Effects of triclosan incorporation into ABS plastic on biofilm communities

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Objective: This study compared the attached biofilm populations on acrylonitrile-butadiene-styrene (ABS) plastic with and without the incorporation of the antimicrobial triclosan [5-chloro-2-(2,4-dichlorophenoxy) phenol] after 1–3 weeks of exposure to drinking water.

Methods: Biofilms were cultivated on triclosan-incorporated (TP) and control plastics (CP) in continuous flow culture reactors with drinking water as the growth medium and inoculum. After 1–3 weeks of exposure, the plastics were removed and the biofilms aseptically harvested. The attached communities were examined with respect to direct cell counts, culturability, triclosan resistance and community composition.

Results: Based on these analyses, no significant differences were observed between the populations attached to TP and CP surfaces. Results from both a bioavailability assay and gas chromatography mass spectrometry analyses, revealed that only trace amounts of triclosan desorbed from the plastic. The lack of biofilm community difference, coupled with this limited desorption of triclosan from the TP indicates that the ABS plastic studied was no more effective at controlling bacterial populations than the control plastic because the antimicrobial was not bioavailable.

Conclusions: These results call into question the long-term utility of triclosan incorporation into ABS plastic and highlight the need for proof of efficacy regarding the antimicrobial properties of such materials.

Keywords: 5-chloro-2-(2,4-dichlorophenoxy) phenol, drinking water, antimicrobials, bis-phenol

Introduction

Triclosan [5-chloro-2-(2,4-dichlorophenoxy) phenol], a non-ionic, broad-spectrum antimicrobial agent has been widely used since its introduction in the 1960s. Recently, however, the use of triclosan as a preservative and disinfectant has seen a dramatic increase in the USA and Europe.¹ Triclosan is also widely marketed in numerous personal care products including a variety of shampoos, hand creams, hand soaps² and toothpastes. Toothpastes and mouthwashes with triclosan have been studied as anti-plaque and anti-gingivitis agents.³⁻⁵ Commercial products containing triclosan include textiles, plastics and other polymers, which may be fashioned into toys, cutting boards and other products (http://www.microban.com).

The widespread use of this compound in household, industrial and hospital settings results in disposal down the drain and ultimately leads to environmental deposition. As a result of such widespread use, triclosan is found in wastewater, deposited in environmental sediments and concentrated in aquatic biota.¹,⁶⁻⁸ Triclosan may also enter the terrestrial environment when triclosan-containing sewage sludges⁹,¹⁰ are land-applied. Additionally, triclosan has been identified in human plasma,¹¹ breast milk and in the bile of fish exposed to municipal waste water.¹² The mechanism of action, consequences of resistance selection and bioaccumulation of triclosan raise concerns over the long-term effects that such pervasive use may have.

Triclosan acts as a broad-spectrum antimicrobial agent by targeting lipid biosynthesis and inhibiting cell growth.¹³⁻¹⁵ The enoyl-ACP reductase (ENR) is a cellular target of triclosan. The minimal inhibitory concentrations for a variety of tested organisms range from less than one part per million to parts per thousand for Pseudomonas.¹⁶ Pseudomonas aeruginosa contains a triclosan-sensitive ENR, but resistance can be mediated by multidrug efflux pumps such as MexAB:OprM, MexCD:OprJ and MexEF:OprM.¹⁷ In addition to efflux mechanisms, specific mutations and degradation have been reported to increase tolerance to triclosan.¹⁸,¹⁹ Similar mechanisms are involved in resistance to antibiotics and may account for increased cross-resistance to antibiotics in triclosan-tolerant organisms.²⁰

