THE BIODEGRADATION AND MICROBIOLOGICAL IMPACTS OF MICROPOLLUTANTS IN METHANOGENIC COMMUNITIES

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Dedication

This dissertation is dedicated to my loving wife, Lindsay, for her unending support throughout graduate school.

Abstract

Pervasive use of chemicals generates micropollutants throughout the environment. The risks micropollutants pose to humans and other multicellular organisms are well studied, but the impacts on microbial processes are not well understood. Fundamental processes, such as carbon and nutrient cycling, in both the built and natural environment rely on healthy functioning of microbial communities that are chronically exposed to a multitude of micropollutants. Anaerobic environments in particular accumulate high levels of hydrophobic micropollutants, and it is estimated that over 200 metric tons of micropollutants are discharged with biosolids each year. The impact of prevalent micropollutants on methanogenic communities must be assessed to better understand the risks associated with their continued usage. Furthermore, research is also needed to understand how emerging treatment processes impact the fate of micropollutants so that we can minimize the discharge of micropollutants into the environment. This dissertation investigates how micropollutants impact methanogenic microbial communities and how an emerging anaerobic treatment process impacts the fate of micropollutants.

In the second chapter of this dissertation, the impact of advanced anaerobic digestion on the fate of nonylphenol and nonylphenol ethoxylates was tested. Thermal-hydrolysis coupled to mesophilic anaerobic digestion (TH-MAD) is a burgeoning treatment process that yields more efficient solids destruction and gas production than convention anaerobic digestion, but the impacts it has on the biodegradation of

nonylphenol ethoxylates, an abundant group of estrogenic compounds in anaerobic digesters, were unknown until now. Batch tests on thermal hydrolysis (TH) of clean water and sludge were performed to establish the abiotic impacts of thermal hydrolysis on micropollutants. Nonylphenol, nonylphenol ethoxylates, and triclosan were not directly destroyed by thermal hydrolysis. Chemical analysis (liquid chromatography-mass spectrometry) of nonylphenol ethoxylates was performed on influent and effluent sludge from i) mesophilic anaerobic digesters (MAD) fed TH pretreated sludge, ii) conventional MAD reactors, and iii) aerobic digesters treating MAD effluent sludge. The thermal hydrolysis-MAD process actually inhibited biodegradation of nonylphenol ethoxylates to nonylphenol relative to MAD with no pretreatment. The ratio of nonylphenol to the sum of nonylphenol ethoxylates + nonylphenol only increased by 24.6±3.1% in TH-MAD reactors compared to a 56% increase following conventional MAD treatment. Neither anaerobic treatment process decreased the sum of nonylphenol ethoxylates + nonylphenol. Aerobic treatment, on the other hand, reduced total nonylphenol ethoxylates by 30-70% and reduced total estrogenicity by approximately 50% as measured by the yeast estrogen screen (YES) assay. Because neither MAD treatment reduced the total sum of nonylphenol ethoxylates + nonylphenol, and aerobic treatment is costly, source control is likely the best option for removing these micropollutants from treatment systems.

The third chapter of this dissertation describeds research to understand the impact of triclosan on the structure and function of methanogenic communities. First,

communities that were not previously exposed to triclosan were amended with environmentally relevant levels of triclosan. Results from automated ribosomal intergenic spacer analysis (ARISA) were used for community fingerprinting to assess shifts in community structure. ARISA profiles indicated that triclosan caused initial community shifts, but communities were then able to adapt back to control communities. Methane production concomitantly increased during initial exposure (71 days), but was stable throughout the rest of the experiment, even in the face of perturbations, suggesting that methanogenic communities are resilient to environmental levels of triclosan.

Experiments were also performed to test the impacts of increased triclosan levels on previously-exposed communities. Initial community shifts in 5 mg (triclosan)/kg (dry solids)—and 50 mg/kg—amended communities were followed by subsequent reconvergence with control communities. Reconvergence did not, however, occur in 500 mg/kg—amended communities. This quantity of triclosan is 4x higher than the current maximum measured environmental concentration in biosolids (133 mg/kg). Methane production was inhibited in the 500 mg/kg reactors, which only produced 56% of the methane produced in the control reactors. Additionally, 50 mg/kg led to the largest variation in methane production, with a relative standard deviation of 57% compared to < 10% relative standard deviation in other reactors, suggesting that 50 mg/kg could be the tipping point for functional collapse. The impacts of triclosan on antibiotic resistance were also quantified. Most noteworthy, 500 mg/kg triclosan amended in previously unexposed communities selected for *mexB,* a constituent on the MexAB-Opr multiple

drug efflux pump, indicating that triclosan can select for antibiotic resistance in mixed anaerobic communities. The results in this chapter demonstrate that communities can adapt to environmental levels of triclosan, but continued usage of triclosan threatens healthy functioning of methanogenic communities and may have broader impacts with respect to antibiotic resistance.

In the fourth chapter of this dissertation the community structure and function impacts of perfluorooctane sulfonate (PFOS), alone and in mixtures, and the importance of exposure time were investigated. 140-day and 14-day experiments were performed with methanogenic communities amended with PFOS, and in some cases also with triclosan. In long-term experiments environmentally relevant levels of PFOS increased specific methane production by approximately 50%. PFOS also caused minor shifts in community structure based on ARISA. Furthermore, in reactors amended with mixtures of PFOS and triclosan, there was an increase in the methane production rate compared to reactors containing only triclosan. PFOS did not alter methane production or augment the impacts of triclosan in short-term exposure experiments. These findings indicate that exposure time, as well as mixture effects, must be considered when appropriating risks of micropollutants.

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Chapter 1 - Introduction and Literature Review

1.1 Introduction

Micropollutants are ubiquitous chemicals that are harmful to multicellular organisms. Micropollutants are found in terrestrial (*e.g.,* Kinney *et al.,* 2008) and aquatic environments, including lakes (*e.g.,* Soares *et al.,* 2008), rivers (*e.g.,* Corada-Fernandez *et al.,* 2011) and estuaries (*e.g.,* Cantell *et al.,* 2010), and they enter the environment with liquid and solid effluents from wastewater treatment plants (WWTP) (*e.g.,* Nelson *et al.,* 2011; Walters *et al.,* 2010). In fact, more than 200 metric tons of micropollutants are annually discharged with biosolids, *i.e.,* the residual solids from wastewater treatment (McClellan and Halden, 2010). Micropollutants in the environment cause concern because they adversely impact wildlife in a variety of ways, including endocrine disruption (Baldwin *et al.,* 1997; Colborn *et al.,* 1993; Kuiper *et al.,* 1998; Masuyama *et al.,* 2000; Raut and Angus, 2010; Rubin *et al.,* 2001), and more specifically fish feminization (Kidd *et al.,* 2007). Although researchers have extensively studied the impact of micropollutants on multicellular organisms (Kidd *et al.,*2007; Matsumura *et al.,* 2005; Ishibashi *et al.,* 2004; Sumpter, 2005; Vajda *et al.,* 2008), both isolated and in chemical mixtures, few researchers have studied their impact on microbial processes. Indeed, critical functions in both natural and engineered systems, such as carbon and nutrient cycling, rely on microbial communities that are chronically exposed to a plethora of micropollutants. Without a fundamental understanding of how micropollutants impact microbial communities, we cannot properly assess the risk that they pose nor can we effectively evaluate whether chronic exposure could lead to a tipping point where normal

ecosystem function is lost. Research is also needed to understand how emerging treatment processes impact the fate of micropollutants so that we can minimize the discharge and associated risks of micropollutants in the environment.

The impact of micropollutants on anaerobic microbial communities is of particular interest since several hydrophobic micropollutants accumulate under anaerobic conditions. Two prevalent micropollutants of concern in anaerobic environments are nonylphenol and triclosan (Figure 1-1). Nonylphenol is a detergent metabolite with a log K_{ow} of 4.48 (Ahel and Giger, 1993) that forms, but degrades very slowly, under anaerobic conditions (Giger *et al.,* 1994; Ferguson and Brownawell, 2003). Therefore nonylphenol accumulates to levels approximately between 100 and 1,000 mg/kg in anaerobic digester biosolids (*e.g.,* Gonzales *et al.,* 2010; Kinney *et al.,* 2006; Kinney *et al.,* 2008; Pryor *et al.,* 2002), and to mg/kg levels in sediments (Ferguson *et al.,* 2003; Cespedes *et al.,* 2004). Triclosan (log K_{ow} of 4.8 [Heidler and Halden, 2009]) is an antimicrobial agent found in 75% of people's urine in the United States (Calafat *et al.,* 2008). As a result of this source and its widespread use in handsoaps and toothpastes, it is discharged in high quantities to wastewater treatment systems (Heidler and Halden, 2007). Triclosan is recalcitrant under anaerobic conditions (McAvoy *et al.,* 2002; Miller *et al.,* 2008; Ying *et al.,* 2007), and is typically found in anaerobic digester biosolids at levels between 0.5 and 50 mg/kg (Cha and Cupples, 2009), and in sediments at μg/kg levels (Singer *et al.,* 2002; Buth *et al.,* 2010). If usage of these chemicals continues to increase, it is not out of the

question that levels could raise by an order of magnitude or more in anaerobic environments, especially in high discharge regions (Phillips *et al.,* 2010).

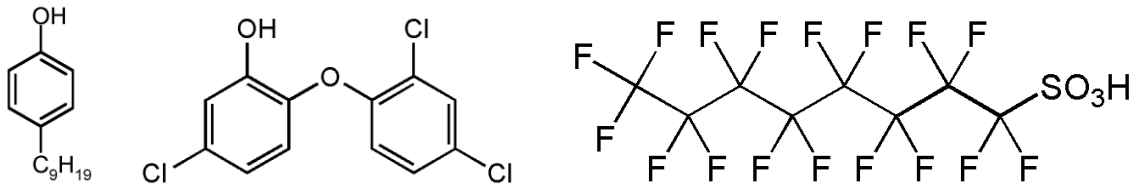

Figure 1-1. Chemical structures of nonylphenol (left), triclosan (middle), and perfluoroctane sulfonate (right).

Micropollutants are often found in the environment as complex mixtures of compounds (Kolpin *et al.,* 2002). Interestingly, studies have shown that mixtures of micropollutants can have additive effects where impacts occur even when no compound is present at high enough levels to ellicit impacts individually (Silva *et al.,* 2008). Certain compounds can also change how other compounds behave. For example, perfluoroalkyl substances (PFAS), such as perfluoroctane sulfonate (PFOS, Figure 1-1), have been shown to alter toxicity of other micropollutants when in mixtures (Boltes *et al.,* 2012). PFAS also tend to accumulate in anaerobic environments (Higgins *et al.,* 2005; Sun *et al.,* 2011) and therefore might impact anaerobic communities directly or indirectly by altering the behavior and toxicity of other micropollutants such as triclosan.

1.2 Anaerobic Systems

Anaerobic processes, in which the transformations of complex organic matter culminate in methane production, are important in engineered and natural systems.

