Evaluating the impacts of triclosan on wastewater treatment performance during startup and acclimation

R. M. Holzem, C. M. Gardner and C. K. Gunsch

ABSTRACT

Triclosan (TCS) is a broad range antimicrobial agent used in many personal care products, which is commonly discharged to wastewater treatment facilities (WWTFs). This study examined the impact of TCS on wastewater treatment performance using laboratory bench-scale sequencing batch reactors (SBRs) coupled with anaerobic digesters. The SBRs were continuously fed synthetic wastewater amended with or without 0.68 μM TCS, with the aim of determining the effect of chronic TCS exposure as opposed to a pulse TCS addition as previously studied. Overall, the present study suggests inhibition of nitrogen removal during reactor startup. However, NH_4^+ removal fully rebounded after 63 days, suggesting acclimation of the associated microbial communities to TCS. An initial decrease in microbial community diversity was observed in the SBRs fed TCS as compared to the control SBRs, followed by an increase in community diversity, which coincided with the increase in NH_4^+ removal. Elevated levels of NO_3^- and NO_2^- were found in the reactor effluent after day 58, however, suggesting ammonia oxidizing bacteria rebounding more rapidly than nitrogen oxidizing bacteria. Similar effects on treatment efficiencies at actual WWTFs have not been widely observed, suggesting that continuous addition of TCS in their influent may have selected for TCS-resistant nitrogen oxidizing bacteria.

Key words | ecological impacts, nitrification, personal care products, triclosan, wastewater treatment

R. M. Holzem
Department of Natural and Applied Sciences,
University of Wisconsin – Green Bay,
Green Bay, WI 54311,
USA

C. M. Gardner
C. K. Gunsch (corresponding author)
Department of Civil and Environmental Engineering,
Duke University,
121 Hudson Hall, Box 90287, Durham,
NC 27708-0287,
USA
E-mail: *ckgunsch@duke.edu*

INTRODUCTION

Triclosan (TCS), also known as 5-chloro-2-(2,4-dichlorophenoxy) phenol, is a broad range antimicrobial agent commonly used in personal care products, such as shampoos, soap, detergent, and toothpaste (Singer *et al.* 2002; Sabaliunas *et al.* 2003), which is heavily used in the USA (Rosenberg 2000). As a result of their use, TCS-containing products have been identified as being the major contributors of TCS to wastewater treatment facilities (WWTFs) (Kolpin *et al.* 2002; Bester 2003; Harrison *et al.* 2006). TCS has been measured in wastewater at concentrations up to 1.94 μM (Lindstrom *et al.* 2002; Thompson *et al.* 2005). TCS has reported maximum aqueous solubilities ranging from 6.9 to 13.8 μM (Grove *et al.* 2003). The log K_{ow} of TCS is 4.8, and its acid dissociation constant (pK_a) is 7.9, suggesting that TCS is fairly bioaccumulative (in the non-ionic form) under ambient conditions and partitions to solids (biological material) in wastewater treatment, as evidenced by the high concentrations (up to 113 mg/kg) reported in biosolids (Harrison *et al.* 2006; Ying & Kookana 2007; Clarke & Smith 2011). Because TCS is an antimicrobial that partitions to solids, or flocs, where much of a WWTF's biological processes occur, it is of interest to understand its potential effects on the beneficial microorganisms in the treatment process.

The mechanism of TCS microbial inhibition has been thoroughly examined. At high concentrations TCS inhibition primarily consists of post-translational inhibition of the enoyl-acyl carrier enzyme (FabI), which blocks fatty acid biosynthesis, and eventually leads to a compromised membrane (McMurry *et al.* 1998; Levy *et al.* 1999). At low concentrations, TCS has been shown to have some impact on membrane-associated gene expression (Escalada *et al.* 2005; Bailey *et al.* 2009). Many studies have examined the general toxicity of TCS, with a few focused on biological wastewater treatment processes and microbial processes in general. In our previous research, we showed that TCS

doi: 10.2166/wst.2017.566

inhibited denitrification in liquid culture, a major component of the nitrogen cycle, at concentrations as low as 1.04 μM (Holzem *et al.* 2014). Others reported median effective concentrations (EC50) on various pure and mixed cultures of microorganisms of 6.29 μM (based on biochemical oxygen demand (BOD) degradation), 69.1 μM (based on oxygen consumption), and 825 μM (based on glucose utilization) (The TCC Consortium 2002; Neumegen *et al.* 2005). Furthermore, examining a continuous-flow activated sludge system, Gatidou *et al.* (2007) and Stasinakis *et al.* (2008) demonstrated that nitrification was temporarily inhibited at a TCS concentration of 1.73 μM, and the EC50 of ammonium (NH_4^+) uptake was 34.4 μM. However, in both instances, TCS was not introduced continuously to the reactors, but rather, TCS inhibition was measured by adding TCS to samples taken from the reactors. Thus, the noted impacts may in fact have been artifacts of solids not acclimated to TCS. In another study, Federle *et al.* (2002) incrementally increased TCS feed from 0.14 to 6.90 μM to sequencing batch reactors (SBRs) inoculated with activated sludge and measured no inhibition of chemical oxygen demand (COD), BOD, or NH_4^+ removal. However, the primary focus of that study was on TCS degradation and the authors did not present any microbial ecological data; thus it is unclear if these data are a result of microbial functional redundancy as has been previously reported in the presence of other antimicrobials (Alito & Gunsch 2014). Another study previously examined the effects of TCS on the anaerobic digestion of wastewater treatment sludge. In that study, McNamara *et al.* (2014) found that methane production through anaerobic digestion significantly decreased in the presence of 500 mg/kg of TCS. The goal of the present study was to examine the impact of continuous TCS dosing on the operation of, and microbial community within, bench-scale wastewater SBRs coupled with anaerobic digesters at startup and steady-state. The SBRs were designed and operated to simulate an activated sludge process. Waste activated sludge was subsequently subjected to anaerobic digestion, a common process used to stabilize the solids generated from wastewater treatment (i.e., biosolids) prior to disposal.

METHODS

SBR design and operation

Four reactors, consisting of 11.5 L Mr. Aqua rectangular glass fish-tanks sealed with silicon (Taiwan), were set up in parallel in a chemical containment hood and were maintained at room temperature (~20 °C) throughout the experiment (Figure 1). To prevent photodegradation of TCS, the walls and top of the reactors were covered in aluminum foil and an aluminum foil-lined plastic container, respectively. The peak volume in each reactor was 9 L and the volume after decanting was 3.2 L. The SBRs were operated on a 6 h cycle, with 30 min of synthetic influent feeding without aeration, 3 h of aeration and complete mixing, 1 h of settling, and 30 min of decanting, and 1 h idle. The SBRs were kept idle (i.e., the mixers were not turned on) during influent feeding to encourage phosphorus removal (Manning & Irvine 1985; Wang *et al.* 2008). A solids retention time (SRT) of 10 days was selected to promote nitrification (Kos 1998). The operating hydraulic retention time was 5.6 h, which is in the range of contact stabilization (USEPA 1999). Once per day, during the last 5 min of idle time, the mixers were turned on and 250 mL of waste was decanted and collected in the anaerobic digester (after 30 days (i.e., ~3 SRTs) of operation when steady-state conditions were assumed to have been reached as others have observed (Wang & Gunsch 2011; Rieger *et al.* 2012). All four of the reactors were fed synthetic wastewater (SWW) as described in a previously published study that contained 450 mg/L COD and 40 mg/L of ammonium (Zeng *et al.* 2003). Two reactors were fed only SWW (named Control SBRs), while the other two reactors were fed SWW with 0.73 μM TCS (named TCS SBRs).

