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Triclosan Enriches for *Dehalococcoides*-Like *Chloroflexi* In Anaerobic Soil at Environmentally Relevant Concentrations

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Abstract

Triclosan is an antimicrobial agent that is discharged to soils with land-applied wastewater biosolids, is persistent under anaerobic conditions, and yet its impact on anaerobic microbial communities in soils is largely unknown. We hypothesized that triclosan enriches for *Dehalococcoides*-like *Chloroflexi* because these bacteria respire organochlorides and are likely less sensitive, relative to other bacteria, to the antimicrobial effects of

triclosan. Triplicate anaerobic soil microcosms were seeded with agricultural soil, which was not previously exposed to triclosan, and were amended with 1 mg kg^{-1} of triclosan. Triplicate control microcosms did not receive triclosan, and the experiment was run for 618 days. The overall bacterial community (assessed by automated ribosomal intergenic spacer analysis and denaturing gradient gel electrophoresis) was not impacted by triclosan; however, the abundance of *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes (determined by qPCR) increased 20-fold with triclosan amendment compared with a fivefold increase without triclosan. This work demonstrates that triclosan impacts anaerobic soil communities at environmentally relevant levels.

Keywords

community fingerprinting, *Dehalococcoides*, emerging contaminants, organochloride respiration, biosolids

Introduction

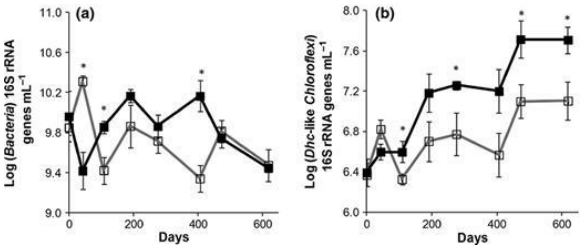
Triclosan is an antimicrobial agent that specifically targets bacteria (McMurry *et al.,* 1998). Following household usage, triclosan collects in wastewater treatment plants where a small portion of the influent mass is discharged with liquid effluent, which can then be converted to dioxin photoproducts found in lake sediments (Anger *et al.*, 2013). Triclosan is hydrophobic and so a much greater portion of the influent mass to a wastewater treatment plant readily partitions with wastewater solids that are sent on for residual solids treatment (McAvoy *et al.*, 2002). Because triclosan is persistent under anaerobic conditions (McAvoy *et al.*, 2002; Ying *et al.*, 2007) and is resistant to advanced wastewater solids treatment processes (McNamara *et al.*, 2012), it is readily discharged to the soil environment with land-applied wastewater biosolids following solids treatment (McClellan & Halden, 2010). Even though triclosan is both predicted (Fuchsman *et al.*, 2010) and detected at near mg kg^{-1} quantities in the soil environment (Kinney *et al.*, 2008; Butler *et al.*, 2012), its impact on soil microbial communities is not well understood, and research has focused only on aerobic communities (Butler *et al.*, 2011; Svenningsen *et al.*, 2011). The impacts of triclosan in anaerobic soils, where it will likely accumulate and persist (Xia *et al.*, 2010), are yet to be elucidated. Triclosan, as an organochloride, may serve as an electron acceptor through reductive dechlorination by organochloride-respiring bacteria. These bacteria are ubiquitous in soil environments and may play a crucial role in natural organochloride cycling (Krzmarzick *et al.*, 2012, 2013) in addition to their bioremediation importance (Maymó-Gatell *et al*., 1997). Additionally, organochloride respirers might become enriched in triclosan-laden environments as these bacteria have shown tolerance for chlorinated pollutants and other antibiotics (Maymó-Gatell *et al*., 1997; Harkness *et al.*, 1999). We hypothesized that triclosan would enrich for the organochloride-respiring *Dehalococcoides*-like *Chloroflexi* in anaerobic microcosms containing agricultural soil – a common sink for triclosan.