Anaerobic digestion has been used to convert organic waste into methane for over 100 years (Van Lier *et al.,* 2001). The production of methane makes this process economically sustainable, and methane generation from anaerobic digesters could contribute 5% of the U.S. energy needs by 2050 (Zaks *et al.,* 2011). Digesters also produce biosolids, which are rich in nutrients and are used as fertilizer. In fact, over half of the biosolids produced in the United States are land applied (USEPA., 2002). Digesters are, however, prone to process upsets because of the sensitive microbial dynamics in operation in these systems (Van Niekerk *et al.,* 1987). These microbial dynamics also occur in the environment, such as wetlands, where approximately 100-230 Tg of methane are produced each year (Pachauri and Reisinger, 2007).

Bacteria and *Archaea* work in unison to convert complex carbonaceous material into methane (Figure 1-2). Hydrolysis is the first (and rate-limiting) step in carbon breakdown, where extracellular enzymes convert particulate matter into soluble products (Noike *et al.,* 1985; Siegrist *et al.,* 2001). *Bacteria* then convert soluble multi-carbon compounds, *e.g.,* sugars and acids, into acetate, hydrogen, and carbon dioxide (Madigan and Martino, 2006). Methanogens, members of the domain *Archaea*, then convert these simple compounds into methane. Methanogens rely on *Bacteria* to generate their energy source and electron donor (hydrogen and acetate), and *Bacteria* rely on methanogens to maintain a hydrogen partial pressure low enough so that hydrogen generation remains thermodynamically feasible and overall function is sustained (Fukuzaki *et al.,* 1990).

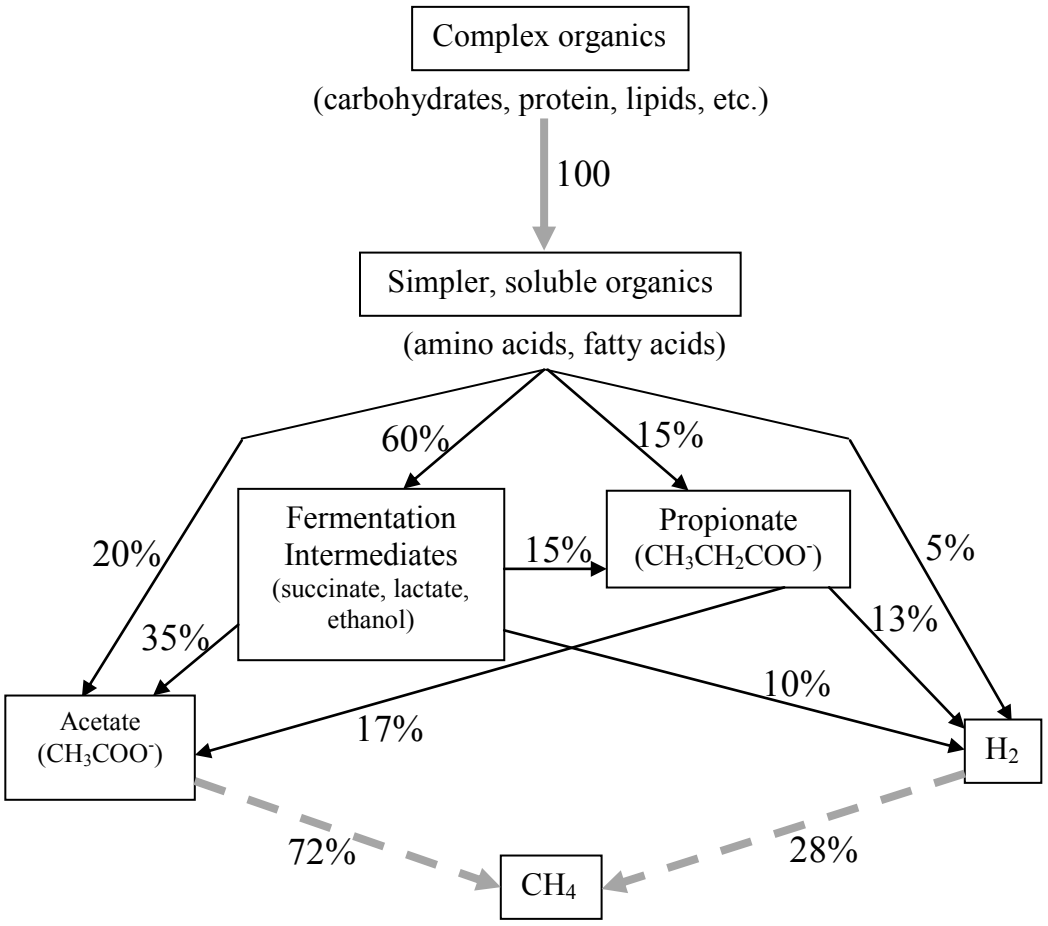

Figure 1-2. Role of *Bacteria* and *Archaea* in converting multi-carbon compounds to methane. The electron flow is shown with the % representing the relative proportions of electrons passing through a given intermediate. The black arrows are steps carried out by *Bacteria* and the grey dashed arrows are steps carried out by *Archaea*. Adapted from McCarty and Smith, 1986.

Stable function in anaerobic microbial communities is thought to stem from a flexible community structure (Fernandez *et al.,* 2000). Functionally stable communities have physiological diversity *i.e.,* different metabolic pathways to degrade the same substrate (Hasham *et al.,* 2000), and have functional redundancies, *i.e.,* different microbes

can perform the same tasks (Allison and Martini, 2008). When communities have high evenness, they can take advantage of their metabolic diversity and functional redundancy to maintain stability (Wittebolle *et al.,* 2009). Indeed, in a study of stable full-scale anaerobic digesters, the Bacterial communities were observed to vary, while evenness consistently correlated to functional stability (Werner *et al.,* 2011). As long as communities do not become dominated by few members, characterized by a strong decrease in evenness, or lose members that are irreplaceable, such as syntrophs in anaerobic digesters, function will be maintained. Consistent with this, function has been lost when uneven communities are stressed (Wittebolle *et al.,* 2009). Micropollutants may serve as stressors that could decrease evenness and lead to functional collapse (Ager *et al.,* 2010; Hickey *et al.,* 1987), or they may simply perturb a flexible community with no negative consequences.

1.3 Importance of Triclosan

As described above, triclosan accumulates under anaerobic conditions and is harmful to multicellular organisms. Triclosan inhibits the growth of aquatic organisms, including algae (Orvos *et al.,* 2002), phytoplankton (Delorenzo and Fleming, 2008), and zebra fish (Oliveira *et al., 2*009) at low µg/L levels. Triclosan also reduces sperm counts in mosquito fish (Raut and Angus, 2010), larval growth in tadpoles (Fort *et al.,* 2010), aggression in fathead minnows (Schultz *et al.,* 2012), muscle functioning in mice (Cherednichenko *et al.,* 2012), and alters estrogen metabolism in humans (Jacobs *et al.,*

2005). The detrimental impacts of triclosan exposure on multicellular organisms are wide-ranging and well-documented; nevertheless, its effects on anaerobic microbial communities at environmentally relevant concentrations are largely unknown.

Because of its antimicrobial activity, the impact of triclosan on microbial community function has been studied, though primarily in aerobic systems. In activated sludge, triclosan (<2 mg/L) reduced oxygen consumption by 50% (Neumegen *et al.,* 2005) and inhibited nitrification by 20% at 1 mg/L (Stasinakis *et al.,* 2008). Of particular interest to the research described in this dissertation, triclosan fed at 10 mg/L to an anaerobic digester increased effluent soluble COD levels for 80 days before function returned to pre-perturbation effluent COD levels (Stamatelatou *et al.,* 2003). None of these studies investigated community structure dynamics. Thus, it remains unknown if triclosan forces communities to adapt.

In *Bacteria* triclosan is known to inhibit the enoyl reductase protein expressed by the *fabI* gene at µg/L concentrations (a gene responsible for biosynthesis of fatty acids) and acts more generally by actually lysing *Bacteria* at mg/L concentrations (McMurry *et al.,* 1998a). In environmental systems where concentrations are low, triclosan is much more likely to inhibit the function of the FabI protein, as opposed to broadly killing microorganisms via lysis. Furthermore, although methanogens lack the *fabI* gene, triclosan is a chlorinated phenol, and several chlorinated phenols are inhibitory to acetoclastic methanogens (Blum and Speece, 1991); yet, no research has been done to study the impacts of triclosan on methanogens. In addition, at environmental

concentrations triclosan might also select for microbial community members with resistance to triclosan (Chen *et al.,* 2009; Chuanchuen *et al.,* 2001; Ghosh *et al.,* 2011; McMurry *et al.,* 1998b).

Other antibiotics that disrupt cell growth by inhibiting gene function in *Bacteria* are better studied for their impacts on anaerobic communities and may provide clues as to how triclosan might impact such communities, particularly with respect to the acquisition of resistance. Erythromycin, a macrolide antibiotic that affects protein synthesis, inhibited gas production in anaerobic digesters when fed at 1 mg/L (Amin *et al.,* 2006). Sulfamerazine, a sulfonamide that inhibits folate synthesis, had similar effects when fed at 90 mg/L (Sponza and Demirden, 2006). Tylosin, also a macrolide, was inhibitory to anaerobic propionate-oxidizing *Bacteria* at levels below 2 mg/L (Shimada *et al.,* 2008) and to methanogens in lagoon communities at 1 mg/L (Loftin *et al.,* 2005). Interestingly, tylosin had no impact on methanogenesis at 1 mg/L during the anaerobic treatment of swine manure, but the manure contained antibiotic resistant *Bacteria* (Angenent *et al.,* 2008). Perhaps the acclimation of anaerobic digesters to triclosan, as observed by Stamatelatou and co-workers was in part a result of *Bacteria* acquiring resistance to triclosan over time. Research is needed to understand if communities change as a result of triclosan, and if antibiotic resistance is a component of this adaptation.

1.4 Importance of PFOS

PFOS is a recalcitrant micropollutant with unknown impacts on anaerobic microbial communities. PFOS directly enters WWTP and is also formed in WWTP from the breakdown of fluorinated surfactants (Boulanger *et al.,* 2005). It was found in anaerobic biosolids at levels over 600 µg/kg in Switzerland, and over 100 µg/kg in the United States (Sun *et al.,* 2011; Sinclair and Kannan, 2006; Sepulvado *et al.,* 2011). PFOS was not degraded in hydrogenotrophic methanogenic assays nor was it toxic to hydrogenotrophic methanogens (Hollingsworth *et al*. 2005). Methanogens, however, reside in the center of sludge flocs (Guoit *et al.,* 1992), and may be protected from PFOS. *Bacteria*, on the other hand, reside on the outside of sludge flocs and may be more susceptible to the direct impacts of PFOS on metabolic processes.

In addition to possible direct impacts, such as uncoupling (Starkov and Wallace, 2002), PFOS has the potential to augment the effects of other harmful chemicals when present as mixtures. In mitochondria cells, PFOS induced uncoupling by causing protons to leak out of the membrane (Starkov and Wallace, 2002). It is unknown whether PFOS can cause similar effects in microorganisms. Nevertheless, PFOS could also impact microorganisms by altering the toxicity of other micropollutants, such as triclosan. Indeed, PFOS was observed to increase cell uptake of pentachlorophenol in algae (Liu *et al.,* 2009). Because PFOS accumulates in anaerobic environments with mixtures of micropollutants, the extent of its synergistic effects is an important area for future research.