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Previous investigations of triclosan-incorporated plastics and polymers involved experimental systems based on pure cultures and were not conclusive as to the antimicrobial utility of such polymers. Triclosan released from poly styrene initially reduced growth of Bacillus thuringiensis and Escherichia coli, but was less effective at growth inhibition over extended time.\textsuperscript{21} Triclosan-incorporated plastic wrap boxes were demonstrated to be effective against \textit{E. coli} when grown in rich liquid medium in contact with the plastic at 30 and 22°C but no difference was observed when grown at 4°C.\textsuperscript{22} Using plate growth assays, it was demonstrated that triclosan-containing polymer coating a food packaging material was effective against \textit{Enterococcus faecalis},\textsuperscript{23} whereas a triclosan-incorporated plastic wrap did not effectively reduce bacterial numbers on refrigerated and vacuum packed meat surfaces.\textsuperscript{24} The presence of triclosan in a soft denture liner did not reduce the adherence of viable \textit{Candida albicans} after 24 h of exposure.\textsuperscript{25} Others have demonstrated that triclosan in solid substrates was deactivated by soil bacteria and this deactivation provided a niche for sensitive bacteria to grow.\textsuperscript{18}

Despite concerns about the development of antibiotic-resistant bacteria, a recent study, investigating the effect of triclosan-containing detergents at standard use concentrations, did not detect any effects on antimicrobial susceptibility of biofilm organisms at normal use levels.\textsuperscript{26} Although direct exposure to triclosan in water had no effect on resistance, it is not clear what effect triclosan incorporation has on the development of mixed biofilm communities grown on plastic containing this compound.

Biofilms can form wherever a solid surface is in contact with water. The cells within the biofilm are surrounded by exopolymeric substances, which protect the cells from predation and microbicides.\textsuperscript{27} Cells residing within a biofilm are typically slow growing and therefore less susceptible to many traditional microbicides that target aspects of rapid cell growth and division.\textsuperscript{28} Additionally, the structure of the biofilm, as well as the growth status of the cells and exopolymeric substances, may limit the diffusion and effectiveness of biocides incorporated into polymers.\textsuperscript{27}

The goal of this study was to determine the effects that triclosan incorporation into acrylonitrile–butadiene–styrene (ABS) plastic has upon adherent bacterial biofilm populations from drinking water.

**Materials and methods**

**Bioreactor design and operation**

Triclosan-incorporated ABS plastic with 5% w/w (5000 mg/kg) triclosan (TP) and control ABS plastic without triclosan (CP) were obtained from Watkins Manufacturing Corp. (Vista, CA, USA). These plastics were cut into 7.5 × 2.5 × 0.3 cm coupons, which were used as a surface for biofilm attachment and to study triclosan desorption. Reactors for biofilm formation were previously described.\textsuperscript{29} Briefly, they consisted of four plastic coupons suspended in a stopped 250 mL glass graduated cylinder with ports for influent, effluent flow and aeration.\textsuperscript{29} The reactors were assembled and sterilized by autoclave. A 10 L polypropylene carboy was sterilized by autoclave and then filled with non-sterile drinking water from the water treatment facility, City of Ithaca, NY (treated by chlorination). The drinking water, which served both as the sole bacterial inoculum and nutrient source, was peristaltically pumped by a Watson-Marlow pump. Each reactor had a retention time of 6 h and the liquid volume was maintained at 195 mL. Airflow was provided by a Second Nature, Whisper 900 aquarium pump, regulated at 0.8 L/min, sterilized by passage through a 0.2 µm PTFE filter (Nalgene, Rochester, NY, USA), and dispersed in the reactor with a Kordon Mist Air fine bubble airstone (Hayward, CA, USA). The reactors were continually operated at bench top conditions between 20 and 22°C for either 7 or 21 days, after which times the plastic coupons were removed, rinsed with sterile water and the biofilm examined by the methods described below.

Two sets of reactor experiments were conducted. The first experiment was operated for 7 days and consisted of two reactors, which contained four coupons of either TP or CP. These reactors were designated T1 and C1, respectively. The biofilms harvested from these coupons were analysed by ribosomal intergenic spacer analysis (RISA).\textsuperscript{30} Automated RISA (ARISA),\textsuperscript{31} restriction fragment length polymorphism (RFLP) analysis of the 16S–23S intergenic spacer region and a clone library. The second set of experiments consisted of triplicate reactors, which were run for 21 days and were operated as indicated above except that each contained two TP and two CP coupons. These were designated reactors 2, 3 and 4. The biofilms that formed in these reactors were examined for culturability and increase in triclosan tolerance. Community fingerprints were also obtained using ARISA.