Materials and methods

Glass serum bottles (160 mL) capped with Teflon-lined septa were inoculated with agricultural soil collected 30 cm below the soil surface (Fairfax, MN). No background concentration of triclosan was detected (Supporting Information, Table S1). Triplicate microcosms were amended with 40 μg of triclosan, and triplicate control microcosms were operated without triclosan amendment. Each reactor received 40 g soil, 40 mL reduced mineral medium (Shelton & Tiedje, 1984), 1.6 μmol of potassium acetate, 1.25 mg yeast extract and were maintained and sampled in an anaerobic glovebag (Coy, Grass Lake, MI) with a 1–3% H_2 headspace. To determine the long-term effects from triclosan amendment, microcosms were run for 618 days. After initial startup and every 3 months following startup, a volume of 1.5 mL of mixed slurry was drawn, and a total of eight time points (Days 0, 42, 108, 190, 275, 406, 472, and 618) were sampled so that no more than 15% of reactor contents were sampled upon conclusion of the experiment. Prior to sampling, the bottles were manually shaken for 5 min to homogenize contents, which were sampled with sawed-off Pasteur pipettes. Additional aliquots of potassium acetate were added after sampling at Days 275 and 406 to model intermittent loading of carbon. DNA

from the slurry was extracted with a PowerSoil DNA kit (MoBio, Carlsbad, CA) according to manufacturer's directions with the exception of the use of a FastPrep FP 120 for bead beating (MP Biomedicals, Santa Ana, CA).

The abundance of *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes (*Dhc-Chl*) and *Bacteria* 16S rRNA genes (*Bac*) was determined by qPCR, as previously described (Krzmarzick *et al.*, 2012; see Supporting Information, Fig. 1) at the end of the experiment. Three samples (triclosan-amended reactor A, control-A, and control-B on day 618) were also analyzed with qPCR after a 1 : 10 dilution in water to test for the presence of inhibitors; the calculated results were within 0.1 log units of the undiluted qPCR results from the undiluted extracts, indicating inhibition was not a factor. Community fingerprints were also taken to test whether triclosan had a general impact on the overall bacterial community composition. Automated ribosomal intergenic spacer analysis (ARISA) and denaturing gradient gel electrophoresis (DGGE) were performed as described previously (LaPara *et al.*, 2011, 2000, respectively, see Supporting Information). DGGE analysis was performed for DNA extracts from the samplings at Days 472 and 618 to compare the microbial communities between triclosan-amended microcosms and unamend microcosms after long incubation times. Each microcosm was also analyzed by ARISA to further investigate whether the communities changed temporally. ARISA was performed for DNA extracts from the samples at Days 0, 108, 275, 472, and 618 to investigate any differences in the microbial communities over time and as a result of triclosan amendment.



Number of *Bacteria* 16S rRNA genes per mL of reactor slurry (a) and number of *Dehalococcoides*-like (*Dhc*-like) *Chloroflexi* 16S rRNA genes per mL of reactor (b) over the duration of the experiment. Samples from triclosan-amended microcosms are depicted by black symbols and black lines; samples from control microcosms are depicted by white symbols and gray lines. Error bars indicate standard error of the mean for triplicate microcosms. Asterisks correspond to statistically significant differences between the triclosan-amended microcosms and control microcosms (Student's *t*-test, $P < 0.05$).

A PCR-restriction fragment length polymorphism (PCR-RFLP) method was used to measure the difference in diversity of the *Dehalococcoides*-like *Chloroflexi* at Day 618 between the triclosan-amended reactor, triclosan-A, and the control reactor, control-A. For PCR-RFLP, *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes were amplified using the primers Chl348F (5'-GAG GCA GCA GCA AGG AA-3') from Fagervold *et al*. (2005) and Dhc1286R (5'-GAT ATG CGG TTA CTA GCA ACT CCA AC-3') from Krzmarzick *et al*. (2012) with thermocycling and PCR conditions, as described by Fagervold *et al*. (2005). Chl348F is specific for a broad range of *Chloroflexi*, while Dhc1286R is specific for *Dehalococcoides myccartyi* spp. This amplified PCR product was then cleaned with the GeneClean Kit II (MP Biomedicals) and cloned into electrocompetent *Escherichia coli* DH5α using the pGEM-T Easy cloning kit (Promega, Madison, WI). A total of 138 clones originating from the triclosan-A reactor and a total of 153 clones from the control-A reactor were picked from selective LB agar plates, lysed by freeze-thaw cycling, and the inserted 16S rRNA gene was again amplified with Chl348F and Dhc1286R as described above using the lysed cells as template. Each reamplified product was digested with AluI enzyme (New England Biolabs, Ipswich, MA) according to manufacturer's recommendations, and the digested fragments were analyzed by electrophoresis on a 1.5% agarose gel dyed with Gel-Red (Biotium, Hayward, CA) with a HyperLadderIV DNA

marker (Bioline USA, Taunton, MA) and imaged with QUANTITYONE software (BioRad, Hercules, CA). Fragment patterns were compared and categorized by manual inspection, and these results are summarized in Table S2.