Ambient air was fed to all the reactors via two PetSmart Top Fin Air 8000 aerators (Phoenix, AZ, USA). Each aerator had four separate tubes attached to PetSmart Top Fin Fine bubble air stone diffusers (Phoenix, AZ, USA) which were placed evenly throughout the reactors in order to maintain

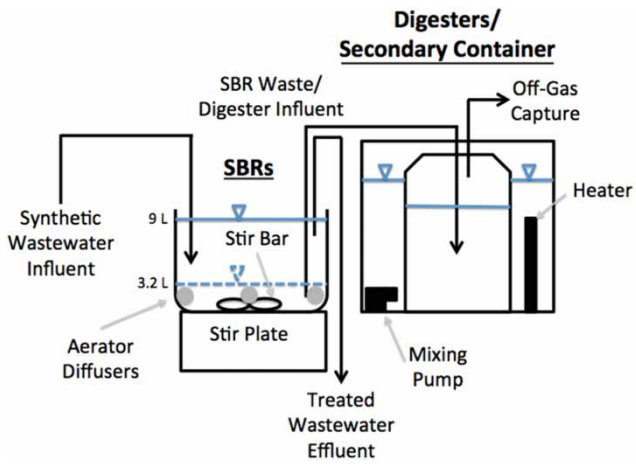

Figure 1 | Schematic of SBR and anaerobic setup.

uniform aeration. The dissolved oxygen (DO) was maintained above 4.2 mg/L, to achieve maximum nitrification (Stenstrom & Poduska 1980). Multi-head peristaltic pumps were used to add the influent and remove the effluent (Cole Parmer, Masterflex L/STM, Vernon Hills, IL, USA). Influent SWW was stored for a maximum of 3 days in a PetSmart Great Choice 76 L rectangular glass tank (76.2 cm × 30.5 cm× 30.5 cm) lined with aluminum foil and covered to prevent photodegradation of TCS (Phoenix, AZ, USA). Nutrient and TCS stability while in the storage tank was verified to ensure no significant degradation occurred during the storage period, using the same analytical methods described in the 'Analytical methods' section. Effluent from each reactor was collected in a separate unlined, but covered, 76 L glass tank. Reactors were mixed with large stir bars on stir plates at 500 rpm. All cycling components were controlled with GE Digital SunSmart digital (Fairfield, CT, USA) and Intermatic TN311C (Grove, IL, USA) timer-controllers. The SBRs were initially spiked with 9 L of activated sludge taken from the North Durham Water Reclamation Facility (NDWRF, Durham, NC, USA), which currently treats 20 MGD and completes biological nutrient removal of BOD, NH_4^+, and phosphorus. Inoculum was added to the SBRs within 2 h of collection from the NDWRF.

SBR analysis began immediately following inoculation and was repeated approximately every 7 days until operation ceased (77 days). COD, NH_4^+, nitrate (NO_3^-), nitrite (NO_2^-), total suspended solids (TSS), volatile suspended solids (VSS), DO, and pH were measured on each sampling day. Phosphate (PO_4^{3-}) and microbial community analyses were completed every two to three sampling events. All analyses were completed in triplicate. Five hundred microlitres was collected from the influent and effluent of each reactor for NH_4^+, NO_2^-, and PO_4^{3-} analyses. The samples were then microcentrifuged for 5 min at 13,000 rpm in a 1.5 mL centrifuge tube using an Eppendorf 5424 centrifuge (Hamburg, Germany) prior to analysis. Triplicate 35 mL samples were obtained from the influent and effluent of each reactor for NO_3^-, COD, pH, TSS, and VSS analyses. Finally, every 10 to 20 days, triplicate 1 mL samples were obtained from the SBRs for microbial community analysis. These samples were centrifuged for 5 min at 13,000 rpm, the supernatant was removed via pipetting, and the pellet was stored at −20 °C for further processing.

TCS addition

A target concentration of 0.73 μM TCS was selected for the present study based on published literature for a WWTF that generates biosolids with high levels of TCS (McAvoy *et al.* 2002; Bester 2003; Chu & Metcalfe 2007). Calculations were carried out based on available published adsorption characteristics of TCS for soil and biosolids as well as published range of concentrations measured in biosolids (Wu *et al.* 2009). The selected target TCS concentration was calculated from a theoretical biosolids concentration of 30 to 50 mg/kg dry weight (dependent upon the adsorption isotherm used). To achieve the desired influent concentration, 1.0 mL of a 4 M stock TCS acetone solution was spiked into the influent tank using a Hamilton Syringe (Hamilton Company, Reno, NV, USA) and mixed using a stainless-steel spoon. Fresh influent with TCS was prepared every 2 or 3 days. No significant degradation was observed within this time frame as verified analytically.

Anaerobic digester design and operation

A single anaerobic digester was assembled to receive the wasted sludge from each SBR. Each digester consisted of a 19 L solvent-rinsed, glass carboy obtained from Learn to Brew, LLC (Moore, OK, USA). The digesters were sealed with a VWR rubber stopper (Radnor, PA, USA) and silicone. The respective waste decanting tube from the SBR was fed into the digester through the rubber stopper. Another tube was placed into the digester through the stopper and was connected to a 5 L SKC Tedlar sampling bag (Eighty Four, PA, USA). Once the SBRs reached steady-state, which was achieved at 3 SRTs on day 30 (Wang & Gunsch 2011), the digesters were inoculated with 100 mL of anaerobically digested sludge from the NDWRF and prepared for operation. After inoculation, the tubing and the stoppers were sealed and the headspace of the carboys was evacuated and replaced with nitrogen gas at atmospheric pressure. The glass carboys were then placed in a 113 L plastic container and weighed down using sand bags and the container was filled with water. A 200 Watt Aqueon aquarium heater (Franklin, WI, USA) was used to maintain the water temperature at 27 °C and a PetSmart 950 Aqueon circulation pump (Phoenix, AZ, USA) was used to continuously mix the water. The container with the digesters was covered in order to minimize photodegradation. An amount of 250 mL was wasted from each SBR to the anaerobic digesters per day for 47 days and, when the SBRs were shutdown, all remaining solids from the SBR were pumped to the digesters. The digesters were then isolated from the SBRs and digestion proceeded for an additional 20 days. The digesters were mixed manually every 3 days throughout the entire operation.

