Effect of triclosan on protozoa in wastewater treating bioreactors
B. Krishnakumar, V. N. Anupama, S. Anju and M. Rugminisukumar

ABSTRACT

The effect of antimicrobial chemical, triclosan (TCS) on protozoa present in aerobic and anaerobic bioreactors for wastewater treatment is reported in this study. Bioreactor sludge ciliated protozoa such as Spirostomum, Cyclidium and Colpoda and flagellated protozoa Mastigella were exposed to TCS at 1 mg/L level in batch cultures. The response of TCS on protozoa was followed by microscopic observation of the sludge at specific time intervals. Among the protozoa in aerobic sludge, Colpoda exhibited strong sensitivity to triclosan and the cells distorted and burst in 20 minutes period. Mastigella and Spirostomum were resistant to triclosan for first two days, but afterwards the mobility was found declined and subsequently mortality increased to 100% in four and five days respectively. Anaerobic sludge ciliate, Cyclidium exhibited morphological distortion and motility decline after 1 hour exposure to triclosan. The sludge flocs were affected and pelagic cell count declined by the presence of triclosan at the tested level.

Key words | antimicrobial compounds, grazing fauna, triclosan, wastewater treatment

INTRODUCTION

Triclosan (5-chloro-2-(2,4-dichlorophenoxy) phenol) (TCS) is a common antimicrobial/ bacteriostatic compound present in a large number of personal care and household products (Daughton & Ternes 1999; Adolfsson-Erici et al. 2002). It is active against many of Gram-positive, Gram-negative-non sporulating bacteria, certain viruses and fungi (Suller & Russell 2000; Schweizer 2001). It can also control the growth of parasitic protozoa – Plasmodium and Toxoplasma (McLeod et al. 2001). The excess use of this compound makes its way to the environment mainly through sewage treatment facilities (Lindstrom et al. 2002; Singer et al. 2002). There is a growing concern about the natural occurrence, environmental fate and effect some of the transformation products of TCS (McMurry et al. 1998). There are a number of reports on the removal of TCS in sewage treatment systems (Sabaliunas et al. 2003; Thomas & Foster 2005). Removal of TCS in wastewater treatment facilities depends on operational parameters of the bioreactor such as Hydrolytic Retention Time (HRT), Solid Retention Time (SRT), pH of the bulk phase, temperature, fat content etc. (Winkler et al. 2007). Meanwhile, the actual mechanism of TCS removal in wastewater treatment facilities has not been clearly elucidated yet. There are different reports on biodegradation, sludge adsorption and effluent release of TCS in wastewater treatment systems. Activated sludge systems removed more TCS (96%) compared to Trickling-filters (58–86%), the treated effluent finally contained TCS at 0.2–2.7 µg/L (McAvoey et al. 2002). In a field study, Singer et al. (2002) have reported biodegradation of 79% TCS, while 15% of the compound adsorbed onto sludge and 6% (42–213 ng/L) released through the treated effluent. In a separate study, >90% removal of TCS was reported where the treated effluent containing ~50 ng/L TCS (Bester 2003). In a conventional treatment system, ~50% of the TCS entering the treatment the system was adsorbed onto sludge, while ~48% biotransformed, the discharge effluent contained ~0.07 µg/L TCS (Heidler & Halden 2007). The concentration of TCS in sludge and biosolid samples reported ranged from <1 mg/kg to 55 mg/kg (Table 1).

The presence of TCS in treated water reduces its reuse potential and studied have been reported on the removal of TCS from aqueous phase. Both pure and mixed microbial
Table 1 | Concentration of Triclosan in sludge and biosolid samples reported

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample</th>
<th>Triclosan level</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Primary &amp; secondary</td>
<td>0.53–15.6 mg/kg</td>
<td>McAvoy et al. 2002</td>
</tr>
<tr>
<td></td>
<td>digested sludge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Wastewater treatment sludge</td>
<td>0.4–8.8 mg/kg</td>
<td>Bester 2003</td>
</tr>
<tr>
<td>3</td>
<td>Digested sludge</td>
<td>Up to 29 mg/kg</td>
<td>US EPA 2003</td>
</tr>
<tr>
<td>4</td>
<td>Digested, dewatered sludge</td>
<td>20–55 mg/kg</td>
<td>Heidler &amp; Halden 2007</td>
</tr>
<tr>
<td>5</td>
<td>Anaerobic wastewater</td>
<td>0.09–16.79 mg/kg</td>
<td>Ying &amp; Kookana 2007</td>
</tr>
<tr>
<td></td>
<td>treatment plant biosolid</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2 | Characteristics of aerobic sludge used in the study