Results and discussion

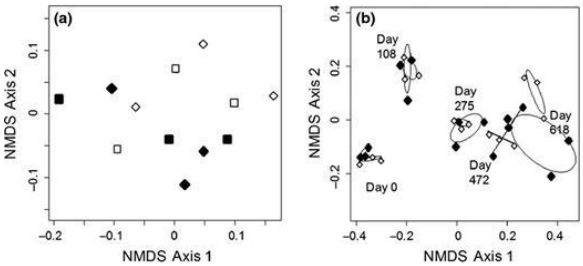
The abundance of *Dhc-Chl* was significantly higher (Student's *t*-test, $P \leq 0.05$) in the triclosan-amended microcosms than in the control microcosms for four of the eight time points analyzed after triclosan amendment (Days 108, 275, 472, and 618). The changes in *Dhc-Chl* in the control microcosms strongly correlated with the changes in *Bac* (Spearman's $\rho = 1.00$, $P < 0.001$). On the contrary, in the triclosan-amended microcosms, the changes of *Dhc-Chl* were independent of changes in *Bac* (Spearman's $\rho = 0.25$; $P = 0.59$). The increase of *Dhc-Chl* in the control microcosms was anticipated as *Dhc-Chl* has been shown to dechlorinate naturally produced organochlorides which are nascent in the soil (Myneni, 2002; Krzmarzick *et al.*, 2012). The impact of triclosan on *Bac* varied. At 108 days, the number of *Bac* was significantly lower in the triclosan-amended microcosms than in the controls, but by 190 days, the triclosan-amended microcosms had significantly greater numbers. By the end of the experiment, however, no significant differences in *Bac* existed.

Over 618 days, the overall growth of *Dhc-Chl* was fourfold greater with triclosan amendment (1.3 ± 0.2 logarithm units in the triclosan-amended microcosms compared with 0.7 ± 0.2 logarithm units in the controls), which was statistically significant (Student's *t*-test, $P = 0.05$). This result indicates that the presence of triclosan was favorable toward the growth of *Dehalococcoides*-like *Chloroflexi. Dehalococcoides*-like *Chloroflexi* using triclosan as an electron acceptor could explain the increased growth. The dehalogenation of triclocarban, a similar antimicrobial compound and common co-contaminant with triclosan (Halden & Paull, 2005), has been observed (Miller *et al.*, 2008). In the study by Miller *et al*. (2008), triclosan and triclocarban concentrations tracked concomitantly in sediment where dehalogenation products of triclocarban were only minimally observed. Sediments from a different source, however, had lower concentrations of triclocarban and higher levels of dechlorinated-triclocarban metabolites, but triclosan was not detected above limits of quantification despite historical contamination (Miller *et al.*, 2008). The lack of triclosan in the sediment that had triclocarban dehalogenation metabolites suggests the possibility that triclosan was removed via reductive dehalogenation. The sediment was also found to contain *Dehalococcoides mccartyi* spp. and was used to rapidly develop a trichloroethene-dechlorinating culture (DehaloR^2; Ziv-El *et al*., 2011). Yet with no direct evidence to date, further research is warranted to determine whether organochloride-respiring bacteria reductively respire triclosan.

The increase in abundance of *Dhc-Chl* might have also resulted from a competitive advantage stemming from the presence of triclosan, that is, bacteria that competed with *Dehalococcoides*-like *Chloroflexi* for nutrients, carbon, or electron donor were lost from triclosan exposure while *Dehalococcoides*-like *Chloroflexi* were comparatively unaffected. Thus, *Dehalococcoides*-like *Chloroflexi*, presumably respiring natural organochlorides, may have gained a competitive advantage for growth and activity compared with the control microcosms. Indeed, it is evident that, although *Bac* decreased in the first 42 days in the triclosan microcosms, the *Dhc-Chl* actually increased, suggesting that *Dehalococcoides*-like *Chloroflexi* may be resistant to triclosan. Additionally, the diversity of *Dehalococcoides*-like *Chloroflexi,* as measured by the PCR-RFLP method, was very similar between the reactors triclosan-A and control-A at Day 618 when triclosan-A contained a 20× higher abundance of *Dhc-Chl* relative to control-A (Table S2). No unique digestion pattern was present in the triclosan-amended reactor compared with the control reactor, and none of the eight identified patterns were predominantly higher in the triclosan reactor compared with the control reactor (Table S2). This similarity in the diversity of *Dehalococcoides*-like *Chloroflexi* suggests that no single member of the *Dehalococcoides*-like *Chloroflexi* grew significantly in response to triclosan, thus leading credence to the hypothesis that the increase in *Dhc-Chl* was from a competitive advantage of this group of organisms as a result of triclosan