1.5 Importance of Nonylphenol

As with triclosan, the release of nonylphenol into the environment poses a variety of risks to multicellular organisms. Nonylphenol is an endocrine disrupting compound that causes developmental abnormalities in oysters (Nice *et al.,* 2000), reduces the ability of *Daphnia magna* to metabolize testosterone (Baldwin *et al.,* 1997), and decreases sperm quality in mice (Kyselova *et al.,* 2003). Nonylphenol is also an estrogenic compound (Jobling and Sumpter, 1993) that can be detrimental to fish populations (Kidd *et al.,* 2007; Vajda *et al.,* 2008). These environmental risks led the European Union to label nonylphenol as a priority organic pollutant in their Working Document on Sludge (Abad *et al.,* 2005).

Nonylphenol is formed when nonylphenol ethoxylates are biodegraded in wastewater treatment plants (Ahel *et al.,* 1994a; Brunner *et al.,* 1988; Giger *et al.,* 1984; Liu *et al.,* 2006). Long-chain ethoxylates (chain lengths of 3-20 ethoxylate groups) readily degrade under aerobic conditions in activated sludge tanks (Ahel *et al.,* 1994a; Jonkers *et al.,* 2001; Staples *et al.,* 2001; Morales *et al.,* 2009). As these long-chain ethoxylates degrade into shorter chain ethoxylates they become more hydrophobic (Ahel *et al.,* 1993), and more readily sorb to solids that undergo treatment processes like anaerobic digestion. Under anaerobic conditions, nonylphenol accumulates as the short-chain ethoxylates are biodegraded (Giger *et al.,* 1984; Ejlertsson *et al.,* 1999; Janex-Habibi *et al.,* 2009; Lu *et al.,* 2008). The extent of this biodegradation, *i.e.,* the fraction of ethoxylates that are converted to nonylphenol, has environmental impacts in that

nonylphenol is more estrogenic than the parent ethoxylate compounds (Routledge and Sumpter, 1996; Rutishauser *et al.,* 2004; Van de Belt *et al.,* 2004). Thus, in anaerobic digesters where nonylphenol accumulates estrogenic loading increases (Holbrook *et al.,* 2002). We need to understand how emerging solids treatment processes impact the biodegradation of nonylphenol ethoxylates to nonylphenol so that, when feasible, treatment processes can be selected to mitigate the estrogenic impacts of nonylphenol in biosolids.

1.6 Objectives

It is important to understand how micropollutants impact anaerobic microbial communities, as well as how anaerobic communities can impact the fate of micropollutants. Usage of consumer products that contain chemicals such as nonylphenol ethoxylates and triclosan will likely continue to increase as populations and affluence rises. Hydrophobic micropollutants will inevitably continue to accumulate in anaerobic environments as a result. The fate of nonylphenol is greatly altered in engineered anaerobic systems where this compound and its precursors accumulate. The objective of **Chapter 2** was to understand how an emerging solids treatment technology, thermal-hydrolysis coupled to mesophilic anaerobic digestion, impacted the fate of nonylphenol ethoxylates and nonylphenol relative to conventional mesophilic digestion.

Triclosan is another prevalent hydrophobic micropollutant in anaerobic systems. While it is possible that triclosan impacts anaerobic microbial communities, we have no

knowledge of how chronic exposure to triclosan affects microbial community structure and function. The objective of **Chapter 3** was to understand how methanogenic microbial communities respond to chronic exposure to triclosan, and determine if a tipping point exists where function is lost.

Lastly, micropollutants are found in chemical mixtures in the environment, and it is necessary to understand how mixtures may alter micropollutant impacts. Mixtures of PFOS and triclosan are of particular interest in anaerobic environments because PFOS could augment or diminish the impacts of triclosan on microbial communities. The objective of **Chapter 4** was to understand how PFOS impacts methanogenic communities in isolation and when combined in a mixture with triclosan. Overall, this work will strengthen our understanding of the dynamic relationship between micropollutants and anaerobic communities and facilitate better risk assessment of these micropollutants.

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Chapter 2 - The Effect of Thermal Hydrolysis Pretreatment on the Anaerobic Degradation of Nonylphenol and Short-Chain Nonylphenol Ethoxylates in Digested Biosolids

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Chris Wilson operated the lab-scale digesters described in this chapter.

2.1 Introduction

Anaerobic digestion has been used worldwide for over 100 years to stabilize wastewater solids (van Lier *et al.,* 2001). As global energy demands continue to increase, it is important to make this process as efficient as possible and to effectively utilize all byproducts of wastewater treatment, including biosolids. A recent study indicated that anaerobic digesters could emerge as an important and useful renewable energy source for electricity generation in the face of new greenhouse gas emission limits (Zaks *et al.,* 2011). Approximately half of the biosolids produced in the European Union (EU) and the United States (US) are used as fertilizer through land application (Commission of European Communities, 2008; USEPA, 2002). According to a life-cycle assessment on the use of biosolids, land application can greatly reduce the carbon footprint of solids handling when compared to the landfilling of biosolids (Peters and Rowley, 2009). Additionally, agricultural phosphorus demand is reduced through the land application of biosolids, helping to alleviate a growing global problem of diminishing phosphorus reserves (Weikard and Seyhan, 2009). Land application of biosolids is not without risks, however, including potential problems associated with the presence of micropollutants, particularly estrogenic compounds, in biosolids.

Of the micropollutants found in wastewater solids, nonylphenol monoethoxylate (NP_1EO), nonylphenol diethoxylate (NP_2EO; these two ethoxylates collectively referred to as NPEO) and nonylphenol (NP) (all three compounds collectively referred to as NPE) are of particular interest as a result of their endocrine disrupting capabilities (Masuyama

et al., 2000) and estrogenicity (Jobling and Sumpter, 1993). Nonylphenol has been detected at levels greater than 1,000 mg/kg in biosolids used for land application (Kinney *et al.,* 2006), and was shown to bioaccumulate in earthworms inhabiting a field fertilized with biosolids (Kinney *et al.,* 2008). In 2003 the EU passed a directive that restricted the sale or use of NPE (European Union, 2003). In the US the Environmental Protection Agency has started a "Safer Detergents Stewardship Initiative" to encourage use of surfactants other than NPE. A report that updated the 2003 working document on land application of biosolids has suggested that NPE should not exceed 450 mg/kg of dry solids (Saludo *et al.,* 2010; Commission of European Communities, 2003); nevertheless, NPE in biosolids have exceeded this level in both the EU (Santos *et al.,* 2007) and the US (Kinney *et al.,* 2006). It is reasonable to expect that the presence of NP in biosolids could result in additional bans or restrictions on the use of NP and NPE within the EU or elsewhere. Therefore, it is critical that we understand how both conventional biosolids handling technologies and emerging technologies impact the fate of NPE in biosolids.

Anaerobic sludge stabilization often results in an accumulation of NP in biosolids (Giger *et al.,* 1984), likely because the anaerobic degradation of NP is very slow compared to its formation from longer-chain ethoxylates (Chang *et al.,* 2008), either NP_nEO (n=3-20) or NP_1EO plus NP_2EO. Anaerobic biodegradation of longer chain NP_nEOs (n=3-20) to short chain NPEO has been demonstrated in the laboratory when fed at very high concentrations (100 g/kg) (Lu *et al.,* 2008); nevertheless, other research has found NP_nEO to be more recalcitrant under anaerobic conditions, with half-lives greater

than 100 days in sediments (Ferguson and Brownawell, 2003). The biodegradation of NPEO to NP under anaerobic conditions, on the other hand, is well documented in full-scale systems (Giger *et al.,* 1984; Ahel *et al.,* 1994; Patureau *et al.,* 2008) and is often the immediate precursor to NP in digesters.

Because NP is more estrogenic than NPEO (Jobling *et al.,* 2003) anaerobic digestion can increase the estrogenicity of biosolids (Holbrook *et al.,* 2002). This production of estrogenicity is of particular concern to wastewater utilities because anaerobic digestion is widely considered a beneficial technology for solids stabilization, producing methane gas and recoverable soluble nutrients, and effectively reducing the number of pathogens and pathogenic indicators in biosolids prior to land application.

An emerging process for solids handling is the advanced pretreatment process of thermal hydrolysis (TH). This process of TH followed by mesophilic anaerobic digestion (TH-MAD) has been implemented in plants worldwide because of benefits such as increased volatile solids reduction, improved sludge dewaterability, and minimization of digester volume requirements (Kepp *et al.,* 2000; Pickworth *et al.,* 2006; Wilson *et al.,* 2011). During TH, sludge is heated to 150°C for 30 min at a pressure of 5-8 bar; under these conditions particulate organic matter is hydrolyzed into soluble organic matter. Because the hydrolytic pretreatment step substantially alters the chemical and physical properties of the sludge, the biodegradation of NPEO to NP during the subsequent digestion stage could also be altered relative to a conventional digestion process with no pretreatment. Carballa *et al.,* (2006) found that autoclaving sludge at 130°C for 60 min

prior to MAD did not improve removal of estrone, 17β-estradiol, or 17α-ethinylestradiol and Barret *et al.,* (2010a) found that thermal treatment of primary sludge did not improve removal of NP. Research is still needed on how TH pretreatment of blended primary and secondary sludge affects the fate of NPE during subsequent anaerobic digestion.

The objectives of this research were to determine the direct impact of TH on NPE, and the impact of TH-MAD, relative to conventional MAD and aerobic digestion, on the biodegradation of NPEO to NP. Laboratory digester studies were conducted to determine if sludge pretreatment alters the efficiency of NPEO and NP biodegradation during downstream anaerobic and aerobic digestion. Batch thermal hydrolysis experiments were conducted to determine if the TH pretreatment step directly destroyed NPE.

2.2 Materials and Methods

Chemicals

Triclosan (sold as Irgasan, ≥97%), 17β-estradiol (E2) (≥98%), nonylphenol (NP, technical grade mixture), bisphenol A (BPA) (97%), NP_1EO and NP_2EO technical analytical standards, and IGEPAL CO – 210 (a mixture of NP_1EO and NP_2EO) were purchased from Sigma-Aldrich (St. Louis, MO). Non-labeled p-n-NP_1EO and p-n-NP_2EO, and labeled $^{13}C_6$-p-n-NP (99%), were purchased from Cambridge Isotope Laboratories (Andover, MA). Cyclohexane, dichloromethane (DCM), acetone, and methanol were all HPLC grade or higher.

Experimental Setup

Thermal Hydrolysis of Micropollutants in Water or Sludge. Thermal Hydrolysis experiments were performed in a general chemical digestion bomb (No. 4745, Parr Instrument Co., Moline IL) to determine if pertinent micropollutants were degraded from the high temperature and pressure conditions of TH. Bisphenol-A, triclosan, NP, NP_1EO, and NP_2EO were dissolved into methanol and were mixed with Milli-Q water to achieve a final methanol concentration of 0.4%; chemical concentrations were approximately 10 μM. Experiments were also performed with neat NP, NP_1EO + NP_2EO, or triclosan (no methanol) mixed with Milli-Q water. Ten mL of water/methanol or water were placed in the bomb, which was then heated for two hours at 150°C in an oven at a pressure of 5.1 bar. Samples were removed from the bomb the following day to ensure that they had cooled sufficiently to avoid volatile loss of compound upon opening the bombs. Additional experiments were performed with NP_1EO + NP_2EO in which the sample was exposed to two consecutive thermal hydrolysis treatments (1 per day) to determine if degradation occurred after multiple thermal treatments. Controls were run in an identical manner except that they were not heated. Initial and final concentrations of the analytes were determined by liquid chromatography-mass spectrometry (LC/MS). Replicate bomb experiments were performed for all compounds tested.