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLSS</td>
<td>2200 mg/L</td>
</tr>
<tr>
<td>Floc size range</td>
<td>50–400 μm</td>
</tr>
<tr>
<td>pH</td>
<td>7.3</td>
</tr>
<tr>
<td>Temperature</td>
<td>30°C</td>
</tr>
<tr>
<td>No. of Spirostomum</td>
<td>280 ± 21/ml</td>
</tr>
<tr>
<td>No. of Mastigella</td>
<td>55 ± 7/ml</td>
</tr>
<tr>
<td>No. of Colpoda</td>
<td>34 ± 4/ml</td>
</tr>
<tr>
<td>No. of Vorticella</td>
<td>NA</td>
</tr>
<tr>
<td>No. of Amoeba</td>
<td>NA</td>
</tr>
<tr>
<td>No. of Flagellates</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA – Not accounted

The present study was conducted with protozoa (ciliates and flagellates) present in both aerobic and anaerobic bioreactor sludge. The protozoa selected were *Spirostomum*, *Mastigella*, *Colpoda* and *Cyclidiun*. triclosan at a concentration of 1 mg/L was tested and the effect was monitored in terms of motility, morphological changes and mortality at different time intervals in batch experiments. triclosan was procured from Sigma-Aldrich and TCS stock solution was prepared by dissolving TCS in methanol (conc. equivalent to 1 mg/ml). All the batch experiments were repeated with duplicate and average values were plotted.

**MATERIALS AND METHODS**

Activated sludge sample collected from a commercial diary effluent treatment plant in Thiruvananthapuram, Kerala (India) was used for conducting batch experiments with aerobic protozoa. Characteristic of the sludge sample is given in Table 2. The sludge was brought to the Laboratory and maintained as a seed culture. It was continuously aerated and synthetic sewage was provided as feed once in a week. The composition of the synthetic sewage was (per litre) Tapioca powder 0.55 g, sodium bicarbonate-0.84 mg, ammonium chloride-0.05 g, stearic acid-2.5 ml, milk-1.1 ml and pH adjusted to 6.8. The MLSS of the sludge sample was estimated through standard methods (APHA 1998).

**Sludge samples and aerobic protozoa**

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Effect of triclosan on protozoa in aerobic reactor sludge

Effect of TCS on three different protozoa from aerobic sludge was conducted in separate batch experiments. Protozoa such as Spirostomum, Mastigella and Colpoda originally present in the sludge were selected for the study. 50 ml of activated sludge sample was taken in a 250 ml conical flask. One of the flasks were kept as control (sludge alone), whereas methanol (MeOH) alone and TCS in MeOH at 1 mg/l were added to the second and third flask respectively. The conical flasks were kept in a shaker maintained at 30°C and 100 rpm. The number of Spirostomum present in test and control bottles was accounted at the beginning of incubation. Samples were withdrawn daily and the total number of Spirostomum was counted. One ml of the sample was taken from the bottles and spread uniformly on a plankton counting chamber and counting was performed under 10X objective of a microscope (Nikon-Alphashot-2 YS2). MeOH and TCS in MeOH at 1 mg/l was added in to the samples once a day for three days and counting was repeated as described. The effect of triclosan on Mastigella and Colpoda were also done in a similar way as described. However, in the case of Colpoda, the samples were withdrawn at 5, 10, 15 and 20 min interval and counting was done under 20X objective of the microscope. Fast moving Colpoda was slowed down by adding 0.6% NiSO4 to the sample.

Effect of triclosan on anaerobic ciliate: Cyclidium

From a stock culture of Cyclidium maintained in our laboratory, 50 ml was taken. Culture was centrifuged (Tomy, Japan) at 1000 rpm for 10 min., supernatant was discarded and pellet was taken. 100 ml of deaerated ciliate mineral media was added to the pellet and maintained. The composition of ciliate mineral medium was (g/L) K2HPO4 – 0.125, NH4Cl–0.025, NaCl–0.4, MgCl2.6H2O–0.2, KCl–0.15, CaCl2 2H2O–0.25. The medium was deaerated by sparging in N2 for 10 minutes and pH was adjusted to 7.0 by adding 1N NaOH. Three 15 ml crimp cap vials were taken and 10 ml of Cyclidium culture was added to each bottle. One of the bottles is maintained as control, the second and third bottles were added with methanol and TCS in methanol respectively. The samples were then kept for incubation under room temperature. The initial number of Cyclidium in the bottle was counted and daily samples were withdrawn from each bottle and counting was repeated till day 5.