Completely mixed samples were obtained from each digester after digestion was complete and were analyzed for TCS concentration, TSS, VSS, total Kjeldahl nitrogen (TKN), phosphorus, potassium, and pH. Triplicate 10 mL samples were obtained from each digester for TSS and VSS analyses. Triplicate 50 mL composite samples were obtained for TKN, phosphorus, potassium, and pH analyses. These samples were sent to Waters Agricultural Laboratories, Inc. (Georgia, USA) for processing. Samples of 40 mL were also obtained for TCS analysis and placed in volatile organic analysis certified Thermo Scientific 50 mL amber vials with a silicone/polytetrafluoroethylene (PFTE) septum in polypropylene caps (Waltham, MA, USA), stored at 4 °C and processed within 24 h.

Analytical methods

COD (mercuric digestion method) and nitrate (cadmium reduction method) were measured using Hach reagents (Loveland, CO, USA). NO_2^- was measured colorimetrically using a modified version of the Griess reagent method as described in previously published literature (Hernández-López & Vargas-Albores 2003; Holzem *et al.* 2014). A modified version of the phenate method from Section 4500 of *Standard Methods for the Examination of Water and Wastewater* (American Public Health Association *et al.* 2005) was used to measure NH_4^+ as described previously (American Public Health Association *et al.* 2005; Holzem *et al.* 2014). PO_4^{3-} was also measured according to a previously published method (Hernández-López & Vargas-Albores 2003). Note that due to a sampling error, PO_4^{3-} was not measured on day 40. TSS and VSS analyses were completed according to Section 2450 of *Standard Methods for the Examination of Water and Wastewater* (American Public Health Association *et al.* 2005). Finally, pH was measured using a Beckman Coulter, Inc. pH/temperature/millivolt meter and probe (Fullerton, CA, USA).

Gas production was also monitored to evaluate digester performance. Triplicate 10 mL gas samples were taken from each gas sample bag using a Poulten & Graf Ltd Fortuna 10 mL gas-tight syringe (Barking, Essex, UK) and Becton Dickinson and Company 25 gauge needles (Franklin Lakes, NJ, USA). The samples were then injected into 9 mL gas vials crimp-capped with butyl stoppers that were flushed with nitrogen gas (N_2) and evacuated as described elsewhere (Groffman *et al.* 1999). Vials, butyl stoppers, and aluminum crimp caps were obtained from Grace Davison Discovery Science (Deerfield, IL, USA). Methane levels were measured with a modified Shimadzu gas chromatograph 17A version 3 (Kyoto, Japan) and Tekmar headspace autosampler (Vernon, BC, Canada) within 7 days.

To verify the TCS dosage, TCS stock concentrations were quantified. TCS stock concentrations were first diluted to have a final total mass between 10 and 100 ng. The diluted samples were then spiked with 100 μL of a 1.0 μg ^{13}C TCS/mL internal standard and filtered through a methanol-rinsed 0.2 μm PTFE filter. The samples were then analyzed using liquid chromatography/tandem mass spectrometry (LC/MS-MS) as previously described (Davis *et al.* 2012).

To measure the TCS concentration in the simulated digested biosolids, the liquid and solids fractions were first separated via centrifugation by an Eppendorf 5810R centrifuge (Hamburg, Germany). The aqueous portion was then transferred to a 50 mL Kinman glass vial (Des Moines, IA, USA) centrifuged tube and extracted with 50:50 dichloromethane (DCM):hexane three times. Briefly, 10 mL of 50:50 DCM:hexane was added to the sample and vortexed for 10 s. The samples were then centrifuged at 2,500 rpm for 5 min. The supernatant was then collected in a VWR 15 mL glass test tube (Radnor, PA, USA). The extraction was repeated a total of three times. The samples were further processed and analyzed using LC/MS-MS as previously described for biosolids samples starting with the sulfuric acid purification step (Davis *et al.* 2012). The solid portion of the samples were analyzed according to the same method, but starting with homogenization with sodium sulfate and pressurized fluid extraction (Davis *et al.* 2012). All solvents were high-pressure liquid chromatography grade and were obtained from Sigma Aldrich (Milwaukee, WI, USA).

DNA extraction and PCR conditions

Total DNA was extracted from all replicates using the MO BIO PowerLyzer PowerSoil DNA Extraction Kit (Carlsbad, CA, USA). All extractions were performed following a modified version of the manufacturer's protocol. Briefly, after the addition of reagent C1, 400–500 μL of 25:24:1 phenol:chloroform:isoamyl alcohol was added to each tube and vortexed. Following this, instead of homogenizing the samples, the samples were placed on a horizontal shaker at maximum speed for 5 min. Twenty-five microlitres of eluent buffer (C6) was added to the filter membrane; the samples were incubated for 5 min and centrifuged for 30 s at 13,000 rpm. This step was then repeated. After DNA extraction was completed, the DNA concentration and purity were verified by spectrophotometry (Thermo Fisher Scientific, Waltham, MA, USA). Polymerase chain reaction (PCR) of the bacterial 16S SSU rRNA gene region was completed

based on the method previously described by Lukow *et al.* (2000) and modified from a previously published method (Alito & Gunsch 2014). 6-FAM labeled 27F was used as the forward primer and unlabeled 1392R was used as the reverse primer (Lukow *et al.* 2000). For each PCR reaction, 1 μL of purified template DNA was used. Following amplification, the presence of the correct length PCR amplicons was confirmed via visualization on a 1 percent agarose gel containing 0.1% ethidium bromide. PCR amplicons were then purified using a Qiagen PCR Purification Kit (Qiagen, Hilden, Germany). The final PCR product concentrations and purity were again verified using spectrophotometry.

T-RFLP analysis

PCR amplicons were digested with restriction enzymes as described in Lukow *et al.* (2000). However, no bovine serum albumin was used in the reactions as preliminary experiments showed interference. One hundred nanograms of purified PCR product and 10U of the restriction enzyme MspI (New England Bio-Labs Inc., Beverly, MA, USA) were used for each reaction. The reactions were incubated for 2 h at 37 °C. Following incubation, samples were stored at −20 °C until further processing by the Duke University DNA Analysis Facility (Durham, NC, USA) where samples were desalted through a spin column filtration and subjected to fragment analysis using an Applied Biosystems 3100 capillary sequencer (Foster City, CA, USA) with POP6 polymer and ROX-labeled MapMarker 1000 size standards (BioVentures, Inc., Murfreesboro, TN, USA).