Thermal hydrolysis experiments were also performed on a sludge matrix to determine if TH treatment in a sludge matrix altered NP concentration. Room temperature (RT) controls were performed with each TH experiment such that the same

source of sludge was held at room temperature for the same amount of time that the TH bomb was heated. Mixed primary and secondary sludge (from the blend tank at the WWTP in Mankato, MN) was used for the sludge source. This sludge had been stored at 4°C for 50 days. Nonylphenol (19,200 ng) was spiked into 100 mL of sludge, hand-mixed in a sealed round bottom flask, and stored at 4°C overnight. The sludge was hand-mixed again and loaded into the bombs for the TH and RT experiments. The bombs were either placed in a GC oven at 150°C for two hours (TH experiments) or placed next to the oven at room temperature for the same period of time (RT controls). Additionally, blanks were tested by loading sand into a bomb and heating the bomb at 150°C for two hours. Upon completion of the experiment, all bombs were stored at 4°C overnight and were sampled the following day. Each sample was put into an aluminum tin container and stored in a freezer until lyophilization. Experiments were performed on three consecutive days so that each treatment (TH or RT) was tested in triplicate. Three aliquots of the raw sludge, *i.e.,* influent samples, were also put into aluminum tin containers and frozen until lyophilization.

Thermal Hydrolysis of Wastewater Sludge Followed by Anaerobic Digestion. Lab-scale experiments were also performed in which raw sludge was subjected to TH followed by anaerobic digestion to determine if TH pretreatment altered the biodegradation of NPEO to NP. Three thermal hydrolysis-mesophilic anaerobic digestion (TH-MAD) reactors were operated. One reactor (TH150-MAD) received sludge that underwent TH at 150°C followed by MAD with an SRT of 15 days; a second reactor

(TH170-MAD) received sludge that underwent TH at 170°C followed by MAD with an SRT of 15 days; a third reactor (TH150-MAD20) received sludge that underwent TH at 150°C followed by MAD with an SRT of 20 days. A conventional mesophilic anaerobic digester (MAD) was fed untreated sludge and served as a control. Biosolids from full-scale MAD reactors at Pepper's Ferry Regional Wastewater Treatment Plant (Radford, VA) were used to seed the lab-scale MAD reactor, and biosolids from a full-scale TH-MAD reactor were used to seed the lab-scale TH-MAD reactors. The reactor operating conditions and general reactor performance are described in Table 2-1. The feed to either the MAD digester or the TH step was a 50/50 mixture (by mass) of primary and secondary (activated) municipal wastewater sludge obtained from the Blue Plains wastewater treatment plant (WWTP) in Washington D.C. Feed for the control MAD reactor was shipped weekly on ice from Blue Plains WWTP to Virginia Tech overnight and stored at 4°C until fed to the digester (less than two weeks). Sludge subjected to TH was shipped on ice overnight from Blue Plains WWTP to the TH pilot scale plant (see below) where it was stored at 4°C until treatment (less than two weeks).

The TH treatment step took place at a pilot-scale system operated by RDP Technologies, Inc. in Norristown, PA. In the pilot system approximately 30-40 L of blended sludge were heated to either 150ºC or 170ºC under high pressure (5-8 bar) for 30 minutes. The reactor was vented to the atmosphere by a quick flash to reduce the pressure. This TH-treated sludge was then shipped to Virginia Tech overnight on ice where it was stored at 4°C until used as the influent feed to the TH-MAD reactors. The

lab-scale anaerobic digesters were Minibrew 6.5 gallon fermenters (Hobby Beverage Equipment Co.). The operational and performance parameters of each reactor are summarized in Table 2-1.

Table 2-1. Average Reactor Operating Conditions and Performance

Sample	SRT (days)	Temp (°C)	Total Solids in Samples (%)	Volatile Solids Reduction (%)	VFA (mg/L as HAc)	Ammonia (mg/L)	Duration of Operation at Sampling Time (SRTs)	Days Past Steady-State at Time of Sampling
[1]MAD Inf			5.5			915		
[1]MAD Eff	15	37	3.1	50	230	1,760	11.8	40
[1]MAD-AER Eff	6	34	2.5	62	36	282	35.4	40
[2]TH150-MAD Inf			9.8 [α];12.8 [β]		30,000	535		
[2]TH170-MAD Inf			9.8		34,950	550		
[3]TH150-MAD Eff	15	42	5.8 [α];6.1 [β]	59	6,180	2,510	5.5 [α]; 21.3[β]	21[α]; 258[β]
[3]TH170-MAD Eff	15	42	5.8	58	4,250	2,470	5.5	33
[3]TH150-MAD20 Eff	20	42	6.7	60	3,230	2,130	7	87
[2]TH-M-AER 20/20 Eff	5	34	4.0	70	760	828	2.5	15*
[2]TH-M-AER 12/12 Eff	6	34	6.0	70	760	171	2	12*

[1]Banjade, 2008; [2]Tanneru 2009; [3]Wilson *et al.,* 2011
[α]TH150-MADa; [β]TH150-MADb
*Days in operation with consistent aeration/anoxic cycling

Influent and effluent samples from the TH170-MAD and TH150-MAD20 reactors were taken once, and influent and effluent from the TH150-MAD reactor were sampled twice, to generate an overall total of four sets of influent/effluent TH-MAD samples. The influent and effluent of the conventional MAD reactor was sampled once. While having

one sample from the MAD reactor might not be representative of the conventional MAD process, much work is already available on the transformation of NPEO to NP in conventional MAD (Giger *et al.,* 1984; Brunner *et al.,* 1988), and thus it was deemed most important to operate and sample multiple TH-MAD reactors. The first set of TH150-MAD samples are labeled with an 'a' and the second set of TH150-MAD samples are denoted with a 'b'; these samples were taken 237 days apart. All influent and effluent digester samples were packed in a cooler with ice packs, stored in plastic bottles, and shipped to the University of Minnesota overnight; once at the University of Minnesota, the samples were stored at 4°C until they were freeze dried for further cleanup and analysis (maximum storage time of any sample at 4°C was 104 days). A test on the effect of storage time on NP showed that there was a 5.5% difference between NP in a sample analyzed immediately (Day 0) and a sample analyzed after 154 days of storage at 4°C. Samples were normalized to amount of influent raw sludge present in the sample to account for variations in solids destruction during digestion.

The samples from the anaerobic digesters (MAD + TH-MAD digesters) were taken after at least one sludge retention time (SRT) following the initiation of steady-state (Table 2-1). Steady-state was defined as a time in operation in which the daily gas production differed by less than 10% from the average, and the pH varied by 0.1 or less from the average.

Aerobic Digestion Following Anaerobic Digestion. Aerobic digesters were set up to determine the benefit, with respect to NPE and total estrogenicity removal, of an

aerobic digestion phase following TH-MAD or MAD. Following MAD, the MAD effluent served as the influent to an aerobic reactor (MAD-AER reactor); therefore, the MAD effluent was the same as the MAD-AER influent. The aerobic digester consisted of a 9.5-L glass reactor (Novak *et al.,* 2011) that was aerated continuously. Dissolved oxygen levels were maintained at 2.5 – 3 mg/L (Banjade, 2008). The MAD-AER reactor was sampled after at least one sludge retention time (SRT) following the initiation of steady-state (Table 2-1).

Following TH-MAD, an aerobic/anoxic reactor was operated under two different conditions. In the first operational setup, aerobic and anoxic phases were alternated every 20 minutes. The influent and effluent to this reactor are called TH-M-AER 20/20 Inf and TH-M-AER 20/20 Eff, respectively. The influent to this reactor was a blend of the digested effluent from the TH150-MAD and TH170-MAD reactors. Under the second set of operational conditions the aerobic and anoxic phases were alternated every 12 minutes. The influent and effluent to this reactor are called TH-M-AER 12/12 Inf and TH-M-AER 12/12 Eff, respectively. The influent to this reactor was a blend of the digested effluents from the TH150-MAD and TH150-MAD20 reactors. The SRTs in these TH-M-AER digesters were held constant for more than nine SRTs, but the cycling scheme of the aerobic and anoxic phase was held constant for just two SRTs. We therefore did not consider the aerobic digesters following the TH-MAD treatments to be at steady-state.

Biosolids Sample Handling and Processing for LC/MS and YES analysis.

Samples were sent for freeze drying to either the National Lacustrine Core Repository

(LacCore) or the Gortner Laboratory located at the University of Minnesota. A fraction of the dried solids was weighed (approximately 1 g) and underwent successive overnight Soxhlet extractions in methanol followed by DCM. The liquid extract was reduced via evaporation using a Rotovap (Rotovapor R-3000, BUCHI Laboratory Equipment). Dichloromethane completely evaporated and the remaining methanol extract was reduced to 10 mL. A quantitative aliquot (approximately 10% by volume) was removed and filtered through a 0.2 μm filter for yeast estrogen screen (YES) analysis. The remaining extract was solvent-exchanged into cyclohexane for further cleanup prior to LC/MS analysis. This fraction was loaded onto a normal phase cleanup column containing sodium sulfate, alumina, and silica, and extracted with cyclohexane, DCM, and acetone. The cyclohexane fraction was wasted, and the DCM and acetone fractions were pooled for further preparation. Samples from the TH170-MAD, TH150-MADa, and TH-M-AER 20/20 reactors were analyzed at this point, whereas samples from the conventional MAD, TH150-MADb, and TH-M-AER 12/12 reactors were loaded onto a gel permeation column (GPC) for further cleanup. The GPC had a 25 mm diameter and 100 cm length, and contained Bio-Beads S-X3 (Bio Rad Co., Hercules CA). The mobile phase, a mixture of cyclohexane/DCM (60/40 by volume), was pumped at a rate of 5 mL per min and the eluent collected from 50 min to 120 min was retained for LC/MS analysis. This final aliquot was exchanged into methanol, blown down under a nitrogen stream to approximately one mL, and filtered through 0.2 μm filters for analysis via LC/MS. The relative standard deviation (RSD) of the ratio of NP/NPE on duplicate samples extracted

from the same freeze-dried aliquot was 11.8% after alumina/silica processing, and 2.8% after GPC processing.

The extraction and cleanup method for the TH experiments in sludge were slightly different. Sludge samples and sand blanks were extracted using a Dionex Accelerated Solvent Extractor (ASE). The samples were extracted with DCM for 5 min at 100°C. The extracted sample was blown down under nitrogen and resuspended in DCM. This aliquot was cleaned with a silica cleanup column containing glass wool and 2 g silica, blown down to dryness, and resuspended in an 80/20 mixture (by volume) of methanol and water.