Effect of TCS on floc structure and pelagic cells

To assess the toxicity of TCS on bacterial cells and flocs, fluorescent dye staining was performed with 4,6- Diamidino-2 Phenyl Indole (DAPI) and Propidium Iodide (PI) (both from Sigma-Aldrich). One ml sludge sample was withdrawn from control, MeOH-control and TCS-MeOH added bottles. The samples were diluted ten times with sterile saline. To 90 μl of diluted samples, 10 μl of triton X-100 was added and mixed well. 10 μl of the above samples was uniformly spread on to alternate wells in a HTC slide (Cell line USA). Slide was dried in an oven at 40°C for 10 minutes. After drying, 5 μl of DAPI was added to the samples and kept for incubation at room temperature for 10 minutes. After incubation, the slide was rinsed with Milli-Q water to remove excess dye, dried mounted on Vectashield (Sigma) and observed under UV filter in an epifluorescent microscope (Leica DM 2500). PI staining was also done in a similar way. The slide was observed under green filter of the microscope.

Statistical significance of the data (protozoa and free floating cell count in control and TCS added cultures) was analyzed through paired T-test (two tailed) using Excel software.

RESULTS AND DISCUSSION

Effect of triclosan on protozoa in aerobic sludge

In the present study the antimicrobial compound TCS was added as dissolved in methanol, due to its comparatively low solubility in water (10 mg/L). Therefore, a methanol control experiment was run in parallel to separate the effect of methanol alone on protozoa studied. Among the four different protozoa studied, each showed different tolerance level to TCS at 1 mg/L. The MLSS of the sludge sample used was 2200 mg/L. When TCS was added to aerobic sludge sample for the first time, no change in the number, shape or mobility of the protozoa was observed. This could be due to adsorption of TCS on suspended solids including flocs and associated extracellular matrix. But, during subsequent addition of TCS, the number of organisms found to decline and there were observable morphological changes. A number of studies have reported the adsorption of TCS to sludge/biosolids (Table 1). In the present study the effect of TCS was tested at 1 mg/L, which is higher than normal TCS level in wastewater, sludge and treated effluents. A slightly higher TCS was chosen in this study to overcome the adsorptive removal of TCS from bulk liquid. The sludge used in the present study was from a diary
effluent treatment plant, possibly with higher fat content and
so more adsorption of TCS is expected. Significant correla-
tion was reported between TCS removal and lipid content of
biomass in Rotating Biological Contactor ($r^2 = 0.986$) and in
oxidation ditch ($r^2 = 0.897$) (Winkler et al. 2007). Moreover, it
was demonstrate that TCS at 30 $\mu$g/L exhibited 2,532 time
bioaccumulation in zebra fish owing to high fat content of the
organism (Orvos et al. 2002). The physicochemical properties
of TCS also justify high solubility in lipids. However, TCS
adsorption to sludge was not quantified in this study.

Spirostomum is a ciliated protozoa, the size may vary
from 200–2000 $\mu$m (Figure 1A). It feeds mainly on suspended
bacterial cells in the sludge. In this study, when TCS was
added to sludge at 1 mg/l daily for two days, initially there
was no change observed in the number, shape or mobility of
the organisms. But after two days, the mobility was affected in
TCS (in MeOH) added cultures. Meanwhile, no change was
observed in MeOH alone added cultures. On the third day,
the number of Spirostomum was declined and on the fifth
day, Spirostomum was completely absent in the medium
(Figure 2). A recent study with pure culture of Spirostomum has
reported no detectable effect up to 50 $\mu$g/L TCS, but
significant effect was observed at 100 $\mu$g/L (Lawrence et al.
2009). There were no previous reports on effect of antibac-
terial or toxic compounds on Spirostomum in wastewater treat-
ing sludge. The present study also showed methanol exerts
mild toxicity to Spirostomum. However, in TCS-MeOH cul-
tures the effect was more and this could be due to the added
toxic effect of TCS. One of the reasons for TCS toxicity could
be direct effect on the ciliate as reported in Plasmodium and
Toxoplasma (McLeod et al. 2001).

Mastigella is a mono flagellated protozoa present in
aerobic sludge (Figure 1B). When TCS was added to sludge,
initially Mastigella exhibited no toxicological responses.
However, after 2 days the mobility was reduced and the
organism showed morphological distortion. The number of
mastigella declines gradually and complete mortality was
observed on the fourth day. The decline in number of
Mastigella in TCS added bottle was statistically significant
($P = 0.16$). In methanol added control after three days, the
number of Mastigella reduced gradually (Figure 3).