resistance. In the enrichment of *Dehalococcoides*, antibiotics are often used as a selective agent to aid in isolation (Maymó-Gatell *et al*., 1997), and in one study, antibiotics have been shown to favor *Dehalococcoides* abundance and activity (Bunge *et al.*, 2008); no study to date has specifically used triclosan.

From the DGGE, no unique bands appeared in the triplicate triclosan-amended microcosms or in the controls, and no bands had significantly different relative intensities between the microcosms at Day 472 or Day 618 (Student's *t*-tests, $P > 0.05$, Fig. S1). The relative band intensities for microcosms at Days 472 and 618 were used for nonmetric multidimensional scaling (nMDS) analysis using the VEGAN package in R. NMDS confirms that the overall structure of the triclosan-amended and control microcosms were similar (Fig. 2a). NMDS analysis of ARISA data corroborated the DGGE results (Fig. 2b); the triclosan-amended and control communities grouped very closely and shifted temporally together throughout the experiment. Thus, triclosan amendment at environmentally relevant levels did not substantially alter the overall bacterial community structure.



Results of nMDS analysis for the bacteria based on DGGE gel analysis (a) and based on ARISA analysis (b). Triclosan-amended microcosms are depicted as solid symbols and control microcosms are depicted as clear symbols. For DGGE panel (a), squares correspond to Day 472 samples and diamonds correspond to Day 618 samples.

While triclosan seemingly had no impact on overall community structure as assessed by fingerprinting techniques, triclosan still impacted the microbial communities through an enrichment of *Dehalococcoides*-like *Chloroflexi*. This result suggests that release of triclosan into the environment may pose inadvertent effects on the biogeochemical cycling of organochlorides in terrestrial environments.

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References

Anger CT, Sueper C, Blumentritt DJ, McNeill K, Engstrom DR & Arnold WA (2013) Quantification of triclosan, chlorinated triclosan derivatives, and their dioxin photoproducts in lacustrine sediment cores. *Environ Sci Technol* 47: 1833–1843.

Bunge M, Wagner A, Fischer M, Andreesen JR & Lechner U (2008) Enrichment of a dioxin-dehalogenating Dehalococcoides species in two-liquid phase cultures. *Environ Microbiol* 10: 2670–2683.

Butler E, Whelan M, Ritz K, Sakrabani R & van Egmond R (2011) Effects of triclosan on soil microbial respiration. *Environ Toxicol Chem* 30: 360–366.

Butler E, Whelan MJ, Sakrabani R & van Egmond R (2012) Fate of triclosan in field soils receiving sewage sludge. *Environ Pollut* 167: 101–109.

Fagervold SK, Watts JEM, May HD & Sowers KR (2005) Sequential reductive dechlorination of meta-chlorinated polychlorinated biphenyl congeners in sediment microcosms by two different Chloroflexi phylotypes. *Appl Environ Microbiol* 71: 8085–8090.

Fuchsman P, Lyndall J, Bock M, Lauren D, Barber T, Leigh K, Perruchon E & Capdeviellek M (2010) Terrestrial ecological risk evaluation for triclosan in land-applied biosolids. *Integr Environ Assess Manag* 6: 405–418.

Halden RU & Paull DH (2005) Co-occurrence of triclocarban and triclosan in U.S. water resources. *Environ Sci Technol* 39: 1420–1426.

Harkness HR, Bracco AA, Brennan MJ, Deweerd KA & Spivack JL (1999) Use of bioaugmentation to stimulate complete reductive dechlorination of trichloroethene in Dover soil columns. *Environ Sci Technol* 33: 1100–1109.