Analytical Methods and Binding Assay

LC-MS and LC-MS-MS Operation. Except for the experiments that quantified the direct impacts of TH on NP in a sludge matrix, analyte quantification was performed on an 1050 Agilent HPLC pump connected to an MS equipped with electrospray ionization (ESI). Chromatography was performed on a Synergi (150 x 2.0 mm, 4μm) RP column that was protected by a guard column (4 x 2.0 mm) (Phenomenex, Inc.; Torrance, CA). Mobile phase was constantly pumped at a rate of 200 μL per min and consisted of an isocratic 23.5%/76.5% mixture of solvent A (90% water, 10% methanol, 2 mM ammonium acetate) and solvent B (100% methanol, 2 mM ammonium acetate). The MS was operated in selective ion monitoring (SIM) mode. The drying gas temperature was 350°C. Nitrogen was the desolvation gas and the nebulizing gas. The MS capillary voltage was set to 4 kV. NP (analyzed in biosolids and clean water TH experiments),

triclosan, and bisphenol-A (clean water only) were quantified in negative mode. NP_1EO and NP_2EO (analyzed in biosolids and clean water experiments) were quantified in positive mode.

Internal standards ($^{13}C_6$-NP for negative mode; p-n-NP_1EO and p-n-NP_2EO for positive mode) were used to account for matrix suppression in biosolids samples and variation in instrument injection volumes. A methanol blank and a standard were injected at least once every 12 samples to check for column contamination and consistency in MS response. The instrument detection limit (IDL; ng injected) for NP, NP_1EO, and NP_2EO were 0.4, 1.25, 0.23, respectively. The corresponding method quantification limits (MQL, based on analyte recovery, sample concentration factors during cleanup, and a ratio of 10/3 for quantification to detection limits) for NP, NP_1EO, and NP_2EO were 0.4, 1.3, and 0.2 mg/kg, respectively. NPE levels were typically 1 to 2 orders of magnitude greater than the MQL. Spike-recovery tests on samples to which analytes were added following lyophilization resulted in recoveries for NP, NP_1EO, and NP_2EO of 94%, 94%, and 112%, respectively. The relative standard deviation (RSD) on duplicate recovery tests for NP, NP_1EO, and NP_2EO were 1.3%, 4.1%, and 10.9%. Ten blanks were extracted along with samples and were also analyzed for NP, NP_1EO, and NP_2EO. Only one blank contained an analyte (NP) with a concentration greater than 10% of that detected in a sample; NP_1EO and NP_2EO were never detected above 10% of the sample concentration.

For the TH of NP in sludge experiments, NP was analyzed via liquid chromatography-tandem mass spectrometry at the UMN Cancer Center. Liquid

chromatography was performed on an Eksigent (Dublin CA) NanoLC Ultra 2D System with a Phenomenix Column (Synergi Polar-RP, 80A, 150 x 5 mm, 4 micron). Mobile phase A was 10 mM ammonium acetate in water and mobile phase B was 10 mM ammonium acetate in methanol. The gradient went from 45% A at time = 0 min to 5% A at time = 16 min, and then back to A = 45% at time = 17 min where the flow was held constant for four minutes for column equilibration. The flowrate was 15 μL/min. Mass spectrometry was performed on a Thermo Scientific Triple Stage Quadrupole Mass Spectrometer (TSQ Vantage). The m/z of the precursor parent compound was 219.4 and the center mass of the product ion was 133. The collision energy was 35 V. Spike-recovery tests showed a recovery of NP in sludge subjected to TH of 6.6 ± 0.2% and in untreated sludge of 9.1 ± 1.5%. An influent sludge sample was extracted in triplicate, and the NP concentration was 6.1 ± 3.8 mg/kg.

YES Assay Procedure. The YES assay was performed as described by Routledge and Sumpter (1996), with minor modifications. Yeast absorbance was measured at 540 nm and 620 nm by a microplate spectrophotometer (Molecular Devices, Inc.; Sunnyvale, CA), and readings were taken after incubation at 32ºC for 3 days or until a standard curve fully developed. Adjusted absorbance values used to quantify response were calculated as shown in Equation 2 below. Plates were shaken for two minutes prior to incubation. YES assay data are generally assumed to be semi-quantitative (Van den Belt *et al.,* 2004) because chemical analysis and the YES assay produce non-identical estrogenic values of the same order of magnitude (Rutishauser *et al.,* 2004). Therefore, comparisons were

made between samples that were tested on plates with a similar response in standard curves. Comparisons between samples were made by obtaining EC50 values from the modeled dose-response curve as described below.

YES Assay Calculations

When a complete dose-response curve was generated, E2-equivalents (EEQ) were calculated from the ratio of EC50 values in the sample and the standard curve (Eq. 1).

Equation 2-1

$$EEQ\ (ngE2/g\text{-}sample) = EC50_{std}(ngE2/L) / EC50_{sample}(g/L)$$

Absorption data at 540 nm were corrected for background absorbance and turbidity using Eq. 2.

Equation 2-2

$$Adjusted\ absorbance = A_{540}total - A_{540}matrix - 1.07*[A_{620}total - A_{620}matrix]$$

Some sludge samples were toxic to the yeast at high concentrations and caused early die off during the YES assay, resulting in no development of a classic dose-response curve. Best-fit models were applied to the useable data in these toxic samples to complete a full curve, as described in detail elsewhere (Stanford and Weinberg, 2010). Briefly, data were fit to the dose-response equation (Eq. 3), where *Y* is adjusted absorbance, *L* is minimum response, *H* is maximum response, slope is the Hillslope of the curve, and *X* is the concentration. *H* and *L* were manually selected based on response

of the standard and the *slope* and *EC50* were automatically generated by Graphpad Prism software (Graphpad Software, Inc.; La Jolla, CA).

Equation 2-3

$$Y = L + (H-L) / (1+e^{(slope*[logEC50-logX])})$$

Calculated estrogenic equivalents (CEEQ), reported in ng E2/g sample, were calculated from the LC/MS-generated NPE concentrations and the relative potencies (*i.e.*, estrogenicity of analyte relative to estrogenicity of E2) of the measured compound (Eq. 4). Relative potencies for NP, NP_1EO, and NP_2EO were taken from Gabriel *et al*., (2008), Cespedes *et al.,* (2004), and Routledge and Sumpter (1996), respectively. These CEEQ values were compared to the measured EEQ values obtained from the YES assay to estimate the fraction of estrogenicity that NPE accounted for in the biosolids samples.

Equation 2-4

$$CEEQ = (ng[X]/g\text{-}sample)*(ngE2\text{-}eq)/(ng[X])$$

2.3 Results and Discussion

Thermal Hydrolysis of Micropollutants in a Water or Sludge Matrix

Initial experiments focused on whether the high temperature and pressure conditions used during TH could directly destroy micropollutants typically found in high

concentrations in solid wastewater residuals, *i.e.*, NP, NP_1EO, NP_2EO, triclosan, and bisphenol A (Citulski and Farahbakhsh, 2010; McClellan and Halden, 2010; Staples *et al.,* 2010). The high temperature and pressure during TH has been shown to transform lipids to volatile fatty acids by fragmentation of the hydrocarbon chain (Wilson and Novak, 2009), thus suggesting that compounds with hydrocarbon chains such as NP might be degraded via TH. No destruction of NP or of the other micropollutants tested was observed during TH (Table 2-2).

Table 2-2. Impact of Thermal Hydrolysis on Micropollutants in Water

Analyte	Replicates	Solution	p-value	Removal Efficiency (η)
			(C_{RT} *v.* C_{THP})[b]	
NP	3	Water	0.555	NS[a]
NP1EO	3	Water	0.940	NS
NP2EO	3	Water	0.516	NS
			(C_o *v.* C_{THP})[c]	
Triclosan	3	Water	0.336	NS
BPA	3	0.4% MeOH	0.724	NS
			($C_{DAY\ 1}$ *v.* $C_{DAY\ 2}$)[d]	
NP1EO	3	Water	0.584	NS
NP2EO	3	Water	0.898	NS

[a]*NS = no significant difference at $p \leq 0.05$ between the two sets of samples*
[b]*t-test on concentrations in room temperature samples vs. TH samples*
[c]*t-test on concentrations in initial sample taken prior to TH vs. sample taken after TH*
[d]*t-test on concentrations in samples taken after one TH experiment vs. samples taken after a consecutive second TH experiment*

In the sludge matrix there was no significant difference at the 95% confidence (Student's t-test) interval between the concentration of NP in the TH-treated and room temperature control systems, although the standard deviation between samples was high

(Figure 2-1). Any effect that TH might have on the transformation of these micropollutants therefore would be a result of altered downstream biodegradation via secondary impacts (*e.g.*, changing bioavailability or soluble carbon), and not a result of direct TH-mediated transformation. Thus, I focus the remainder of the discussion on the effect of TH on downstream biological processes and what occurs during anaerobic and aerobic digestion following TH.

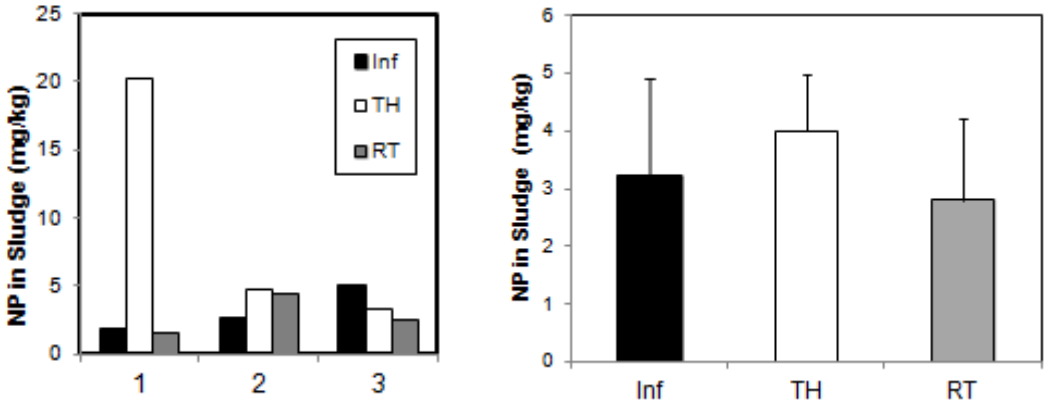

Figure 2-1. Effect of thermal hydrolysis treatment in a sludge matrix on the concentration of NP. Left plot: all replicates shown, numbers 1, 2, 3 refer to replicate experiments. 'Inf' is influent, 'TH' is thermal hydrolysis treated sludge, 'RT' is room temperature. Right plot: Average NP concentrations shown plus standard deviation. Sample TH-1 was removed from analysis.

Impacts of TH Pretreatment to Anaerobic Digestion on NPE Mass in Digester Effluent

The amount of NPE in the influent sludge to the TH-MAD reactors was consistently lower than the NPE in the influent sludge to the MAD reactor. This loss is not likely a result of chemical destruction, as demonstrated by the TH experiments in

water (Table 2-2) and TH experiments in sludge (Figure 2-1); rather, this loss is likely a result of volatilization during the flashing process at the pilot-scale plant. In a full-scale operation, the sludge cooling process occurs in a closed system, and thus NPE will not be removed via volatilization.