**Microscopy and cell enumeration**

Immediately after harvesting, the plastic coupons were rinsed with sterile water. Direct observation of attached cells was carried out on one TP and CP coupon using Acridine Orange staining and epifluorescence microscopy using a Nikon Eclipse E600 with an Acridine Orange filter, illuminated by a Mercury 100 W lamp (Chiu Technical Corporation). Images were captured with a Hamamatsu Colour Chilled 3CCD camera. From the three remaining coupons, cells were removed by scraping the entire surface with a sterile razor blade. These cells were suspended to a volume of 1 mL with sterile water and mixed by vortexing. Microscopic observation revealed that this method appeared to be sufficient to break-up biofilm aggregates, leaving only individual cells in suspension. The scraped cells were pooled for each plastic type within a given reactor. A 100 µL aliquot of resuspended cells was stained with 1 µL of 0.05% Acridine Orange (Electron Microscopy Sciences, Fort Washington, PA, USA) for 3 min. Direct counts were then carried out in a Levey Counting Chamber (Hauser Scientific, Blue Bell, PA, USA) and examined with epifluorescence microscopy. From each plastic type, 30 fields were examined.

Culturability studies were carried out using cell suspensions that were diluted in series and plated on R2A agar (Difco Laboratories, Detroit, MI, USA) and diluted 1/10th strength R2A agar with 50, 25, 5, 0.5 and 0.0 mg/L triclosan (Ciba Specialty Chemicals, High Point, NC, USA). A triclosan stock solution was prepared in 100% ethanol and added to the warm medium after autoclaving, the apparent solubility of triclosan in agar is greater than 25 mg/L. Concentrations that exceeded the agar solubility were used to limit the effect that tolerant organisms may have by reducing the local concentration of triclosan. R2A agar was selected because it is widely used for enumerating heterotrophic organisms from treated potable water. Plated colonies were counted after incubating for 5 days at 22°C. Statistical significance was assessed using the Student’s \textit{t}-test (\textit{p} = 0.05) and compared between the plastic types as well as between the media used for enumeration.

**Fingerprint analysis and cloning**

PCR was carried out using the HotStarTaq Master Mix kit from Qiagen (Valencia, CA, USA) and a PTC-200 DNA Engine thermocycler from MJ Research Inc. (Reno, NV, USA). RISA was carried out after PCR amplification of whole cells with primers 1055F (5′-ATGCGTGCTCAGCT-3′)\textsuperscript{31} and 23SR (5′-GGGTBCCCATCGR-3′).\textsuperscript{30} Thermocycler conditions were based on a modified touchdown protocol.\textsuperscript{19} Briefly, 15 min at 95°C followed by 10 cycles with 95°C for 45 s annealing at 65°C for 15 s and extension at 70°C for 60 s. For touchdown, the annealing temperature was decreased 1°C for the first 10 cycles and then maintained at 55°C for the remaining 20 cycles. After a total of 30 cycles,
the final elongation was 3 min at 70°C. PCR products amplified from the ribosomal intergenic spacer region were separated in a 1.5% agarose gel by electrophoresis at 80 mV using a Bio-Rad PowerPac 300 (Hercules, CA, USA) and visualized by ethidium bromide staining.

Clone libraries were generated by ligation of RISA fragments into pGEM T-Easy (Promega, Madison, WI, USA) and electroporated into E. coli strain JM109 using a BTX, Electro Cell Manipulator 600 (Genetronics Biomedical LTP, San Diego, CA, USA). Electro-competent JM109 cells were grown in complete Luria-Bertani (LB) medium and prepared by standard methods.33 Cells were stored in 20% glycerol at –80°C. Following electrophoresis, transformants were allowed to recover in LB for 1 h and were selected using blue-white screening on LB/ampicillin plates containing 40 mg/L X-Gal (International Biotechnologies Inc., New Haven, CT, USA). Ampicillin was used at a concentration of 100 mg/L. The clone library was stored in 20% glycerol at –80°C.

