exposure dynamics is too coarse to be able to

363 calculate the time weighted average concentrations. At the same time, this is also the
364 possible reason why both algal biomass and the total biomass of river biofilm decreased only
365 by exposure to 100 μg/L TCS in the present study.

366 Moreover, derived from the changes of total biomass and chlorophyll-a content during
367 the recovery period, the biofilm exposed to 100 μg/L TCS showed similar growth rates as the
368 other treatments and control, implying that the low level of biomass after the exposure period
369 is the cause for the lack of recovery in the 100 μg/L TCS treatment (Figure 1)

370 Molecular analyses provide detailed information on the shift of microbial community
371 composition initiated by environmental stressors. As shown in Figure 3, exposure to TCS
372 levels significantly altered the bacterial community composition of river biofilm. For
373 instance, Figure 3A shows that the relative abundance of *Methylotenera* sp. increased
374 significantly in all treatment levels with a decreasing relative abundance of "Others",
375 indicating the negative influence of TCS on biofilm biodiversity. Besides, *Methylotenera* sp.
376 are obligatory or restricted facultative methylamine-utilizing bacteria within the family
377 Methylophilaceae (Figure 2A) (Kalyuzhnaya et al., 2012; Paul et al., 2015) and played a key
378 role in microbial degradation of water pollutants (Yang et al., 2018). In the present study, the
379 increased relative abundance of *Methylotenera* sp. implied its function in the biodegradation
380 of TCS. Furthermore, *P. mexicana* belonging to γ-Proteobacteria, *S. alaskensis* and *S.*
381 *wittichii* belonging to α-Proteobacteria, significantly increased their relative abundance
382 resulting from exposure to higher concentrations (10 μg/L and 100 μg/L) of TCS (Figure
383 3B). Lubarsky et al. (2012) also reported that some species in γ-Proteobacteria appeared
384 insensitive to TCS at 2-100 μg/L. Niculae et al. (2016) observed that a strain of *P. mexicana*
385 degraded aromatic and aliphatic hydrocarbons in different environments.. In addition, several
386 studies confirmed that *S. alaskensis* and *S. wittichii* own the ability to utilize a wide range of
387 organic compounds (Godoy et al., 2003; Nishiyama et al., 1992). Thus TCS and its
388 degradation products, as aromatic compounds, might be utilized by these species as a source
389 of carbon for growth, subsequently leading to the increase of their relative abundance in the
390 present study. To the contrary, two species in β-Proteobacteria, *R. pickettii* and *M.*
391 *universalis*, were inhibited by 10-100 μg/L TCS (Figure 3B). The decreased relative
392 abundance of β-Proteobacteria was observed previously in cultivated river biofilm exposed
393 to 2-100 μg/L TCS (Lubarsky et al., 2012) and in sediment spiked with 12 mg/kg TCS
394 (Drury et al., 2013), indicating they are species sensitive to TCS.

395 The lack of recovery of the river biofilm during the post-exposure period was
396 previously observed under different experimental designs. For instance, Proia et al. (2011)
397 showed that TCS strongly inhibited phosphate uptake (-71%), which did not return to normal
398 values until 2 weeks post-exposure. Lawrence et al. (2015) found that the biomass
399 component patterns of bacteria, cyanobacteria and algae in river biofilms still presented
400 significant differences between the treatments after 2-6 weeks of recovery and the control. In

401 the present study, the growth of biofilm exposed to 100 μg/L TCS was still significantly
402 inhibited compared to the control after the 7-day recovery period (Figure 1). Meanwhile, it
403 can be seen from Figure 3B that the bacterial species composition of biofilms exposed to 10-
404 100 μg/L TCS did not completely return to the control level. The adsorbed TCS might have
405 prevented the recovery as hydrophobic aromatic compound, like TCS, partitions into organic
406 matter, such as extracellular polymeric substances (Branda et al., 2005). Although the
407 dissolved TCS in artificial river water was removed, adsorbed TCS would remain in the river
408 biofilm and continued to affect the microorganisms in the biofilm.