In all of the anaerobic reactors, with one exception (TH150-MAD20), the total masses of NPE in the influent and effluent were within 10% of each other (Figure 2-2). This indicates that, in general, (1) NPEO was transformed to NP and (2) neither substantial loss of NP nor (3) long chain NP_nEO (n=3-20) degradation to NPE occurred during anaerobic digestion, regardless of TH pretreatment. This result was expected, as work has shown that NP_nEOs are long-lived, with half-lives greater than 100 days in anaerobic sediments (Ferguson and Brownawell, 2003), while the biodegradation of NP_2EO to NP_1EO and NP_1EO to NP readily occurs under anaerobic conditions (Giger *et al.,* 1984, Soares *et al.,* 2008; Ferguson and Brownawell, 2003). In addition, although NP degradation under anaerobic conditions is possible (Chang *et al.,* 2005; Chang *et al.,* 2008), the rate is slow, and under typical digester conditions with large quantities of carbon available, NP degradation is not thought to readily occur (Giger *et al.,* 1984).

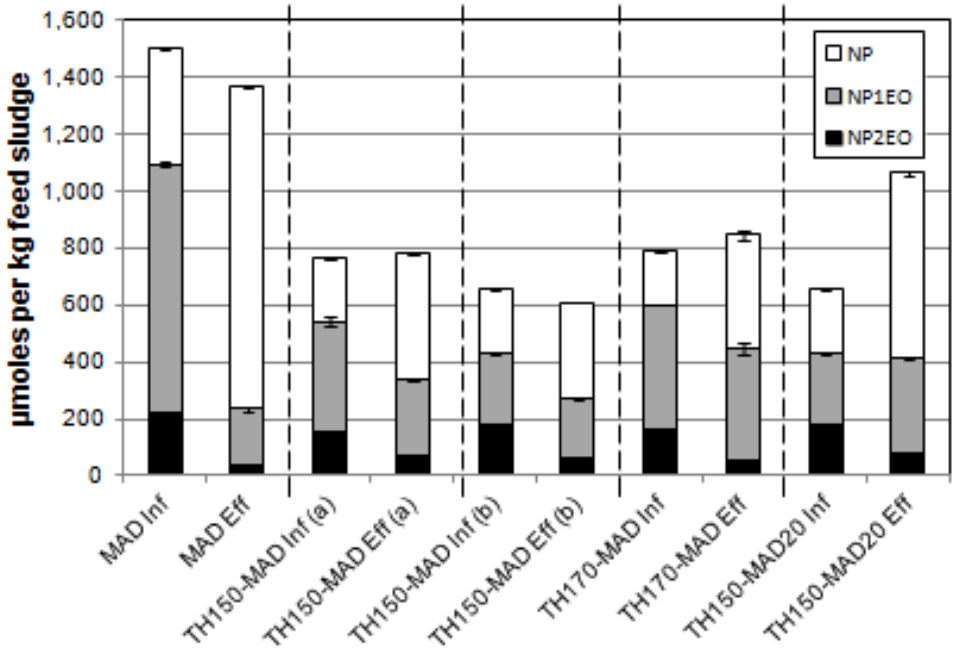

Figure 2-2. Impact of TH pretreatment to anaerobic digestion on total NPE in biosolids. Each bar represents the total NPE in each sample, with the individual concentrations of NP2EO, NP1EO, and NP represented as labeled on the plot. Error bars refer to standard error of the mean (SEM) between triplicate runs on the LC/MS, with the exception of the MAD Eff sample where the error bars represent standard error of the mean on duplicate extractions.

In the TH150-MAD20 reactor, however, the mass of NPE increased by 62% during digestion. Longer chain ethoxylates (NP_3EO – $NP_{20}EO$, not analyzed in this study) are parent compounds of NPE. Although not observed in the MAD, TH150-MAD, and TH170-MAD reactors, the increase of total NPE seen in the TH150-MAD20 effluent suggests that higher chain ethoxylates can be broken down to NPE under these typical digester conditions. As a result, further investigation on the transformation of long-chain NP_nEOs during TH-MAD with an extended SRT is warranted.

Impacts of TH Pretreatment to Anaerobic Digestion on the Ratio of NP to NPE in Digester Effluent

The conventional MAD treatment process produced biosolids with a higher ratio of NP:NPE than the TH-MAD treatments (Figure 2-3). The ratios of NP to NPE increased from influent to effluent in all anaerobic digesters as a result of the biodegradation of NPEO to NP (Figure 2-3). The biodegradation efficiency of NPEO to NP varied, however, depending on whether TH preceded MAD. In conventional MAD the ratio of NP:NPE increased 56% following digestion, whereas in the three sets of samples from TH-MAD reactors operated at 15d SRT, the ratio of NP:NPE only increased 23.9% ± 3.4% (average ± standard deviation). The ratio of NP:NPE in the TH150-MAD20 reactor increased 26.6%. It is interesting that the conventional MAD treatment process was more efficient at biodegrading NPEO to NP than the advanced TH-MAD process, even though the TH-MAD reactors outperformed the conventional MAD reactor based on volatile solids reduction. Thus, digester performance based on classical criteria was not directly related to the biodegradation efficiency of NPEO to NP. These results, showing reduced NPEO biodegradation to NP following TH, are similar to others' work in which PCB and PAH removal during anaerobic digestion decreased after TH of the feed sludge (Barret *et al.,* 2010a).

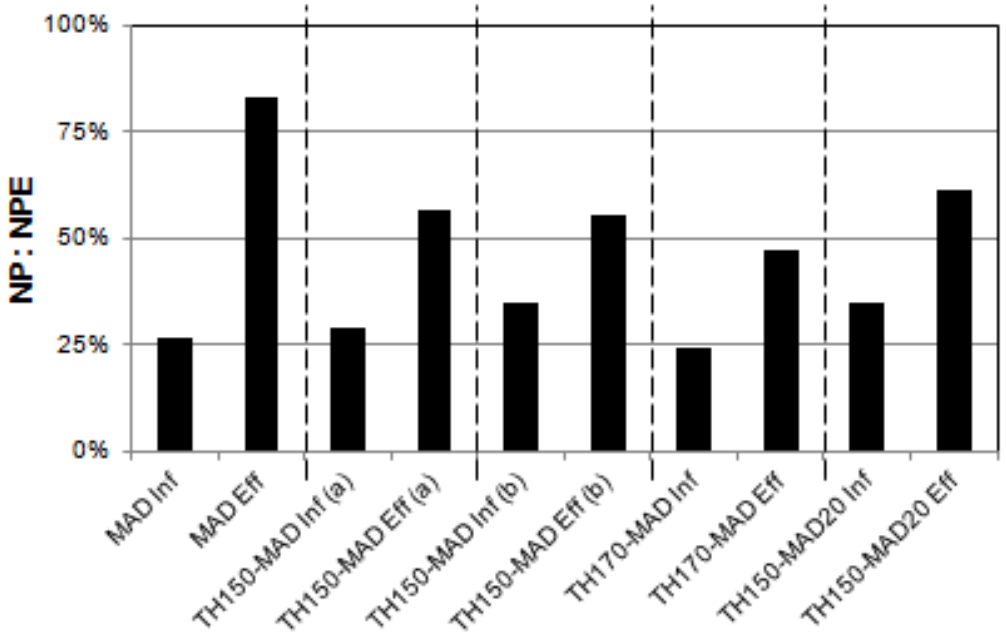

Figure 2-3. Impact of TH pretreatment to anaerobic digestion on the fraction of NP in NPE.

Barret *et al.,* (2010c) have suggested that PAH degradation during anaerobic digestion can be limited by bioavailability or cometabolism. The fact that the TH-MAD reactors had higher solids destruction, an indicator of microbial activity, but lower conversion of NPEO to NP, indicates that, either 1) cometabolism was not the limiting factor, or 2) overall metabolism of the entire reactor was not an indictor of the relevant metabolism for NPEO biodegradation. Indeed, the TH process generated much higher levels of volatile fatty acids, perhaps resulting in the inhibition of ethoxylate-degrading organisms or greater substrate competition (Russell, 1992; Chang *et al.,* 2008). The TH process also resulted in higher ammonia levels from the rapid conversion of soluble proteins (Wilson and Novak, 2009), which could inhibit the degradation of NPEO to NP.

Thermal hydrolysis reduces the bioavailability of hydrophobic micropollutants (Barret *et al.,* 2010b), which could explain the reduction of NPEO biodegradation seen in the TH-MAD reactors with a 15 day SRT.

This trend of less NPEO conversion to NP in the TH-MAD setups was also observed when comparing results from my research to that of published full-scale studies (Figure 2-4). The average ratio of NP:NPE in 36 full-scale conventional mesophilic anaerobic digesters was 82%; in this study the NP/NPE ratio was 83% for the MAD reactor. Because the conversion of NPEO to NP in a few of the full-scale conventional MAD reactors was lower than in the TH-MAD reactors in this study, it cannot be stated that TH will always hamper the biodegradation of NPEOs to NP in downstream digestion. Nevertheless, the difference between the NP:NPEO ratio in all of the 36 full-scale reactors depicted in Figure 2-4 and the 4 sets of samples from the TH-MAD reactors in this study was statistically significant ($p = 0.0006$; Student's t-test).

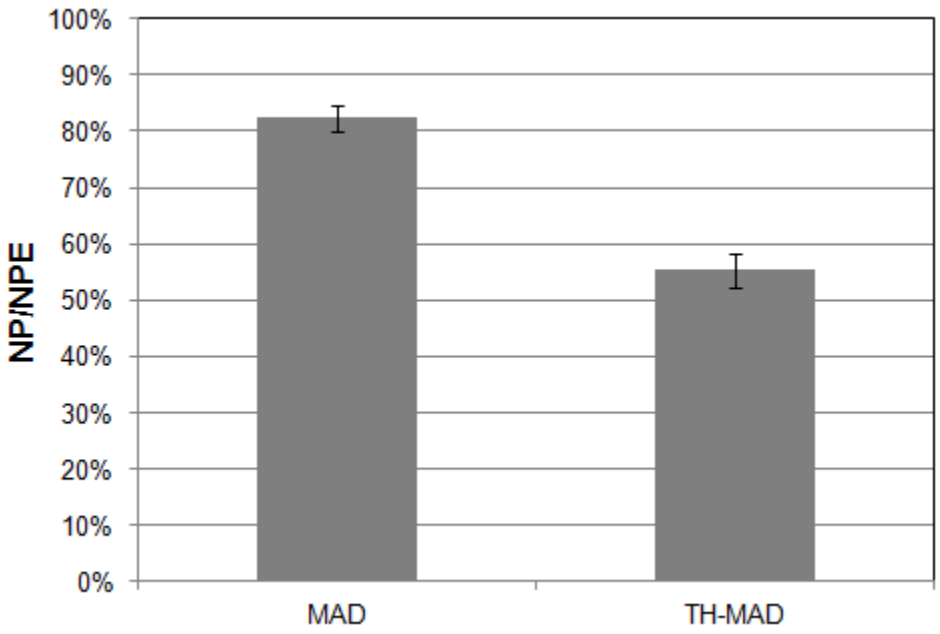

Figure 2-4. Impact of TH-MAD on ratio of NP to NPE relative to full-scale MAD digesters. MAD samples (n =36) taken from González *et al.*, 2010 and Brunner *et al.*, 1998; TH-MAD samples (n = 4) taken from this study. Error bars depict standard error of the mean.