The cloned RISA fragments were PCR amplified from the pGEM T-Easy vector with primers T7F (5′–ACTAGTACGACTCACTATAGG–3′) and M13R (5′–AACGCTATGACCATG–3′) and subsequently digested for 2 h at 37°C with HaeIII (Promega, Madison, WI, USA). These digests were analysed on a 2% agarose gel after electrophoresis at 80 mV for 2 h and visualized by ethidium bromide staining. The resulting RFLP patterns were analysed by visual inspection. DNA sequencing was carried out at the BioResource Center, Cornell University, Ithaca, NY on an ABI3700 DNA Analyser using the Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase. Sequencing results were analysed using the BLASTN algorithm34 on the NCBI homepage (http://www.ncbi.nlm.nih.gov/BLAST/). Unique 16S fragments were submitted to GenBank and are available as accession numbers AF484045–AF484065. A phylogenetic tree was generated with the ARB software package (www.arb-home.de), which used the Phylib, N-J method with Fitch correction.

ARISA was carried out on PCR amplicons obtained from both whole cells and cloned RISA fragments using primers 1406f–5FAM (5′–TGGACACACCGCGCTC–3′) and 23SR.35 The ARISA fragments were separated and analysed on a 5% Long Ranger, 6 M urea, 1x TBE polyacrylamide gel on an ABI 377 DNA Sequencer equipped with GeneScan 3.1.2 and Genotyper 2.1 software (Perkin-Elmer Inc., Boston, MA, USA).

In order to quantitatively compare the ARISA fingerprints, electrophoretogram data were assembled into a binary matrix. All bands of a discrete and discernable base pair (bp) length that consisted of more than 5% of the largest peak were scored as 1, bands smaller than 5% were scored as 0. This matrix was then analysed for each of the microbial communities using a distance matrix described by Schloss et al.35

**Triclosan desorption**

The release of triclosan from consumer products into water was examined by analysing chloroform extracts via gas chromatography coupled with a mass selective detector (GC-MS). TP, a dishwetol with UltraFresh (Charles Craft Kitchen Products, Laurinburg, NC, USA), and a shower curtain (Home Essentials, Troy, MI, USA) were all examined. Desorption was examined in triplicate by adding 4.7 g of each material to 100 mL of sterile deionized distilled water in a glass 250 mL Erlenmeyer flask. This suspension was shaken (150 rpm) at 22°C for 50 h. Samples consisting of 3 mL of water were taken at 0, 22 and 50 h and then extracted with 1 mL of chloroform by shaking for 10 min. The organic phase was removed and dehydrated by passage over sodium sulphate (Fisher Scientific, Pittsburgh, PA, USA). A standard curve of six concentrations was generated by serial dilution of triclosan in chloroform and then evaporating the chloroform with a sterile stream of N2 (AirGas East Inc., Salem, NH, USA). These standards were suspended in water and extracted with chloroform as described above. Samples and standards were analysed on a Hewlett Packard 6890 series GC equipped with a Hewlett Packard 5973 MS detector using a HP-5MS column (29.2 m × 0.25 mm × 0.25 µm), initial oven temperature = 60°C, final temperature = 300°C, ramp rate = 30°C/min. Selected ion monitoring was carried out using m/z 218, 288 and 292. A six-point standard curve (R2 = 0.99) for triclosan extracted from water was determined from concentrations of 10 mg/L to 0.1 µg/L, and was used to calculate the concentration of triclosan in the samples. Glass containers and pipettes were used for all quantitative analyses of triclosan desorption and bioavailability.