Kinney CA, Furlong ET, Kolpin DW, Burkhardt MR, Zaugg SD, Werner SL, Bossio JP & Benotti MJ (2008) Bioaccumulation of pharmaceuticals and other anthropogenic waste indicators in earthworms from agricultural soil amended with biosolid or swine manure. *Environ Sci Technol* 42: 1863–1870.

Krzmarzick MJ, Crary BB, Harding JJ, Oyerinde OO, Leri AC, Myneni SCB & Novak PJ (2012) Natural niche for organohalide-respiring Chloroflexi. *Appl Environ Microbiol* 78: 393–401.

Krzmarzick MJ, McNamara PJ, Crary BB & Novak PJ (2013) Abundance of organohalide-respiring Chloroflexi in lake sediments across a geographical sulfur gradient. *FEMS Microbiol Ecol* 84: 248–258.

LaPara TM, Nakatsu CH, Pantea L & Alleman JE (2000) Phylogenetic analysis of bacterial communities in mesophilic and thermophilic bioreactors treating pharmaceutical wastewater. *Appl Environ Microbiol* 66: 3951–3959.

LaPara TM, Burch TR, McNamara PJ, Tan DT, Yan M & Eichmiller JJ (2011) Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth–Superior Harbor. *Environ Sci Technol* 45: 9543–9549.

Maymo-Gatell X, Chien Y, Gossett JM & Zinder SH (1997) Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. *Science* 276: 1568–1571.

McAvoy DC, Schatowitz B, Jacob M, Hauk A & Eckhoff WS (2002) Measurement of triclosan in wastewater treatment systems. *Environ Chem* 21: 1323–1329.

McClellan K & Halden RU (2010) Pharmaceuticals and personal care products in archived biosolids from the 2001 EPA national sewage sludge survey. *Water Res* 44: 658–668.

McMurry LM, Oethinger M & Levy SB (1998) Triclosan targets lipid synthesis. *Nature* 394: 531–532.

McNamara PJ, Wilson CA, Wogen MT, Murthy SN, Novak JT & Novak PJ (2012) The effect of thermal hydrolysis pretreatment on the anaerobic degradation of nonylphenol and short-chain nonylphenol ethoxylates in digested biosolids. *Water Res* 46: 2937–2946.

Miller TR, Heidler J, Chillrud SN, DeLaquil A, Ritchie JC, Mihalic JN, Bopp R & Halden RU (2008) Fate of triclosan and evidence for reductive dechlorination of triclocarban in estuarine sediments. *Environ Sci Technol* 42: 4570–4576.

Myneni SCB (2002) Formation of stable chlorinated hydrocarbons in weathering plant material. *Science* 295: 1039–1041.

Shelton DR & Tiedje JM (1984) Isolation and partial characterization of bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoic acid. *Appl Environ Microbiol* 48: 840–848.

Svenningsen H, Henriksen T, Priem e A & Johnsen AR (2011) Triclosan affects the microbial community in simulated sewage-drain field soil and slows down xenobiotic degradation. *Environ Pollut* 159: 1599–1605.

Xia K, Hundal LS, Kumar K, Armbrust K, Cox AE & Granato TC (2010) Triclocarban, triclosan, polybrominated diphenyl ethers, and 4-nonylphenol in biosolids and in soil receiving 33-year biosolids application. *Environ Toxicol Chem* 29: 597–605.

Ying G-G, Yu X-Y & Kookana RS (2007) Biological degradation of triclocarban and triclosan in a soil under aerobic and anaerobic conditions and comparison with environmental fate modelling. *Environmental Pollution* 150: 300–305.

Ziv-El M, Delgado AG, Yao Y, Kang D-W, Nelson KG, Halden RU & Krajmalnik-Brown R (2011) Development and characterization of DehaloR^2, a novel anaerobic microbial consortium performing rapid dechlorination of TCE to ethene. *Appl Microbiol Biotechnol* 92: 1063–1107.

Supplementary data

Appendix S1. Supplemental Information.

Table S1. Background levels of triclosan in soil, measured in triplicate, were below limit of quantification.

Table S2. Number of clones exhibiting each PCR-RFLP digestion pattern.

Fig. S1. DGGE fingerprints of initial samples (Day 0) from triclosan-amended microcosm A (Tric) and control microcosm A (Ctrl) and fingerprints of triplicate triclosan-amended and control microcosms at Day 472 and Day 618.

Fig. S2. DGGE profiles of the *Bacteria* 16S rRNA genes for each reactor during the duration of the experiments.