Impact of a Post-Aerobic Digestion Phase on Fate of NPE

The total NPE decreased in the additional aerobic phases that followed the MAD reactor and in the anoxic/aerobic phases that followed TH-MAD (Figure 2-5). Under aerobic conditions, the total NPE decreased by nearly 70%, whereas in the post-digestion reactors (with anoxic/aerobic cycling) that followed TH-MAD, 30-45% of total NPE mass was removed. Nonylphenol is mineralized under aerobic conditions (thus accounting for a decrease in total NPE), but nonylphenoxy carboxylates (NPECs) can also form during aerobic degradation of NPEO (Hesselsoe *et al.,* 2001; Ahel *et al.,* 1994). NPEC were not analyzed and might have accounted for some loss of NPE during aerobic

digestion.

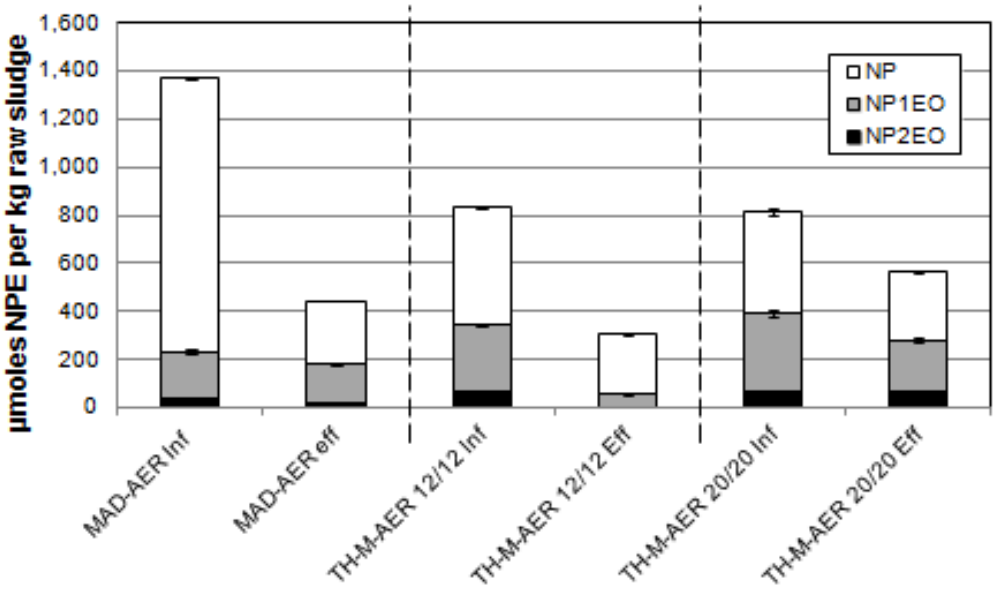

Figure 2-5. Impact of post aerobic digestion following anaerobic digestion on total NPE in biosolids. Error bars refer to standard error of the mean between triplicate runs on the LC/MS, with the exception of the MAD-AER Inf sample where the error bars represent std error of the mean of duplicate extractions.

The LC/MS results showing a decrease in total NPE following aerobic or anoxic/aerobic treatment were corroborated by an observed reduction in total estrogenicity as measured via the YES assay. Total estrogenicity was reduced by approximately 50% during aeration following conventional MAD (Figure 3-6). Similar results have been observed by Knudsen *et al.,* (2000), who found that aerobic treatment following anaerobic digestion reduced NPE concentrations by over 75%. These results indicate that an additional treatment phase that includes aeration of some fashion would decrease total NPE regardless of whether TH pretreatment was used.

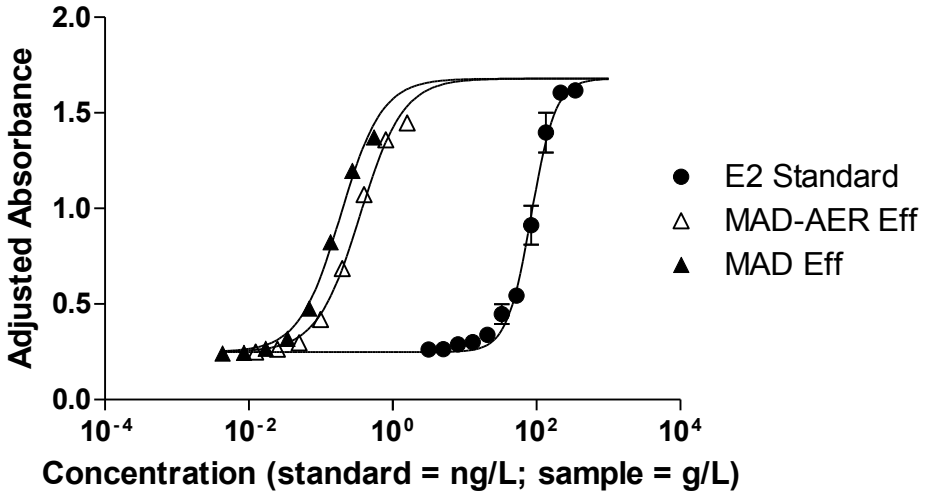

Figure 2-6. Impact of a post-aeration digestion following anaerobic digestion on total estrogenicity in biosolids as depicted by YES assay results. A sample curve shifted to the left is more estrogenic than a sample curve on its right. The "MAD Eff" sample was the feed to the MAD-AER reactor.

Contribution of NPE to Total Estrogenicity

The NPE are only weakly estrogenic (Routledge and Sumpter, 1996). As a result of the high concentrations of NPE that accumulate in biosolids, however, NPE may still represent a notable fraction of the total estrogenicity in biosolids. Indeed, this study found the contribution of estrogenicity from NPE in anaerobically and aerobically treated biosolids was 21±27% and 32±16%, respectively. These results indicate that NPE represent a marked fraction of the total estrogenicity in biosolids. The NPE were shown to account for less than 4% of total estrogenicity in sewage treatment plant liquid effluent in two different studies (Rutishauser *et al.,* 2004; Zhang *et al.,* 2011). Conversely, Holbrook *et al.,* (2002) estimated that nonylphenol and octylphenol accounted for >100%

of the estrogenicity in biosolids. While NPE might be less of a concern regarding impacts in liquid discharge, their contribution to the estrogenicity of biosolids must be considered when evaluating the environmental impacts of land application. Even though NPE have been shown to readily degrade in soil (Gomez-Rico *et al.,* 2008), NP was detected at mg/kg levels in soils that consistently received biosolids (Xia *et al.,* 2010). Certainly the concentration of NPE in soil will depend on when biosolids are land applied and the concentration of NPE in the biosolids.

It is likely that low concentrations of natural estrogens (estrone and 17β-estradiol) contributed to the total estrogenicity of the biosolids (Janex-Habibi *et al.,* 2009). Estrone and 17β-estradiol are several orders of magnitude more potent than NP in terms of estrogenicity (Jobling and Sumpter, 1993; Matsui *et al.,* 2000), so even low concentrations (ng/kg levels) of natural estrogens could greatly increase the estrogenicity of biosolids. Yet, whereas the production and discharge of natural estrogens cannot be controlled or decreased, NPE usage and discharge can be limited. Our work showed that additional aerobic digestion, or perhaps composting (Jones and Westmoreland, 1998), is needed to remove total NPE after MAD (with or without an advanced TH process). Either widespread additional treatment of biosolids, concomitant with greater energy use and a larger carbon footprint, or a ban on NPE might therefore be needed to mitigate the impacts of estrogenicity from NPE in biosolids.

2.4 Conclusions

- The high temperature and pressure conditions used for TH were not sufficient to degrade NP in sludge. Similarly, NPE were not degraded during TH in a water matrix (nor were other micropollutants tested, such as BPA and triclosan). While TH might indirectly impact the fate of NPE in digested biosolids by altering physico-chemical properties in sludge (*e.g.,* bioavailability), this process does not directly degrade NPE abiotically.
- The total NPE in the influent and effluent of the anaerobic digesters (15 day SRT) was approximately equal, regardless of whether TH pretreatment was used or not. Under anaerobic conditions, NPEO were biodegraded to NP more rapidly than NP was biodegraded; this resulted in a decrease in NPEO and an increase of NP in digester effluents, with similar amounts of total NPE in the influent and effluent.
- The NPE in the effluent of the TH-MAD-20d reactor increased relative to the influent. This increase in NPE suggests that longer chain NP_nEO were broken down to NPE during this extended SRT (20 days).
- The biodegradation of NPEO to NP was greater in the conventional MAD reactor than in the TH-MAD reactors, as evidenced by the ratios of NP to NPE in the digester effluents. The ratio of NP to NPE increased 56% following conventional MAD, while the average increase of NP to NPE was only 23.9% in the three samples from TH-MAD reactors with a 15 day SRT. One possible explanation for

this reduced biodegradation of NPEO to NP is that the TH process reduced the bioavailability of NPEO.

- The addition of a post-digestion treatment step that included either alternating aerobic/anoxic conditions or strictly aerobic conditions reduced NPE by 30 to 70%. This additional treatment step would be costly as a result of oxygen requirements, but would help reduce NPE in biosolids used for land application.
- The NPE accounted for 21±27% and 32±16% of the total estrogenicity in the biosolids after anaerobic and aerobic digestion, respectively.
- A ban on NPE would undoubtedly reduce NPE present in biosolids. Alternatively, additional wastewater treatment steps such as aerobic digestion or composting could be used to help mitigate the impact of NPE in biosolids.

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Chapter 3 - The Impact of Triclosan on Methanogenic Community Structure and Function

3.1 Introduction

Triclosan is a widely-used antimicrobial agent that collects in wastewater treatment plants. The heavy use of this compound can clearly be seen in the fact that it is detected in the urine of 75% of the United States population (Calafat *et al.,* 2008). Following household usage, triclosan flows with sewage to wastewater treatment plants (Singer *et al.,* 2002; Heidler and Halden, 2007), where some is degraded, but the remaining triclosan is discharged to surface water with liquid effluent (Singer *et al.,* 2002; Buth *et al.,* 2010) or to soil with land-applied biosolids (Kinney *et al.,* 2006; Cha and Cupples, 2009; McClellan and Halden, 2010). Indeed, approximately half of the influent triclosan mass to a wastewater treatment plant remains in solid residuals (Heidler and Halden, 2007). Concentrations are particularly high in anaerobically digested biosolids (Heidler and Halden, 2007; Cha and Cupples, 2009), up to 133 mg (triclosan)/kg (dry solids) in biosolids (USEPA, 2009), because triclosan is typically recalcitrant under anaerobic conditions (McAvoy *et al.,* 2002; Miller *et al.,* 2008; Ying *et al.,* 2007).

Triclosan poses a wide variety of risks to organisms. Concern regarding triclosan usage stems in part from its effects on multicellular organisms, including decreased strength in mice and decreased aggression in fathead minnows (Cherednichenko *et al.,* 2012; Schultz *et al.,* 2012). Though not as widely studied, triclosan also affects prokaryotes in the environment and in engineered systems, which may be cause for concern. It is a biologically active compound that specifically targets *Bacteria* (McMurry *et al.,* 1998a); therefore, not unexpectedly, triclosan has been shown to decrease oxygen

uptake and inhibit nitrification in activated sludge biomass (Neumegen *et al.,* 2005; Stasinakis *et al.,* 2008). The effects of triclosan on the structure and function of anaerobic communities have not been established, although similar biologically active compounds, such as pharmaceuticals, have been shown to hinder anaerobic wastewater treatment processes (Amin *et al.,* 2006; Sponza and Demirden *et al.,* 2008; Shimada *et al.,* 2008; Loftin *et al.,* 2005). Finally, triclosan is also suspected to select for resistant microorganisms (McMurry *et al.,* 1998b), though no work describes the effect of triclosan in anaerobic communities where antibiotic resistance genes (ARGs) have been found to be abundant (Diehl and LaPara, 2010; Wa *et al.,* 2011). This last point may be particularly important because ARGs are considered emerging contaminants with negative public health consequences (Pruden *et al.,* 2006).