**Bioavailability of triclosan**

The release of triclosan from the products mentioned above was also examined by measuring the induction of mineralization of [14C]triclosan by a triclosan-degrading bacterium that has been previously described.36 Briefly, a dense culture (OD600 = 0.450) of Sphingomonas strain RD1 pBBR1MCS-2 was grown on LB supplemented with rifampicin (100 mg/L) and kanamycin (50 mg/L). Then 5 mL of this culture was inoculated into a sterile 40 mL glass EPA vial. For each of the consumer products, 0.5 g of an autoclave sterilized sample was introduced to the culture. The positive control had 10 mg/L unlabelled triclosan added to the culture. Negative controls consisted of a treatment with no cells but with 10 mL of triclosan, and a treatment with cells but with no labelled triclosan added. To each triclosan/cell suspension, 40000 dpm of [14C]triclosan (Ciba Specialty Chemicals) was added. In previous work, this concentration of 14C-labelled triclosan, equivalent to approximately 0.25 mg/L, was shown to be insufficient to induce triclosan mineralization activity.36 In order to trap any 14CO2, a sterilized 8 mL borosilicate glass vial with 500 µL of 1 N NaOH was introduced aseptically to the conical tube. The system was capped and shaken at 150 rpm for 50 h. After incubation, the NaOH was removed and added to 5 mL of ScintiSafe Econol scintillation cocktail (Fisher Scientific, Pittsburgh, PA, USA), allowed to stabilize for 4 h and read using a Beckman LS5000CE scintillation counter.

**Results**

**Examination of biofilm community structure**

The biofilms from reactors T1 and C1 were examined by RISA, ARISA and clone library. In total, 109 clones were selected from the CP and 84 from the TP. After PCR amplification of each clone with primers T7F and M13R, the products were HaeIII digested and the resulting RFLP pattern from each clone was assigned into one of 43 unique pattern groups. Representatives from each pattern group were then sequenced. The number of clones from each phylogenetic group is represented as a percentage of the total (Figure 1).

Clones representative of unique RFLP patterns were sequenced and analysed for fragment size using ARISA. Sequencing results indicated that the most abundant clones on both TP and CP were from the α-, β- and γ-Proteobacteria. These accounted for approximately 77% of the total. Other clones were related to the Cytophaga, Flavobacterium and Bacteroides (CFB) group (20%), members of the high G+C Gram-positive organisms (1%), Chlamydiales group (<1%) and the Fibrobacter group (<1%). From control of the clones, almost 84% of the RFLP patterns were common to both the TP and CP, however 16% of the patterns were unique to either one plastic or the other. A phylogenetic tree was generated using partial 16S rDNA sequences from the clone libraries (Figure 2). ARISA (Figure 3) of biofilm cells from reactors 2, 3 and 4 are represented as base pair length and relative intensity for the PCR amplified products. The results of the ARISA indicated that the biofilms from different plastics and reactors had some unique bands, however the majority of bands were common to both TP and CP communities.
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from the triplicate reactors (Figure 3a and b). For comparison, the size of the cloned RISA fragments from reactors T1 and C1 are presented in Figure 3(c) but are not scaled with respect to relative abundance.

The ARISA data from reactors 2, 3 and 4 were converted into a binary matrix and assembled as a distance matrix (Table 1). This distance matrix demonstrates the relatedness of the communities found on the plastic surfaces as determined by ARISA. The means of the inter-treatment and intra-treatment distance matrix scores were not significantly different as determined by Student’s t-test (α = 0.05). The biofilms attached to the TP and CP from reactors 2, 3 and 4 were visualized by epifluorescence microscopy and were morphologically similar (data not shown). Direct counts of biofilm cells scraped from the surface of TP and CP showed that there was no significant difference between the numbers of organisms on the different plastic surfaces. Comparison of direct counts with the number of colony forming units also revealed no significant differences in the percentage of biofilm bacteria that were culturable between the two plastics. In addition, the percentage of triclosan-resistant strains was not significantly different at any of the tested concentrations (Table 2).