Terminal restriction fragment length polymorphism (T-RFLP) profiles were evaluated using Applied Biosystem's Genescan v3.7.1 analysis software (Foster City, CA, USA), as previously described (Alito & Gunsch 2014). Manual inspection of each profile was completed to ensure only true peaks were chosen for statistical analyses. The raw data were then imported into T-REX in the following format: Dye/Peak, Sample File Name, Size, Height, Area in Point, Area in BP (Culman *et al.* 2009). All peaks less than 50 bp in length were excluded from the analysis because they were considered to be possible primer dimer fragments. Only T-RFs with a minimum peak height threshold of 50 relative fluorescent units were used in the analysis. T-REX was used to align the profiles based on the replicates using the 'Environments' function in the software. Presence/absence data files were imported into PAST (Paleontological Statistics software) for data analysis (Hammer *et al.* 2001). This analysis method was selected as peak area tends to bias towards the abundance of a specific species (i.e., each T-RF), whereas presence/absence gives all species (i.e., each T-RF) the same weight during analysis. Thus, presence/absence is especially important when species of low abundance are significantly impacted more by the treatments. After the data were analyzed, the relative species abundance was calculated based on the number of peaks present in each sample. The relative abundance values were then compared between the Control and TCS SBRs over time. The data were then analyzed using principal nonmetric multidimensional scaling (NMS) coupled with Jaccard similarity distance for group clustering. The Jaccard similarity distance was used because it has been shown to work well for binary data, such as the presence/absence data compared herein (Choi *et al.* 2010).

Statistical analysis

Experimental values are reported as the mean ± standard error. To analyze statistical differences between treatments, one-way and two-way analysis of variance coupled with Tukey's post-hoc analysis using the open source statistical software R (v.2.15.1) was used. Differences were considered significant for p-values ≤ 0.05 (R Development Core Team 2008). Marginal significance was considered for p-values ≤ 0.10.

RESULTS AND DISCUSSION

TCS concentrations

TCS stock concentrations were quantified to verify that the expected dosage was delivered to the SBRs. On average, 93.6 ± 1.0% of the spiked stock compound was recovered corresponding to influent concentrations of 0.68 ± 0.01 μM, which was approximately 93% of the intended loading (0.73 μM). The concentrations measured in the digested Control and TCS SBR biosolids were significantly different from each other (p-value = 0.0023). Measured TCS concentrations were 2.85 ± 0.69 mg/kg for the Control SBR biosolids and 82.7 ± 11.1 mg/kg from the TCS SBR biosolids. The final TCS concentration in the TCS SBR biosolids was slightly higher than our intended target (30 to 50 mg/kg) but still falls within the published TCS levels measured in biosolids, which range from 0.09 to 133 mg/kg (McAvoy *et al.* 2002; Bester 2003; Morales *et al.* 2005; Chu & Metcalfe 2007; Gatidou *et al.* 2007; Ying & Kookana 2007; Pothitou & Voutsa 2008; Stasinakis *et al.* 2008; Cha & Cupples 2009; USEPA 2009; Davis *et al.*

2012). The higher measured TCS concentration was attributed to differences between the SBR treatment conditions and the available published adsorption characteristics of TCS for soil and biosolids (e.g., pH) used to develop the adsorption isotherms. The TCS concentration of the Control SBR biosolids was also higher than expected (i.e., TCS concentration was significantly different than zero). This was attributed to: (1) background concentrations in the anaerobically digested sludge used for inoculum; (2) background TCS levels from inoculum activated sludge that remained in the SBR after steady-state was reached; and (3) external contamination due to high use of TCS-containing domestic products (dishwashing detergent, etc.).

Reactor characteristics and treatment performance

Throughout the experiment, TSS, pH and DO were monitored and average values are shown in Table 1. Conditions were not significantly different between the Control and TCS SBRs ($p > 0.05$).

Removals of COD, PO_4^{3-}, and NH_4^+ are shown in Figure 2. COD removal was maintained between 86 and 100% throughout the entire operation in both the Control and TCS SBRs. COD removal was not impacted by the addition of TCS, which was consistent with a previous study showing only a minor effect on organic substrate removal at much higher concentrations (6.9 μM) (Stasinakis *et al.* 2007). Similarly, PO_4^{3-} was not impacted by the addition of TCS and there was no significant difference in PO_4^{3-} removal at steady-state conditions between the Control and TCS SBRs ($p > 0.05$). The low PO_4^{3-} removal is likely attributed to the inoculum activated sludge as well as the presence of oxygen. Because biological phosphorus removal only occurs under anaerobic conditions, the high DO during aeration may have led to higher DO during the anoxic/anaerobic cycles of the SBRs and decreased removal of NO_3^- and, therefore, decreased phosphorus removal. While a static influent feed was used to promote phosphorus removal in this study, simultaneous nitrogen and phosphorus removal has been shown to be difficult to achieve in an SBR (USEPA 1999).

NH_4^+ removal was significantly impacted by the addition of TCS from day 28 to 63. Although prior to day 28, NH_4^+ removal in the TCS SBR was highly variable, starting at 93% at startup, dropping to 81% after 12 days, increasing

Table 1 | SBR characteristics (mean values ± standard error)

Characteristic	Control	TCS
TSS (mg/L)	3,084 ± 202	3,327 ± 275
DO (mg/L)	4.21 ± 0.66	
Influent pH	6.38 ± 0.05	6.42 ± 0.14
Reactor pH	7.37 ± 0.07	7.33 ± 0.06
Effluent pH	7.74 ± 0.13	7.74 ± 0.12

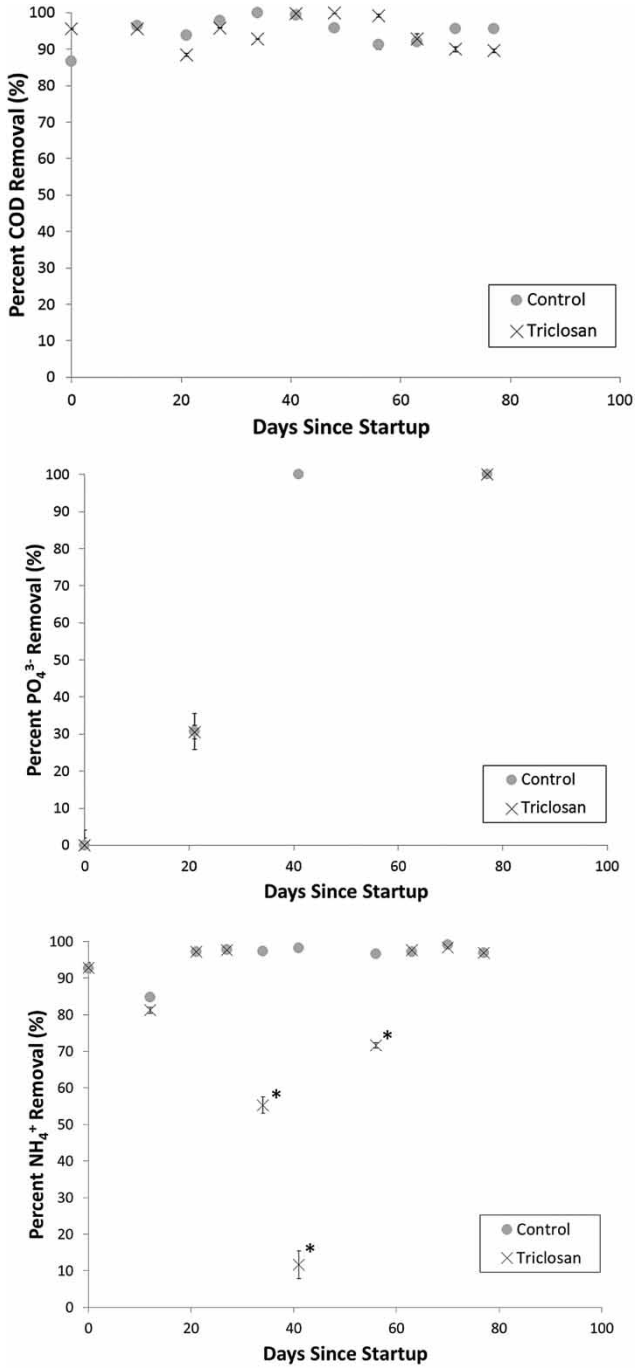

Figure 2 | Average COD, PO_4^{3-}, and NH_4^+ percent removal ($n = 3$). * indicates a significant difference (p-value ≤ 0.05) from the Control. Error bars represent one standard error.