Colpoda was found to be highly sensitive to triclosan,
when compared to other protozoa present in aerobic sludge
in this study (Figure 4). Normally Colpoda is a bean shaped
ciliate, but after treatment with TCS its shape changed into
spherical and burst after 20 minutes. The mobility was
affected within ten minutes of exposure. Analysis of the
data revealed highly significant decline in number of Colpoda
in the test bottle ($P = 0.042$). Time lapse image of the effect of
TCS addition on Colpoda is shown in Figure 5. In MeOH
added samples, there was no change in the mortality or
mobility of Colpoda.

Effect of triclosan on anaerobic ciliate: Cyclidium

Cyclidium is a typical ciliate present in micro-aerophilic and
anaerobic bioreactors for wastewater treatment and in envi-
ronmental samples (Figure 1C). When TCS at 1 mg/L level
was added to Cyclidium cultures, the number was found to
decline after 1 hour. The motility was affected and the shape
changes in to spherical. Within 4 hours 100% mortality was
observed in TCS added samples (Figure 6). In simultaneous
methanol alone added cultures, the toxicity was compara-
tively low. There were no previous reports on the effect of
TCS or other antibacterial chemicals on Cyclidium or similar
ciliates in wastewater treatment systems.

Effect of TCS on floc structure and pelagic cells

In epifluorescent microscopic observation of the activated
sludge after treatment with TCS, the flocs became looser and
an increase in number of small flocs was observed (Table 3).
This could be a toxic response to TCS at 1 mg/L level. In a previous study, activated sludge floc size and structure were not affected by TCS addition at 1 mg/L, but a minor decline in COD removal was reported (Stasinakis et al. 2007). There was a significant (P = 0.0001) decline in free swimming cell (pelagic) count in the sludge declined after TCS treatment (Table 3).

Apart from the antibacterial activity, antimicrobial chemicals such as TCS affect higher trophic organisms such as protozoa in bioreactor sludge as evident in the present study. In a previous study it was reported that TCS inhibits Plasmodium falciparum and Toxiplasma gondii through inhibiting a specific protein synthetic pathway (McLeod et al. 2001). Environmental concentrations of TCS are not considered to be acutely toxic to protozoa (Miyoshi et al. 2003). A recent study using pure cultures of protozoa like Euploetes, Dileptus, Blepharism, Stentor, Spirostomum, Euglena and Paramecium indicated no detectable effects of TCS up to 50 μg/L, but significant effect was observed at 100 μg/L (Lawrence et al. 2009). Studies conducted by Miyoshi et al. (2003) found TCS toxicity P. trichium and P. caudatum at 0.75 μg/L and 0.47 μg/L respectively. In a different study, TCS was reported toxic to a number of aquatic flora and fauna (Orvos et al. 2002). The bioreactors are normally operated around neutral pH. This leads to un-ionized TCS in the liquid phase that imposes more toxicity to protozoa community. The non observed effective concentration (NOEC) of TCS to Ceriodaphnia dubia at pH 7 for survival and reproduction were 50 and 6 μg/L respectively, where NOEC at pH 8.5 were 339 and 182 μg/L respectively (Orvos et al. 2002). Therefore, in sewage treating bioreactors the continued presence of TCS like chemicals can adversely affect protozoa, which in turn affect the treatment process and deteriorate effluent quality. In natural environments, the presence of triclosan and similar compounds can affect the trophic structure and dynamics of the ecosystem. Therefore, more controlled usage of these chemicals is needed. The present study also shows that triclosan and similar chemicals may find application in removing pathogenic protozoa in treated effluent. However, more studies are required to evaluate this.

CONCLUSIONS

The following conclusions can be made from the present study

1. Presence of bacteriostatic/antimicrobial chemicals like triclosan affects higher trophic organisms like ciliate and
flagellate protozoa in aerobic and anaerobic reactor sludge. The response towards triclosan was exhibited in terms of motility slow down, morphological changes and ultimately mortality of the protozoa.

2. Among the protozoa studied, *Colpoda* was most sensitive to triclosan at 1 mg/L level and *Spirostomum* comparatively less sensitive. Also, anaerobic reactor protozoa (*Cyclidium*) was more sensitive than aerobic (*Spirostomum, Mastigella*).

3. The continued presence of these chemicals can affect the growth of protozoa which can directly affect the treatment process and deteriorate effluent quality. In natural environments, the presence of triclosan and similar compounds can affect the trophic structure and dynamics of the ecosystem.

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REFERENCES