409 In the present study, PRC analyses indicated that exposure to TCS in the range of 0.1-
410 100 μg/L did not show significant effect on eukaryotic community composition of river
411 biofilms, which is consistent with the findings that the exposure to 1.4-2621 μg/L TCS
412 affected the total biomass rather than specific pigments (Johansson et al., 2014). However,
413 being the most susceptible taxa to TCS, microalgal species are probably affected through
414 multiple target sites and the differences in sensitivity of microalgae cover three orders of
415 magnitude (Franz et al., 2008). Thus, it is reasonable to deduce that low levels of exposure to
416 TCS may shift the community composition of microalgal systems, as Lawrence et al. (2015)
417 observed significant changes in pigment composition of algal and cyanobacterial populations
418 in river biofilm exposed to 1.8 μg/L TCS. Therefore, the question of whether the community
419 composition of microalgal species in biofilms is affected significantly by TCS needs to be
420 investigated further by more research work.

421 4.*2 Removal of TCS by river biofilms*

422 TCS is quite stable against hydrolysis and photolysis is a major removal pathway in
423 natural aquatic environment (Aranami et al., 2007; Lindstrom et al., 2002; Tixer et al., 2002).
424 In the toxicity experiment of the present study, more than 88% of the initial TCS in the
425 artificial river water was eliminated within 48 h under illumination. In the following
426 experiment, illumination was shielded and the removal efficiency of TCS by river biofilms
427 was 36% to 60% at a series of pH levels within 120 h, indicating that the photolysis process
428 of TCS was effectively minimized. The half-life of TCS was 3.7 d to 7.8 d in the present
429 degradation study, which was relatively fast compared to the half-life of 18 d for the
430 biodegradation of TCS in aerobic soil (Ying et al., 2007). This could be explained by
431 relatively high relative abundance of TCS-degrading bacteria detected in the present study,
432 such as *S. alaskensis* and *S. wittichii,* (Figure 3). A similar finding was reported by Chen et
433 al. (2011) who found that in a laboratory-scale activated sludge reactor, 75% of the TCS was
434 removed under aerobic conditions within 150 h. Meanwhile, a much higher removal rate of
435 96% in 5 days using an exposure concentration of 10 mg/L, was previously reported
436 (Gangadharan Puthiya Veetil et al., 2012) when using isolated batch cultures of TCS tolerant
437 bacterial strains.

In this removal experiment, biodegradation and adsorption might be the main processes which removed TCS from the liquid phase. TCS deprotonates to its negatively ionic phenolate form at pH > 8.1 (Nietch et al., 2013). Therefore, the higher fraction of neutral TCS was present in the lower pH conditions of this removal experiment. Because of its hydrophilic characteristic, neutral TCS tends to adsorb to river biofilms which contain organic carbon. However, it was observed that the lower pH conditions resulted in lower removal efficiency of TCS, implying that adsorption was not so important for the removal of TCS from the water phase in the present study. Hence, we deduce that the biodegradation by river biofilms might play the vital role. As the pH levels increased from 6.5 to 8.5, the ionized form of TCS replaced the neutral form which is responsible for the majority of TCS's toxic effects (Orvos, 2002). Thus, at higher pH levels, TCS had a lower toxic effect on the growth of river biofilm, subsequently resulting in stronger ability of the biofilm communities to degrade TCS.

In the present study, the bacterial species in the river biofilm communities were most important for the biodegradation of TCS. The results of toxicity experiment indicate that bacterial species, such as *P. mexicana*, *S. alaskensis* and *S. wittichii*, were stimulated by higher concentrations of TCS. Gangadharan Puthiya Veetil et al. (2012) isolated 3 bacterial strains which were tolerant to TCS up to 1 g/L and able to degrade 95% of TCS in 5 days, which were identified as *Pseudomonas* sp. Mulla et al. (2016) confirmed the biodegradation of TCS by *Sphingomonas* sp. which was able to catabolize TCS to intermediates with a lower toxicity. *Sphingopyxis* strain KCY1 is capable of dechlorinating TCS with a stoichiometric release of chloride (Lee et al., 2012). In the present study, these bacterial species survived during exposure to high dose of TCS, utilized or catabolised TCS, and increased their abundance (Figure 3). By contrast, as discussed above, the high concentration of TCS exceeded the tolerance level of most fresh-water algae. Meanwhile, little information is currently available on the degradation potential of algal species for TCS. We may conclude that in this experiment, the algal species in the river biofilm were not able to degrade TCS. On the other hand, based on TCS bioaccumulation factor (BAF) value of 1600 in algal species (Coogan et al., 2007), the adsorption process to algal community in river biofilms might also contribute to the removal of TCS.