Figure 1. Distribution of clones based upon the 43 RFLP patterns from the control ABS plastic (black bars) and triclosan-containing plastic (white bars), respectively. Percentages were calculated with respect to the total number of clones from each plastic type (CP, n = 109; TP, n = 84) and are grouped by phylogenetic relatedness: α-α-Proteobacteria; β-β-Proteobacteria; CFB, Cytophaga-Flavobacter-Bacteroides; Chly, Chlamydiales; Fibro, Fibrobacter; γ-γ-Proteobacteria; G+C, high G+C Gram-positive.

Figure 2. Phylogenetic tree of partial 16S rDNA sequences from clones of organisms grown on triclosan (clone T) and control plastics (clone P) in reactors T1 and C1, respectively. ARB software was used to build the tree with the Phylip distance matrix using the Fitch method and Kimura/Dayhoff correction.
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Desorption and bioavailability of triclosan

Desorption of triclosan from consumer products including the triclosan plastic coupons was examined. The concentration of triclosan in sterile water that had been incubated with TP coupons for 50 h was approximately 12 \( \mu \)g/L. Water from all of the triclosan plastic reactors was tested for the presence of triclosan but none was detected (<0.1 \( \mu \)g/L). The concentration of triclosan in water incubated with fragments of the dish-towel was approximately 1.1 mg/L at 50 h, whereas the concentration of triclosan in water incubated with the shower curtain was approximately 18 \( \mu \)g/L.

The bioavailability of triclosan desorbing from the plastic was also investigated using a *Sphingomonas* strain that is capable of degrading triclosan to \( \text{CO}_2 \). The assay demonstrated that triclosan bioavailability was below the 1 mg/L detection limit of the assay in medium incubated with the triclosan plastic and the shower curtain when compared to the negative controls. There was however sufficient bioavailable triclosan in medium incubated with the UltraFresh towel to induce higher triclosan mineralization than observed in the positive control (Figure 4). This increased mineralization may have been due to the reservoir of additional triclosan in the towel fibres, as the 10 mg/L of triclosan in the control would have been rapidly degraded by the test organism, thereby depleting the inducer.

Discussion

Given recent reports about the antibacterial effects of triclosan incorporation into a variety of consumer products, we hypothesized that triclosan incorporation into ABS plastic would affect the community structure, cell numbers and triclosan tolerance of attached biofilm cells. Biofilm from triclosan and control plastics were examined by direct cell count, culturability on R2A agar and diluted 1/10th strength R2A agar, and resistance to 0.5, 5, 25 and 50 mg/L triclosan.

**Table 2.** Biofilm cells attached to triclosan and control plastics were examined by direct cell count, culturability on R2A agar and diluted 1/10th strength R2A agar, and resistance to 0.5, 5, 25 and 50 mg/L triclosan

<table>
<thead>
<tr>
<th></th>
<th>Biofilm from control plastic</th>
<th>Biofilm from triclosan plastic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct cell count average (cells/mm²)</td>
<td>3.89 ( \times 10^3 )</td>
<td>3.58 ( \times 10^3 )</td>
</tr>
<tr>
<td>% Culturability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R2A agar</td>
<td>4.98b</td>
<td>6.41b</td>
</tr>
<tr>
<td>1/10th R2A agar</td>
<td>5.42c</td>
<td>7.88c</td>
</tr>
<tr>
<td>1/10th R2A + 0.5 mg/L triclosan</td>
<td>9.60 ( \times 10^{-3} )</td>
<td>2.03 ( \times 10^{-3} )</td>
</tr>
<tr>
<td>1/10th R2A + 5 mg/L triclosan</td>
<td>3.55 ( \times 10^{-3} )</td>
<td>4.18 ( \times 10^{-3} )</td>
</tr>
<tr>
<td>1/10th R2A + 55 mg/L triclosan</td>
<td>9.11 ( \times 10^{-5} )</td>
<td>3.05 ( \times 10^{-5} )</td>
</tr>
<tr>
<td>1/10th R2A + 50 mg/L triclosan</td>
<td>0</td>
<td>0</td>
</tr>
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</table>

Direct cell counts are expressed as average cell number per surface area. Growth results are expressed as percentage culturability of the direct cell counts. Although culturability was significantly (\( \alpha = 0.05 \), comparisons indicated by superscripts) affected by triclosan in the medium, there were no significant differences in the number of culturable organism from the triclosan and control plastics.