to 97% and 98% percent on days 21 and 27, respectively, before dropping again. The Control SBR reached greater than 92% removal within the first 3 weeks of operation, whereas the TCS SBR took 63 days to reach and maintain similar NH_4^+ removal. Inhibition of NH_4^+ removal could be attributed to the bacterial communities slowly acclimating to TCS; however, inhibition continued well past the typical time frame for reaching steady-state (i.e., three SRTs corresponding to 30 days in the present study) when the majority of the solids from the inoculum should have already been removed. Temporary inhibition of nitrification upon the introduction of TCS has been previously observed albeit at a slightly higher TCS concentration (1.73 μM) (Gatidou *et al.* 2007; Stasinakis *et al.* 2007). Similar to the present study, Gatidou *et al.* (2007) and Stasinakis *et al.* (2007) observed a recovery of nitrification. The increased sensitivity to external changes and slower growth rate of autotrophic nitrifiers compared to the heterotrophic bacteria responsible for COD removal, likely explains why TCS impacted NH_4^+ removal but not COD removal in the present study (Blum & Speece 1992; Eddy 2003).

Effluent NO_3^- and NO_2^- concentrations are shown in Figure 3. For both the Control and the TCS SBRs, the effluent NO_3^- and NO_2^- concentrations were much more variable than COD and PO_4^{3-} removal, and, unlike NH_4^+, treatment performance for both NO_3^- and NO_2^- was more variable in both the Control and TCS SBRs until approximately day 58. At that time, the NO_3^- effluent concentration for the TCS SBR was 3.7 ± 0.23 mg/L, which was ~2 times greater than the NO_3^- effluent of the Control SBR (1.6 ± 0.08 mg/L), was significantly different between treatments (p-value < 0.05) and remained significantly higher for the remainder of the experiment. Similarly, after day 58, the NO_2^- effluent of the TCS SBR reached a steady-state concentration of 9.7 ± 0.60 mg/L. The mean NO_2^- concentration of the Control SBR after day 58 was approximately four times lower (2.2 ± 0.95 mg/L) but was still trending towards 0 when the reactor operation was ceased. The difference between the NO_2^- levels in the Control and TCS SBRs after day 58 were statistically significant (p-values < 0.05).

Overall, these data suggest that ammonium oxidizing bacteria (AOB), which complete the first step of nitrification (i.e., oxidation of NH_4^+ to NO_2^-) were inhibited temporarily by the addition of TCS. However, nitrite oxidizing bacteria (NOB), which complete the second step of nitrification (i.e., oxidation of NO_2^- to NO_3^-) and denitrifiers were inhibited on a more long-term basis. This was unexpected, as Dokianakis *et al.* (2004) showed a full rebound of NO_2^- oxidation in less than 3 days in the presence of TCS as high as 34.5 μM in a continuous-flow activated sludge system, with mixed cultures. The concentration used in the present study was also lower than the TCS concentration shown to inhibit NO_2^- oxidation by mixed cultures in batch reactors (6.91 μM TCS), denitrification by *Paracoccus denitrificans* (1.04 μM TCS), and NH_4^+ removal by mixed culture (Dokianakis *et al.* 2004; Stasinakis *et al.* 2007; Stasinakis *et al.* 2008; Holzem *et al.* 2014). Mixed microbial communities, as opposed to pure cultures, within the SBR would be expected to be more resistant due to functional redundancy as compared to pure cultures as previously reported for other antimicrobials.

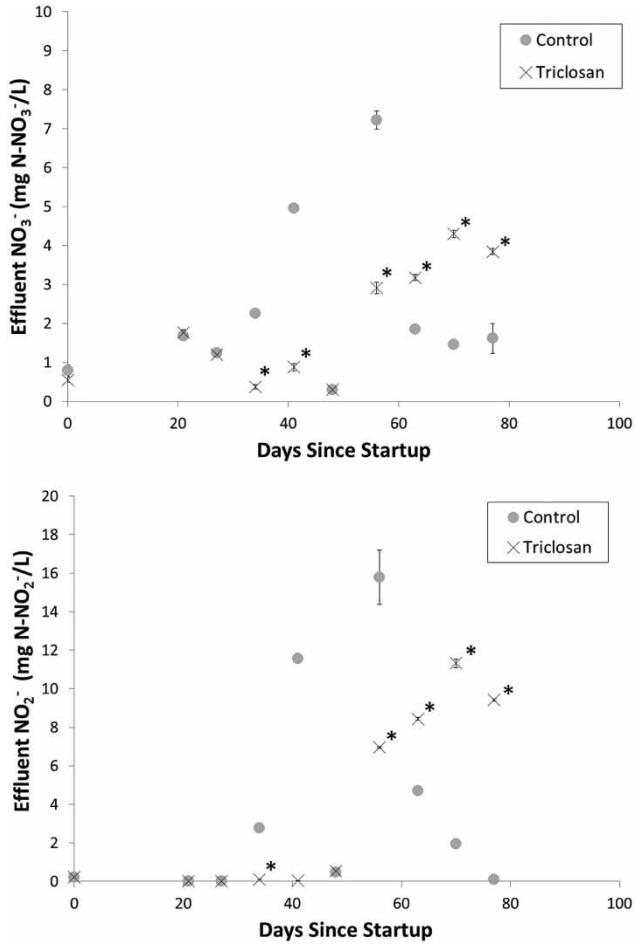

Figure 3 | Average effluent nitrate and nitrite concentrations. * indicates a significant difference (p-value ≤ 0.05) from the Control. Error bars represent one standard error.