Ionized TCS may be degraded by direct environmental photolysis into 2,8-dichlorodibenzo-p-dioxin (2,8-DCDD) and 2,4-dichlorophenol (2,4-DCP) (Latch et al., 2005). However, the photolysis process of TCS was eliminated during our removal experiment. Thus, no 2,4-DCP and 2,8-DCDD could be detected in the river water phase. In addition, methyl-triclosan (Me-TCS) is a metabolite of TCS (Coogan et al., 2007). It has been detected in active sludge (Chen et al., 2011), biosolids-amended soil (Waria et al., 2011) and cultures of certain bacteria in laboratory (Lee et al., 2013). Nevertheless, Me-TCS is more lipophilic and environmentally persistent than the parent compound (Coogan et al.,

2007). Therefore, in our removal experiment, most of the degradation products probably partitioned in the river biofilms. Besides, Chen et al. (2011) found that only 1% of TCS was catalyzed to be Me-TCS with a TCS removal rate of 75% in active sludge. In our removal experiment, it can be deduced that less TCS was transformed to Me-TCS under a relatively low removal efficiency. Thus it should be a better option to detect the degradation products adsorbed by river biofilm instead of those dissolved in the artificial river water. Another reason for the lack of detection of degradation products in our study might be the existence of bacterial species which can utilize organic compounds for growth, such as *Methylotenera* sp., *Methyloversalitis universalis* and *Methylobacterium aquaticum* (Figure 3). They are methylotrophic bacteria which are capable of utilizing single carbon compounds as sole sources of carbon and energy (Kittichotirat et al., 2011; Vuilleumier et al., 2009).

5 Conclusions

The results from this study showed significant inhibition of river biofilm at the high dose treatment by 100 μg/L TCS. The incubation material was collected from a subtropical river in Guangdong Province, so a subtropical community has been tested. Besides, although the lab conditions are not typically subtropical, we can find similar temperatures for subtropical rivers in the research area in March, April, October and November. Exposure to TCS from 0.1 to 100 μg/L resulted in the shift of the taxonomic composition of river biofilms especially in terms of bacteria species. The inhibited biofilm could not completely recover within 7 d of no TCS exposure. Besides, river biofilm could efficiently remove TCS from the aqueous phase through biodegradation and adsorption. Our findings indicate that TCS may pose risks on river ecosystems, and the adaptation of biofilm community to exposure may promote its resistance and removal ability to TCS. Further research is needed to understand the cause of TCS degradation in the system.

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682 the receiving environment. Journal of Hazardous Materials 179(1-3), 215-222.

683 Table 1 *P* values derived from Monte Carlo permutation tests on the differences of prokaryotic
684 community composition between each treatment and the control. T01, T1, T10 and T100 stand
685 for the treatments exposed to triclosan at 0.1 μg/L, 1 μg/L, 10 μg/L and 100 μg/L, respectively.

	T01	T1	T10	T100
Day 14	0.02	0.02	0.02	0.05
Day 21	0.14	0.02	0.02	0.02

686

687 Table 2 The first-order removal parameters and the resulting half-life of triclosan by river
688 biofilms at different pH values

Treatment	a	k (d^{-1})	$T_{1/2}$ (d)	R^2
pH6.5	0.9690	0.0890	7.788	0.9430
pH7.0	0.9475	0.1159	6.796	0.8941
pH7.5	0.9585	0.1020	5.981	0.8849
pH8.0	0.9666	0.1358	5.104	0.8972
pH8.5	0.9544	0.1867	3.713	0.8495