Figure 4. Induction of \(^{14}\text{C}\)triclosan mineralization to \(^{14}\text{CO}_2\) by *Sphingomonas* sp. RD1 pBBR1MCS-2. Negative (Neg) Control 1: cells, \(^{14}\text{C}\)triclosan; Neg control 2: no cells, \(^{14}\text{C}\)triclosan and inducing triclosan; Neg control 3: NaOH in scintillation cocktail; positive (Pos) control: cells, \(^{14}\text{C}\)triclosan and inducing triclosan. The indicated experimental materials were incubated in the presence of cells and \(^{14}\text{C}\)triclosan.
organisms. In order to investigate this hypothesis, two sets of reactors were assembled. The ABS plastic used in this study has triclosan at 5% w/w. Such plastics have been moulded into many products including sinks, showers, spas and hot tubs (http://www.microban.com). Therefore, the reactor and growth conditions were designed to mimic the use of such plastics in household settings where they would be exposed to non-sterile aqueous conditions, at 20–22°C, with ambient light exposure and be used for multiple weeks. No exogenous nutrients were added to the reactor system. The biofilms from the first set of reactors (T1, C1) were examined by RISA, ARISA and clone library. Results from gel electrophoresis showed a few differences in the RISA fingerprints (data not shown), but community differences were found to be minimal upon sequence analysis of the clone library.

The sequencing results indicated that diverse communities were associated with both plastics. Results of the BLASTN search of GenBank indicated sequence similarity of clones to *Comamonas* which have been detected in waste water systems. Clones had similar sequence similarity to *α*-Proteobacteria (*Rhodo- bacter capsulatus*), suggesting the presence of phototrophic bacteria in the biofilm reactors. The library also had clones with sequence similarity to *Parachlamydia acanthamoebae*, an obligate intracellular coccoid parasite of *Acanthamoebae*, which is related to *Chlamydia* spp. The eubacterial specific RISA primers could not have detected the presence of protozoa directly, however the detection of obligate endosymbionts has been used as an indicator for the presence of protozoa in environmental samples. At first glance, the presence of *Sphingomonas* and *Pseudomonas* species is of interest as these organisms are phylogenetically related to others that have been reported to tolerate and/or degrade high levels of triclosan. The significance of these findings with respect to the effects of triclosan incorporation is not clear, however, as both genera were also found on the control plastic. The breadth of community diversity as represented by clones is apparent in the phylogenetic tree (Figure 2).

As a result of low biomass yields, the analyses of bacterial communities from reactors T1 and C1 were conducted on pooled samples. For this reason, it was not clear if the small differences between the communities were a result of natural variation or due to selection by the triclosan which had been incorporated into one of the plastics. To more closely address this question, replicate conditions in reactors 2, 3, and 4, where both plastic types were in the same vessel, were used to examine the direct effects that the different plastic types have upon the adherent bacterial communities. Analysis of ARISA results revealed variations between communities forming on CP and TP; however, the differences between the plastic types were small when compared to the variation observed between replicate samples (Figure 3 and Table 1).

Cell viability and triclosan tolerance studies were conducted on the biofilms from reactors 2, 3 and 4. There were no significant differences detected in the direct cell counts, culturability, or numbers of triclosan-resistant organisms that attached to these plastic surfaces. Given the overall lack of significant phylogenetic and phenotypic variation between the bacteria colonizing the two plastics, we hypothesized that actual triclosan exposure from TP may be insufficient to cause any biologically meaningful changes.