Digestion characteristics and performance

Solids were wasted to the anaerobic digesters for 47 days from both the Control and TCS SBRs. On the final day of SBR operation (day 77), the entire idle-volume of sludge

from the SBRs was emptied into the corresponding digester, mixed, and digested further, as stated in the 'Methods' section. Following digestion, the TKN, phosphorus (as P_2O_5), and potassium (K_2O) concentrations and pH were measured on the Control SBR biosolids and were 4,667 ± 273 mg/kg, 1,028 ± 22 mg/kg, 182 ± 3.1 mg/kg, and 6.68 ± 0.04, respectively. The corresponding TCS SBR biosolids concentrations were 3,777 ± 82 mg/kg, 781 ± 7.9 mg/kg, 153 ± 0.64 mg/kg, and 6.71 ± 0.02, respectively. The TSS of the Control and TCS SBR biosolids were 5,877 ± 46.4 and 6,223 ± 132 mg/L, respectively. The TKN, phosphorus (as P_2O_5), and potassium (as K_2O) values were significantly higher in the Control SBR biosolids compared to the TCS SBR biosolids on a dry weight basis and TSS was significantly lower. In addition, the TKN, phosphorus, and potassium values of both the Control and TCS SBR biosolids generated in this study were approximately an order of magnitude lower than those reported for typical biosolids (Lu *et al.* 2012).

The total gas produced over 67 days (i.e., 47 days of SBR operation and 20 days of additional digestion) by the Control and TCS SBR digesters were ~19.3 and 14 L, respectively. The concentration of methane within the digester gas was 15.1 ± 1.5 and 13.9 ± 1.9 g/m^3, respectively. Neither the volume of gas nor methane concentration was significantly different between the Control and TCS SBR digesters ($p > 0.05$) suggesting little impact of TCS on methanogenic activity. This is consistent with a previous study that showed no significant difference between the cumulative methane production by microbial consortia exposed to 50 mg/kg TCS, which was comparable to the TCS concentration in the TCS SBR biosolids used in the present study (82.7 ± 11.1 mg/kg), and the control (i.e., 0 mg/kg TCS) (McNamara *et al.* 2014). Interestingly, McNamara *et al.* (2014) did observe a significant increase in the cumulative methane production at a minimum TCS concentration of 5 mg/kg, which was slightly greater than the TCS concentration in the Control SBR biosolids in the present study (2.85 ± 0.69 mg/kg) and a significant decrease in cumulative methane production at a TCS concentration of 500 mg/kg.

Impact of TCS on microbial community structure

T-RFLP community structure analysis using the 16S rDNA housekeeping gene was completed to compare microbial community structure between the Control and TCS SBRs and to assess the impact of TCS on overall community structure. The relative abundance of T-RFs in the TCS SBR compared to the Control SBR is shown in Figure 4. For both the Control and TCS SBRs, the number of species was observed to increase over time from day 0 to day 41, but then decrease from day 41 to day 46. Whereas the Control and TCS SBRs were not significantly different at day 0 (i.e., prior to the addition of TCS), they became significantly different thereafter. Specifically, the TCS SBR had a marginally significant overall lower number of species on day 41 ($p = 0.06$) and significantly overall lower number of species on day 34 ($p = 0.037$) and 56 ($p = 0.004$). This suggests that treatment time and TCS concentration affected community structure. However, species abundance data alone do not provide information regarding any differences in composition of the microbial communities as different treatments with the same number of species (i.e., T-RFs) may not necessarily have selected for the same species. Thus, to examine the differences between the microbial communities, an NMS ordination analysis was completed.

The convergent final NMS ordination solution for 16S rDNA is shown in Figure 5. The stress of the NMS plot solution was 0.082, which suggests a strong correspondence between the distances among points. R-values for the first and second axes were 0.61 and 0.31, respectively, and account for 92% of the data variability. At day 0, the Control and TCS SBR communities were similar, but became less similar until day 41. This is consistent with both SBRs starting off with the same communities obtained from the inoculum, but differences between the communities developing due to the introduction of TCS into the TCS SBR influent. On day 41, the community structure starts converging between the Control and TCS SBRs and remains more

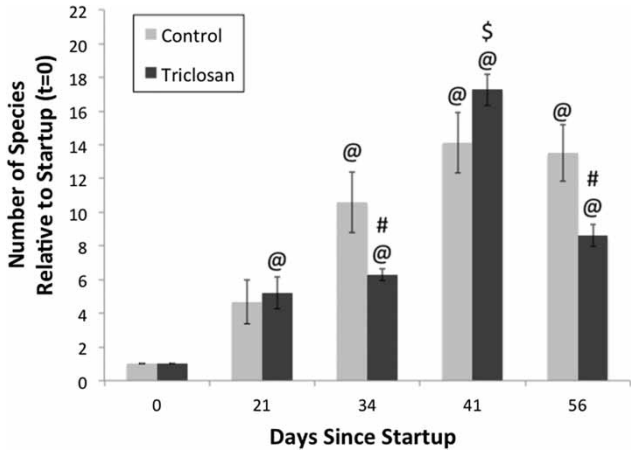

Figure 4 | Relative abundance of microbial species in SBRs over time. @ indicates a significant difference from day 0 (p-value ≤ 0.05). # indicates a significant difference from the Control at the same sample day (p-value ≤ 0.05). $ indicates a marginal significant difference from the Control at the same sample day (p-value ≤ 0.10). Values >1 indicate an increase in the number of species compared to day 0. Error bars represent one standard error.

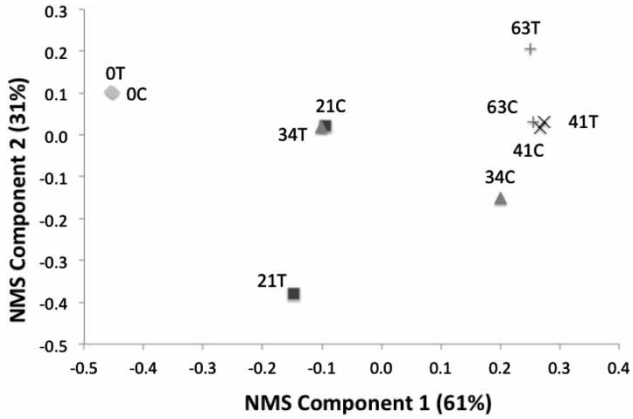

Figure 5 | NMS ordination analysis of 16S rDNA in SBRs. 'C' indicates Control SBR samples. 'T' indicates TCS SBR samples.

similar up to the end of the experiment (day 63) as demonstrated by the observed clustering shown in Figure 5. Similar observations have been previously reported, albeit for bacteria previously exposed to and previously unexposed to TCS under conditions simulating anaerobic digestion (McNamara *et al.* 2014). Specifically, McNamara *et al.* (2014) showed that for bacterial communities previously exposed to TCS and then exposed to 5, 50, and 500 mg/kg of TCS, communities of bacteria diverged from the control communities (i.e., communities that were not exposed to TCS) and reconverged with the control communities over time. The length of time required for reconvergence increased as TCS concentration increased. A similar trend was observed for bacterial communities not previously exposed to TCS and then exposed to TCS at concentrations less than 70 mg/kg. McNamara *et al.* (2014) further suggested that TCS concentrations ≥50 mg/kg TCS resulted in substantial community structure changes. The TCS concentration used in the present study, calculated on a dry weight basis of solids in the TCS SBR from Table 1, was 59.2 mg/kg TCS, which is consistent with the results reported by McNamara *et al.* (2014). Thus, the results herein suggest that the microbial community in the TCS SBR may have acclimated to TCS around day 41, which enabled communities similar to the Control SBR to re-emerge. Because a rebound in NH_4^+ removal was also observed around this time, AOB may also have benefited from TCS acclimation, although additional molecular analyses are needed to confirm this. The rebound could also be due to degradation of TCS by AOB or heterotrophic microorganisms to a point where TCS no longer inhibited NH_4^+ removal, and other microbial populations present in the original inoculum could re-emerge (Roh *et al.* 2009). AOB and heterotrophic microorganisms have been shown to degrade TCS at concentrations of 1.73 to 6.9 μM. However, NH_4^+ removal as well as NO_2^- oxidation was still inhibited in the presence of TCS (Roh *et al.* 2009; Lee *et al.* 2015). This is especially important because an effect on performance efficiencies has not been observed at actual WWTFs, and thus the continued addition of TCS in the influent to a WWTF may either be selecting for TCS-resistant bacteria or the negative impacts of TCS are being masked by functional redundancy.