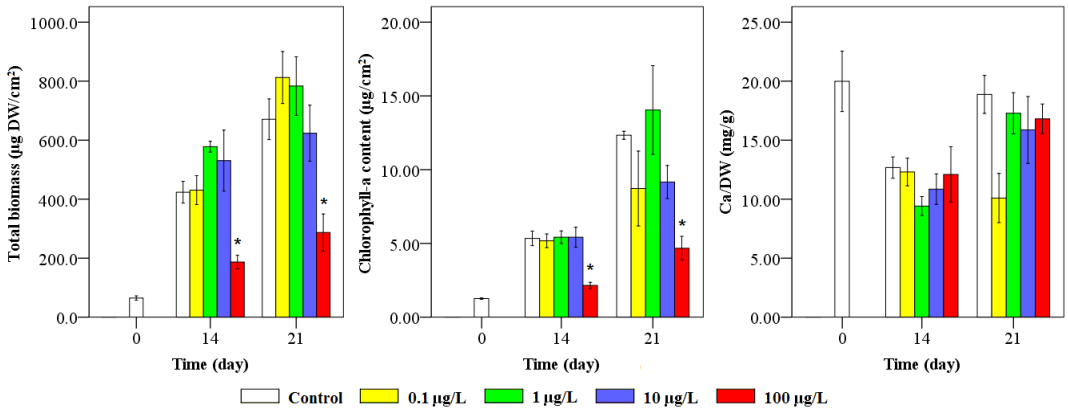
689

690 Table 3 NOECs derived from Williams test for the residual of triclosan (%) at a series of pH
691 levels

692

Time	pH6.5 [a]	pH7.0	pH7.5	pH8.0	pH8.5	NOEC
24 h	85.5	83.3	77.5	80.7	77.6	pH8.5
72 h	76.4	71.2	69.5	69.3	61.3	pH8.0
120 h	60.0	57.4	52.0	46.6	35.8	pH8.0

693 [a] Treatment at pH6.5 was taken as control in Williams test



694

695 Figure 1 Effects of triclosan on the growth of river biofilms in terms of dry weight (DW),
696 chlorophyll-a (C_A), and C_A/DW. The error bars indicate standard errors. The asterisk labels the
697 treatment with significant difference ($P < 0.05$) from the corresponding control at each
698 sampling time (day 14 and day 21).