It was not possible for us to determine the concentration of triclosan available at the plastic surface or within the biofilm. Therefore, we resolved to determine the concentration of triclosan that desorbed from the plastic into a known volume of water and the bioavailability of the triclosan in the plastic. To assess the potential for exposure, we allowed TP to incubate with water and found that the desorption of triclosan from the TP into water after 50 h resulted in an aqueous concentration of approximately 12 µg/L, well below the 10 mg/L solubility limit of triclosan in water. Whereas limited desorption of triclosan into water was observed at 50 h, this concentration was below the MIC for all bacteria studied to date. Therefore, the desorption of triclosan from this ABS plastic into both a batch and a continuous flow system apparently did not result in aqueous concentrations that would be sufficient to effect the microbial populations and numbers in the system.

This supposition is supported by data from the bioavailability assay using *Sphingomonas* strain RD1, which indicated that desorption of triclosan from ABS plastic resulted in aqueous concentrations that were less than the induction threshold for the assay (Figure 4). Taken together, the results from the desorption experiment and bioavailability assay suggest that although triclosan did desorb slightly from the ABS plastic, it was not available at concentrations sufficient to inhibit the growth of any known bacteria, and was therefore unlikely to effect any change in the community structure. While others have shown that the addition of triclosan in more bioavailable forms can reduce bacterial species diversity, the concentrations utilized in that study were far in excess of those likely to have been encountered in this study.

Hypothetically, as triclosan desorbed from the ABS plastic, it may have transiently accumulated at the surface and reached concentrations sufficient to have a biological effect. Although it is unlikely that this occurred in our well-mixed systems, the possibility that this could occur in a more static system cannot be completely ruled out. Given the nature of biofilm growth, however, it is not clear that modest theoretical increases in local triclosan concentrations would be sufficient to affect the number and type of organisms that colonize TP. Even if triclosan did accumulate at the plastic surface, the development of an abiotic conditioning film may have provided a physical separation between the adherent community and the antimicrobial, thereby reducing its effectiveness. The slow growth rate inherent to biofilm organisms would also be expected to render them less sensitive to triclosan, whose mode of action involves the inhibition of fatty acid synthesis and is most effective against rapidly growing cells. The supposition that growth rate is an important consideration with respect to triclosan tolerance is consistent with published observations regarding the reduced antimicrobial effectiveness of triclosan in low nutrient environments and during refrigeration. Slow growth rate may also help to explain the lack of resistance development in biofilm organisms exposed to triclosan in domestic detergents.

In pure culture studies, and where the bioavailability of triclosan from polymers is high, the presence of intrinsically resistant organisms may provide physical protection to susceptible organisms, whereas others capable of degrading or detoxifying the microbicide, may aid in creating a hospitable environment where the concentration of the compound is reduced. The data presented here, however, indicate that the long-term usefulness of triclosan-incorporated ABS plastic is limited with respect to biofilm prevention, and the alteration of the microbial community composition in oligotrophic environments.

The widespread and apparently indiscriminate use of antimicrobial agents is of concern given increased reports of antimicrobial and antibiotic cross-resistance. This is of particular concern given that...
the transfer of genes encoding resistance mechanisms may occur at increased rates in biofilms. Although we found no selection for resistant organisms in our biofilm population, this may have been because of low bioavailability due to limited desorption.

Despite the reports of its effectiveness in toothpastes and mouthwashes, the antimicrobial activity of polymers impregnated with triclosan, against pure cultures in batch studies has been questioned. Confounding factors may include slow growth as observed in refrigeration and in biofilms, or possible degradation of the active compound. The results of this research extend these findings and suggest that limited bioavailability from ABS plastic to naturally occurring biofilm organisms is another potential cause of reduced antimicrobial efficiency. Whereas reduced bioavailability may assure fears concerning the development of antimicrobial resistance, it reinforces the need to validate the efficacy of purported antimicrobial products that are being marketed to the increasingly ‘microbephobic’ public.

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References


4. Mann, J., Vered, Y., Babayof, I. et al. (2001). The comparative anticaries efficacy of a dentifrice containing 0.3% triclosan and 2.0% copolymer in a 0.243% sodium fluoride/silica base and a dentifrice containing 0.243% sodium fluoride/silica base: a 2 year coronal caries clinical trial on adults in Israel. Journal of Clinical Dentistry 12, 71–6.