CONCLUSIONS

Overall the results of this study suggest that biological processes associated with nitrogen removal (i.e., nitrification and denitrification) may be temporarily impacted by TCS in non-acclimated WWTFs especially during startup. NH_4^+ removal was shown to be temporarily inhibited and elevated levels of NO_3^- and NO_2^- in the effluent of the TCS SBR suggest longer-term impacts on NOB and denitrifiers than AOB. This may be due to a positive association between AOB and TCS. Specifically, some AOB and heterotrophic bacteria have been previously shown to degrade TCS and thus may be able to acclimate to TCS faster than NOB. Overall, this study suggests that nutrient removal may be transiently affected, especially during the startup phase, by TCS concentrations as low as 0.68 μM. However, long-term performance effects are minimized possibly due to the selection of TCS-resistant bacteria and development of microbial communities with high functional redundancy. Further work should also be completed on actual activated sludge to determine the effects of the myriad of chemicals present in that matrix.

ACKNOWLEDGEMENTS

This work was supported by the National Science Foundation (NSF) under grant no. CBET 0854167. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of NSF. We thank Dr Heather Stapleton's laboratory at Duke University, particularly Dr Elizabeth Davis and Thomas (Mingliang) Fang for providing the analytical support. We also thank Tony Luz, Carley Gwin, Ross Taggart, and Shanice Mclean of Duke University for their help with operating and sampling the reactors.

REFERENCES

Alito, C. L. & Gunsch, C. K. 2014 Assessing the effects of silver nanoparticles on biological nutrient removal in bench-scale activated sludge sequencing batch reactors. *Environmental Science & Technology* **48** (2), 970–976.

American Public Health Association, Eaton, A. D., American Water Works Association & Water Environment Federation 2005 *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association/American Water Works Association/Water Environment Federation, Washington, DC, USA.

Bailey, A. M., Constantinidou, C., Ivens, A., Garvey, M. I., Webber, M. A., Coldham, N., Hobman, J. L., Wain, J., Woodward, M. J. & Piddock, L. J. V. 2009 Exposure of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium to triclosan induces a species-specific response, including drug detoxification. *Journal of Antimicrobial Chemotherapy* **64** (5), 973–985.

Bester, K. 2003 Triclosan in a sewage treatment process – balances and monitoring data. *Water Research* **37** (16), 3891–3896.

Blum, D. J. W. & Speece, R. E. 1992 The toxicity of organic chemicals to treatment processes. *Water Science and Technology* **25** (3), 23–31.

Cha, J. & Cupples, A. M. 2009 Detection of the antimicrobials triclocarban and triclosan in agricultural soils following land application of municipal biosolids. *Water Research* **43** (9), 2522–2530.

Choi, S. S., Cha, S.-H. & Tappert, C. C. 2010 A survey of binary similarity and distance measures. *Systemics, Cybernetics and Informatics* **8** (1), 43–48.

Chu, S. & Metcalfe, C. D. 2007 Simultaneous determination of triclocarban and triclosan in municipal biosolids by liquid chromatography tandem mass spectrometry. *Journal of Chromatography A* **1164** (1–2), 212–218.

Clarke, B. O. & Smith, S. R. 2011 Review of 'emerging' organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids. *Environment International* **37** (1), 226–247.

Culman, S., Bukowski, R., Gauch, H., Cadillo-Quiroz, H. & Buckley, D. 2009 T-REX: software for the processing and analysis of T-RFLP data. *BMC Informatics* **10** (1), 171.

Davis, E. F., Klosterhaus, S. L. & Stapleton, H. M. 2012 Measurement of flame retardants and triclosan in municipal sewage sludge and biosolids. *Environment International* **40**, 1–7.

Dokianakis, S. N., Kornaros, M. E. & Lyberatos, G. 2004 On the effect of pharmaceuticals on bacterial nitrite oxidation. *Water Science and Technology* **50** (5), 341–346.

Eddy, M. 2003 *Wastewater Engineering: Treatment and Reuse*. McGraw-Hill, Boston, USA.

Escalada, M. G., Harwood, J. L., Maillard, J.-Y. & Ochs, D. 2005 Triclosan inhibition of fatty acid synthesis and its effect on growth of *Escherichia coli* and *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy* **55** (6), 879–882.

Federle, T. W., Kaiser, S. K. & Nuck, B. A. 2002 Fate and effects of triclosan in activated sludge. *Environmental Toxicology and Chemistry* **21** (7), 1330–1337.

Gatidou, G., Thomaidis, N. S., Stasinakis, A. S. & Lekkas, T. D. 2007 Simultaneous determination of the endocrine disrupting compounds nonylphenol, nonylphenol ethoxylates, triclosan and bisphenol A in wastewater and sewage sludge by gas chromatography–mass spectrometry. *Journal of Chromatography A* **1138** (1–2), 32–41.

Groffman, P., Holland, E., Myrold, D., Robertson, G. & Zou, X. 1999 *Standard Soil Methods for Long-Term Ecological Research*. Oxford University Press, Oxford, UK.

Grove, C., Liebenberg, W., Du Preez, J. L., Yang, W. Z. & De Villiers, M. M. 2003 Improving the aqueous solubility of triclosan by solubilization, complexation, and in situ salt formation. *Journal of Cosmetic Science* **54** (6), 537–550.

Hammer, O., Harper, D. A. T. & Ryan, P. D. 2001 PAST: Paleontological statistics software package for education and data analysis. *Paleontologia Electronica* **4**, 9.

Harrison, E. Z., Oakes, S. R., Hysell, M. & Hay, A. 2006 Organic chemicals in sewage sludges. *Science of the Total Environment* **367** (2–3), 481–497.

Hernández-López, J. & Vargas-Albores, F. 2003 A microplate technique to quantify nutrients (NO_2^-, NO_3^-, NH_4^+ and PO_4^{3-}) in seawater. *Aquaculture Research* **34** (13), 1201–1204.

Holzem, R. M., Stapleton, H. M. & Gunsch, C. K. 2014 Determining the ecological impacts of organic contaminants in biosolids using a high-throughput colorimetric denitrification assay: a case study with antimicrobial agents. *Environmental Science & Technology* **48** (3), 1646–1655.