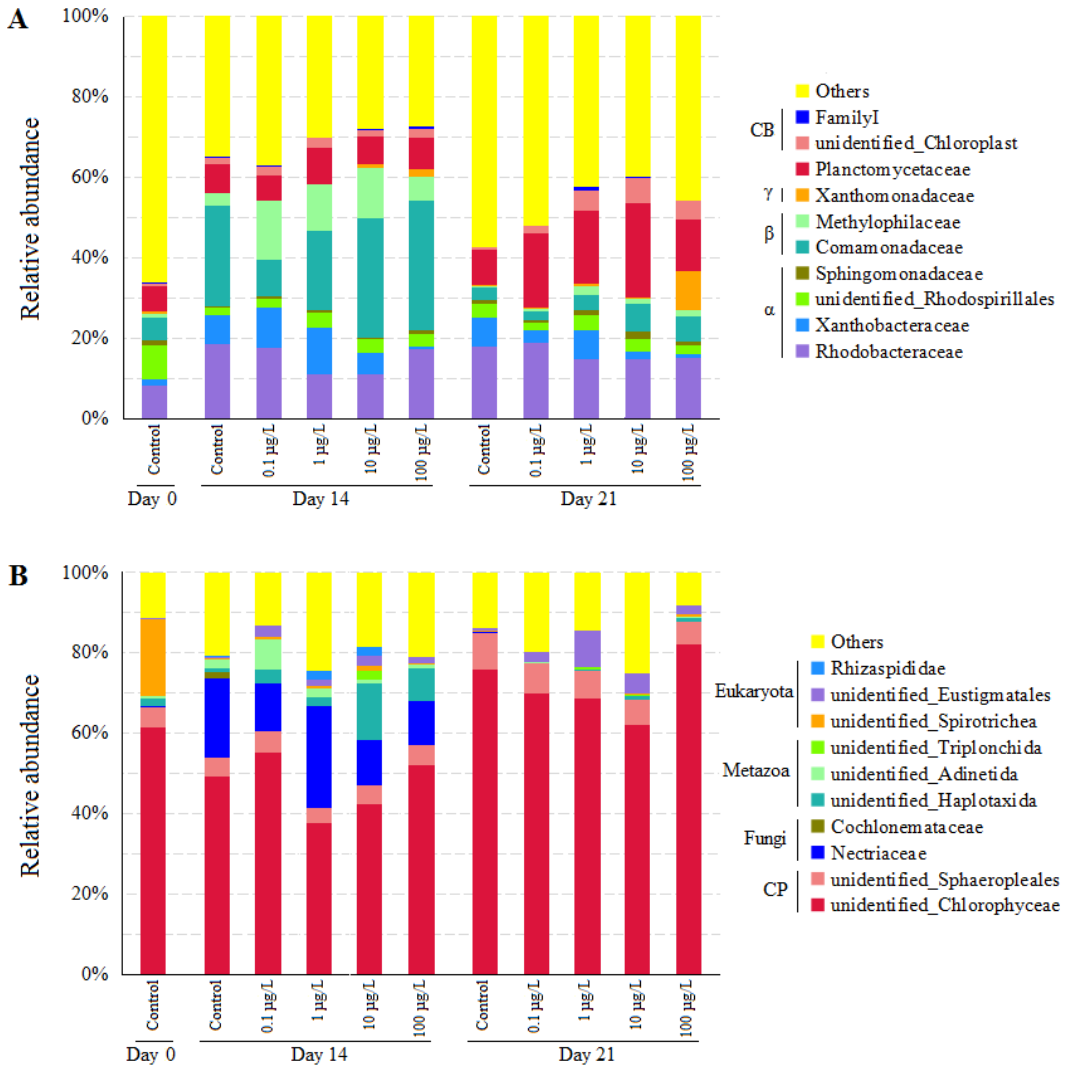
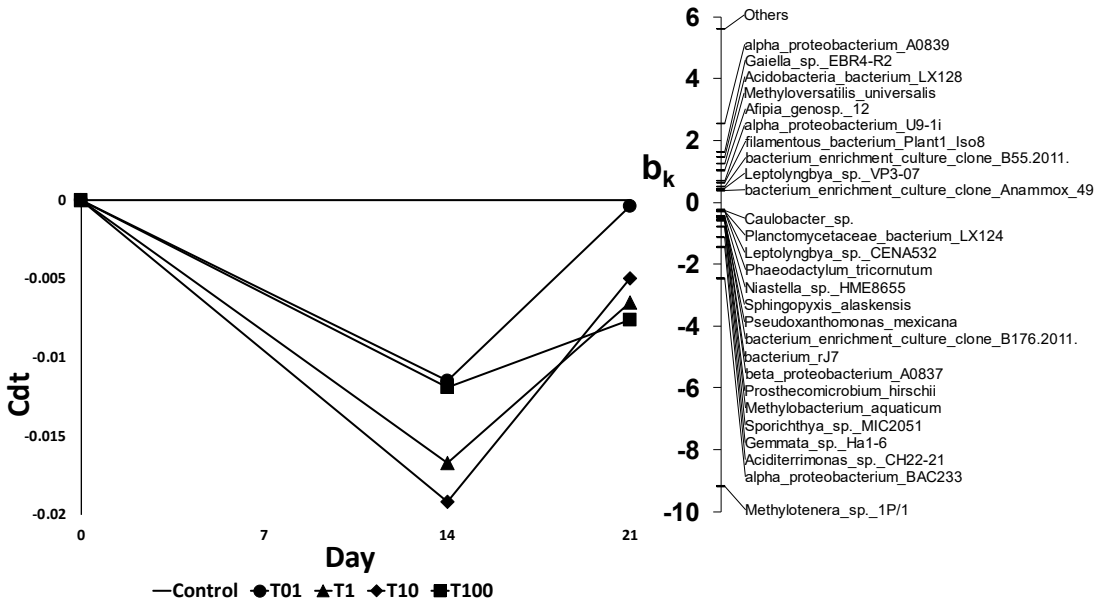
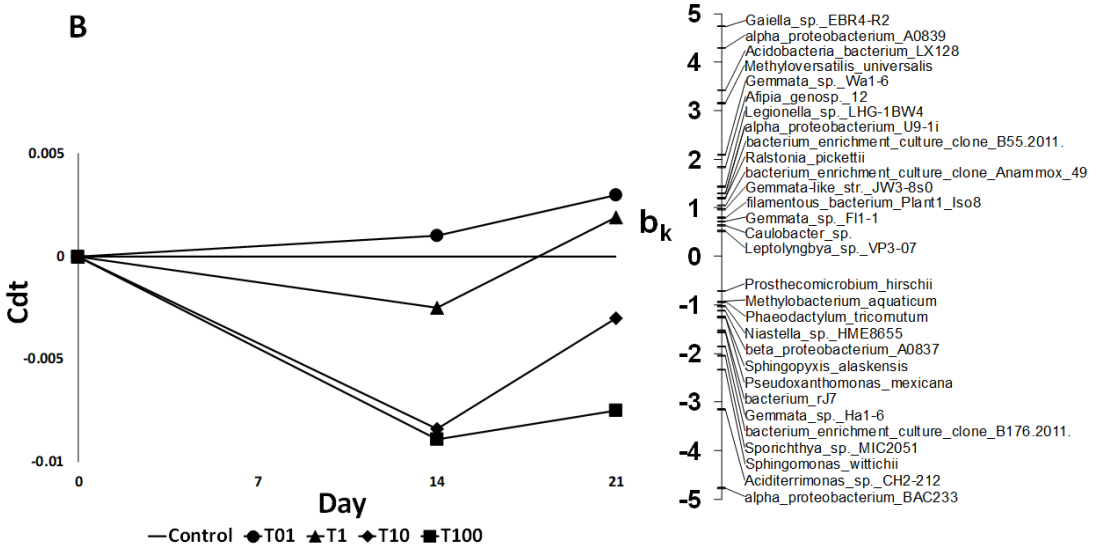


Figure 2 Bar charts of family-level relative abundances of prokaryotes (A) and eukaryotes (B) in river biofilms affected by triclosan exposure (Day 14) and recovery (Day 21). α, alpha-Proteobacteria; β, beta-Proteobacteria; γ, gamma-Proteobacteria; CB, Cyanobacteria; CP, Chloroplastida (green algae)

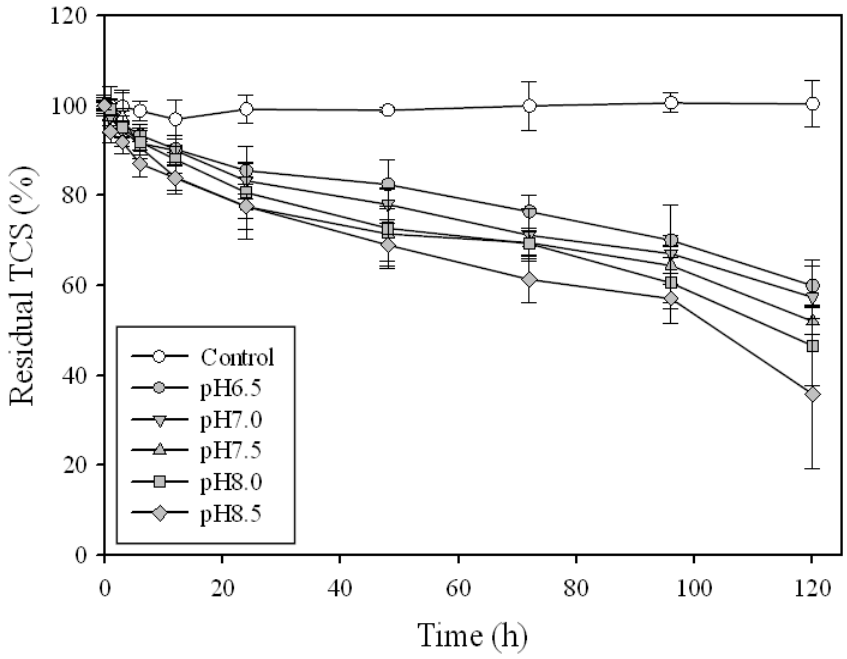


705



706

707 Figure 3. PRC resulting from the analysis of the prokaryotic data set, indicating the effects of
708 TCS on the prokaryotic community (A) and on the prokaryotic community without the taxa
709 Methylotenera_sp._1P/1 and 'Others' (B), respectively. The lines represent the course of the
710 treatment levels in time. Cdt stands for basic response pattern of certain treatment (d) at
711 sampling time (t). The species weight (b_k) can be interpreted as the affinity of the taxon (k)
712 with the PRC. For clarity, only taxa with a weight higher than 0.25 or lower than -0.25 are
713 shown in A and only taxa with a weight higher than 0.5 or lower than -0.5 are shown in B.



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715 Figure 4 Removal of triclosan by river biofilm in artificial river water at a series of pH levels

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