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B. & Buxton, H. T. 2002 Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: a national reconnaissance. *Environmental Science & Technology* **36** (6), 1202–1211.

Kos, P. 1998 Short SRT (solids retention time) nitrification process/ flowsheet. *Water Science and Technology* **38** (1), 23–29.

Lee, D. G., Cho, K.-C. & Chu, K.-H. 2015 Removal of triclosan in nitrifying activated sludge: effects of ammonia amendment and bioaugmentation. *Chemosphere* **125**, 9–15.

Levy, C. W., Roujeinikova, A., Sedelnikova, S., Baker, P. J., Stuitje, A. R., Slabas, A. R., Rice, D. W. & Rafferty, J. B. 1999 Molecular basis of triclosan activity. *Nature* **398** (6726), 383–384.

Lindstrom, A., Buerge, I. J., Poiger, T., Bergqvist, P. A., Muller, M. D. & Buser, H. R. 2002 Occurrence and environmental behavior of the bactericide triclosan and its methyl derivative in surface waters and in wastewater. *Environmental Science & Technology* **36** (11), 2322–2329.

Lu, Q., He, Z. L. & Stoffella, P. J. 2012 Land application of biosolids in the USA: a review. *Applied and Environmental Soil Science* **2012**, 11.

Lukow, T., Dunfield, P. F. & Liesack, W. 2000 Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. *FEMS Microbiology Ecology* **32** (3), 241–247.

Manning, J. F. & Irvine, R. L. 1985 The biological removal of phosphorus in a sequencing batch reactor. *Journal (Water Pollution Control Federation)* **57** (1), 87–94.

McAvoy, D. C., Schatowitz, B., Jacob, M., Hauk, A. & Eckhoff, W. S. 2002 Measurement of triclosan in wastewater treatment systems. *Environmental Toxicology and Chemistry* **21** (7), 1323–1329.

McMurry, L. M., Oethinger, M. & Levy, S. B. 1998 Triclosan targets lipid synthesis. *Nature* **394** (6693), 531–532.

McNamara, P. J., LaPara, T. M. & Novak, P. J. 2014 The impacts of triclosan on anaerobic community structures, function, and antimicrobial resistance. *Environmental Science & Technology* **48** (13), 7393–7400.

Morales, S., Canosa, P., Rodríguez, I., Rubí, E. & Cela, R. 2005 Microwave assisted extraction followed by gas chromatography with tandem mass spectrometry for the determination of triclosan and two related chlorophenols in sludge and sediments. *Journal of Chromatography A* **1082** (2), 128–135.

Neumegen, R. A., Fernandez-Alba, A. R. & Chisti, Y. 2005 Toxicities of triclosan, phenol, and copper sulfate in activated sludge. *Environmental Toxicology* **20** (2), 160–164.

Pothitou, P. & Voutsa, D. 2008 Endocrine disrupting compounds in municipal and industrial wastewater treatment plants in Northern Greece. *Chemosphere* **73** (11), 1716–1723.

R Development Core Team 2008 *R: A Language and Environment for Statistical Computing.* R Foundation for Statistical Computing, Vienna, Austria.

Rieger, L., Gillot, S., Langergraber, G., Ohtsuki, T., Shaw, A., Takacs, I. & Winkler, S. 2012 *Guidelines for Using Activated Sludge Models,* IWA Publishing, London, UK.

Roh, H., Subramanya, N., Zhao, F. M., Yu, C. P., Sandt, J. & Chu, K. H. 2009 Biodegradation potential of wastewater micropollutants by ammonia-oxidizing bacteria. *Chemosphere* **77** (8), 1084–1089.

Rosenberg, S. 2000 Consumer and market use of antibacterials at home. *The Pediatric Infectious Disease Journal* **19** (10), S114–S116.

Sabaliunas, D., Webb, S. F., Hauk, A., Jacob, M. & Eckhoff, W. S. 2003 Environmental fate of triclosan in the River Aire Basin, UK. *Water Research* **37** (13), 3145–3154.

Singer, H., Muller, S., Tixier, C. & Pillonel, L. 2002 Triclosan: occurrence and fate of a widely used biocide in the aquatic environment: field measurements in wastewater treatment plants, surface waters, and lake sediments. *Environmental Science & Technology* **36**, 4998–5004.

Stasinakis, A. S., Petalas, A. V., Mamais, D., Thomaidis, N. S., Gatidou, G. & Lekkas, T. D. 2007 Investigation of triclosan fate and toxicity in continuous-flow activated sludge systems. *Chemosphere* **68** (2), 375–381.

Stasinakis, A. S., Mamais, D., Thomaidis, N. S., Danika, E., Gatidou, G. & Lekkas, T. D. 2008 Inhibitory effect of triclosan and nonylphenol on respiration rates and ammonia removal in activated sludge systems. *Ecotoxicology and Environmental Safety* **70** (2), 199–206.

Stenstrom, M. K. & Poduska, R. A. 1980 The effect of dissolved oxygen concentration on nitrification. *Water Research* **14** (6), 643–649.

The TCC Consortium 2002 *High Production Volume (HPV) Chemical Challenge Program Data Availability and Screening Level Assessment for Triclocarban: CAS #: 101-20-2.* Prepared for the HPV Challenge Program, US Environmental Protection Agency.

Thompson, A., Griffin, P., Stuetz, R. & Cartmell, E. 2005 The fate and removal of triclosan during wastewater treatment. *Water Environment Research* **77** (1), 63–67.

USEPA 1999 *Wastewater Technology Fact Sheet: Sequencing Batch Reactors*. USEPA, Office of Water, Washington, DC, USA, EPA 832-F-99-073.

USEPA 2009 *Targeted National Sewge Sludge Survey Sampling and Analysis Technical Report*. USEPA, Office of Water, Washington, DC, USA.

Wang, S. & Gunsch, C. K. 2011 Effects of selected pharmaceutically active compounds on treatment performance in sequencing batch reactors mimicking wastewater treatment plants operations. *Water Research* **45** (11), 3398–3406.

Wang, S., Holzem, R. M. & Gunsch, C. K. 2008 Effects of pharmaceutically active compounds on a mixed microbial community originating from a municipal wastewater treatment plant. *Environmental Science & Technology* **42** (4), 1091–1095.

Wu, C., Spongberg, A. L. & Witter, J. D. 2009 Adsorption and degradation of triclosan and triclocarban in soils and biosolids-amended soils. *Journal of Agricultural and Food Chemistry* **57** (11), 4900–4905.

Ying, G.-G. & Kookana, R. S. 2007 Triclosan in wastewaters and biosolids from Australian wastewater treatment plants. *Environment International* **33** (2), 199–205.

Zeng, R. J., Lemaire, R., Yuan, Z. & Keller, J. 2003 Simultaneous nitrification, denitrification, and phosphorus removal in a lab-scale sequencing batch reactor. *Biotechnology and Bioengineering* **84** (2), 170–178.

First received 28 August 2017; accepted in revised form 24 October 2017. Available online 7 November 2017