Given the potential for triclosan to disrupt function in microbial communities and its abundance in anaerobic digesters, the objective of this research was to understand the effects of triclosan on the structure and function of anaerobic communities, including whether exposure causes the proliferation of ARGs. Experiments were performed with microbial communities that were i) not previously exposed to triclosan ("previously unexposed") and ii) previously exposed to triclosan. Communities were spiked with triclosan at concentrations spanning those currently detected in digesters, as well as at concentrations that might be expected in the near future (4x current maximum detected concentrations). It was hypothesized that at current concentrations triclosan would shift the *Bacterial* composition, and lead to a community that was less resilient as a result of

stress enduced from triclosan. At higher concentrations it was expected that communities would lose function and ARGs would proliferate. This research will strengthen understanding of the risks associated with continued triclosan usage and its subsequent accumulation in anaerobic environments.

3.2 Methods

Part I. Impact of triclosan on structure and function in previously unexposed methanogenic communities

This experiment was designed to test the hypothesis that environmentally relevant concentrations of triclosan would shift community structure in previously unexposed communities and render them less resilient. The communities were operated under stressful conditions to test if triclosan exposure would result in communities that were more susceptible to collapse. This experimental approach differed from previous perturbation-community structure studies (*e.g.* Fernandez *et al.,* 1999) in that a steady-state was not achieved between perturbations; this experimental design enabled the determination of whether perpetual stress in combination with triclosan led to collapse.

***Methanogenic Source Reactors*.** Three bench-scale 3-L methanogenic source reactors were operated to provide innocula for daughter anaerobic serum bottle reactors. These 3-L source reactors were originally seeded with sludge from a full-scale manure-fed anaerobic digester (Haubenschild Farms, Princeton MN) that had not previously been exposed to triclosan (Appendix A, Table A1). These source reactors were fed a synthetic

blend of organic acids, alcohols and glucose (Table 3-1) in minimal media (Shelton and Tiedje, 1984). The organic loading rate was 0.18 g COD/L-day and the HRT was 60 days.

Table 3-1. Synthetic Feed to Source Reactors

Organic Components	% COD
ethanol	40.5
potassium acetate	10.9
sodium acetate	9.4
propionic acid	11.4
glucose	10.8
butyric acid	8.5
valeric acid	2.8
isopropanol	4.0
methanol	1.6

***Anaerobic Serum Bottle Reactors*.** Four sets of serum bottle reactors were operated in triplicate (Table 3-2). Two sets of reactors were exposed to triclosan at environmental levels (concentrations shown in Figure 3-1), and two sets of control reactors were not exposed to triclosan. One set of the control reactors and one set of the triclosan-exposed reactors were perturbed over the course of the experiment (Table 3-3). All reactors (perturbed and unperturbed) experienced an initial temperature drop to 5°C for 72 hrs and a final temperature increase to 42°C for 24 hrs.

Reactors were constructed from 160-mL serum bottles with 50 mL of active volume. Reactors were originally filled with 20 mL of sludge (12 g VS/L) from the 3-L source reactors and 30 mL of minimal media (Shelton and Tiedje, 1984). Once a week seven mL of the reactor contents were removed with a cut-off 1-mL pipet tip, and 7 mL minimal media were added; the HRT was 50 days. Each bottle was fed 0.2 mL of acetate

and glucose (3:2 based on COD) and 0.03 mL of methanol on triclosan feeding days. For control reactors the methanol did not contain triclosan, and for exposed reactors the methanol contained triclosan. Triclosan (sold as Irgasan, Sigma Aldrich, ≥ 97% (HPLC)) was measured gravimetrically and dissolved in a methanol solution. The methanol solution was loaded into the serum bottle reactors with a gas tight syringe. The mass of triclosan in the reactor for the duration of the experiment was calculated from the dilution rate as shown in Equation 3.1 and this mass was then normalized to total solids to generate the concentration of triclosan (Figure 3-1). For the first three weeks of operation ethanol was also added to the feed (20% of total COD). The average organic loading rate was 0.27 g COD per L-day. The bottles were purged with N_2 gas on the benchtop, capped with Teflon-lined septa, and crimp-capped with aluminum seals. Bottles were incubated at 37°C resting upright in the dark. A 1.5-mL aliquot of sludge was periodically taken from each reactor for DNA extraction and subsequent microbial analysis; the experiment lasted for 245 days.

Table 3-2. Experimental Parameters for Each Reactor Set in Part I

Set	Name	Abbreviation	Triclosan	Non-Temperature Perturbations
1	Control	C	No	No
2	Perturbed	P	No	Yes
3	Triclosan	T	Yes	No
4	Triclosan-Perturbed	TP	Yes	Yes

Equation 3-1

$$C_t = C_o e^{\frac{t}{\tau}}$$

Where t = time since triclosan feed, C_t is concentration of triclosan in reactor at time = t, C_o = initial triclosan concentration following feed, τ = HRT.

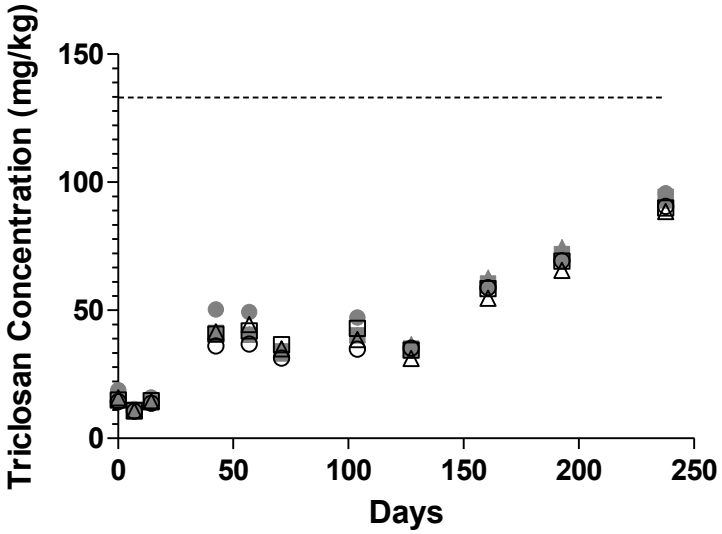


Figure 3-1. Calculated concentrations of triclosan during the Part I experiment. The dotted line corresponds to the maximum triclosan concentration measured in actual biosolids samples (USEPA, 2009). The grey circles, squares, and triangles correspond to concentrations in triclosan-perturbed reactors A, B, and C, respectively. The open circles, squares, and triangles correspond to concentrations in triclosan unperturbed reactors A, B, and C, respectively.

Table 3-3. Perturbations in Part I

Day	Perturbation Name	Perturbation Details	Bottles Impacted
22	Cold Shock	5°C for 72 hr	C,T,P,TP (all)
49	Mixing	125 rpm, 6 Days	P,TP
57	Low Ammonia	NH_4 Spike, 1 g/L	P,TP
71	Organic Overload	5X Glucose	P,TP
104	High Ammonia	NH_4 Spike, 10 g/L	P,TP
196	Diverse Feed	Mixed C3-C6 Acids (Appendix A)	C,T
196	Disinfectant	HOCl (20 μM, pH=7.5; 1.5hr)	P,TP
231	Heat Shock	42°C for 24 hr	C,T,P,TP (all)

Part II. Impact of triclosan on structure and function in previously exposed methanogenic communities

This experiment was designed to test the hypothesis that increased usage of triclosan will impact the community structure and function of methanogenic communities already exposed to triclosan. The previously exposed reactors in this experiment were seeded with biosolids from a bench-scale anaerobic digester (SRT of 35 days) that was processing blended primary and secondary municipal sludge and was originally seeded with biosolids from a full-scale municipal anaerobic digester. Background levels of triclosan in this seed sludge were 3.96 (±0.83) mg/kg (Appendix A, Table A2). Triclosan was added to triplicate reactors at concentrations of approximately 0, 5, 50, or 500 mg/kg. Triclosan concentrations were calculated as described in Part 1 using Equation 3-1; calculated triclosan concentrations are shown Table 3-4. A published survey on triclosan concentrations in biosolids throughout wastewater treatment plants in the U.S. (n=74) found the median concentration to be 3.9 mg/kg (U.S.E.P.A. 2009), the 95th percentile triclosan concentration to be 62.2 mg/kg, and the maximum triclosan concentration to be

133 mg/kg. Therefore, the reactors spanned this range and offered an opportunity to investigate community structure and function in the case of the current maximum triclosan concentration increasing four-fold.

Table 3-4. Calculated Concentrations of Triclosan Normalized to Total Solids in Previously Exposed Reactors in Part II

Day	5_A	5_B	5_C	50_A	50_B	50_C	500_A	500_B	500_C
1	7.2	7.6	7.8	77.1	73.0	79.7	644	796	815
7	5.5	5.7	5.5	57.4	53.9	54.7	492	532	546
15	3.2	3.0	3.0	30.2	31.4	30.8	274	361	325
24	2.2	2.4	2.1	29.0	22.8	23.1	236	227	214
33	1.2	1.3	1.6	15.0	12.4	12.1	107	115	105
42	5.2	4.7	5.5	60.2	46.7	38.7	398	337	448
51	8.3	7.3	7.3	80.8	72.0	63.4	671	648	622
57	8.2	7.6	7.4	78.3	73.0	70.4	686	689	704
Average	**5.1**	**5.0**	**5.0**	**54**	**48**	**47**	**438**	**463**	**472**

Reactors consisted of 160-mL serum bottles with 60 mL of active volume. Reactors were originally filled with 20 mL of seed sludge and 40 mL of minimal media. The initial total solids and volatile solids of the reactors were 1.8% and 1.0%, respectively. Each bottle was fed a synthetic acid-alcohol mixture (Table 3-5) at 1 g COD/L-day plus glucose at 1 g COD/L-day, for a total organic loading rate of 2 g COD/L-day. Every three days 12 mL of the reactor contents were removed and 12 mL of minimal media/synthetic feed were added; the HRT was 15 days. The initial pH was 8, and the pH was adjusted to <7.5 after each feeding with sodium carbonate. The bottles were purged with N_2 gas on the benchtop, capped with Teflon-lined septa, and crimp-capped with aluminum seals. Bottles were incubated at 37°C in the dark in a shaking

incubator (125 rpm). Samples for microbial analysis were taken in triplicate from each reactor periodically throughout the experiment; the experiment lasted for 57 days.

Table 3-5. Composition of Synthetic Feed for Part II Serum Bottle Reactors

Organic Components	g COD/L
sodium acetate	406
ethanol	406
isopropanol	113
propionic acid	294
butyric acid	226
valeric acid	113
caproic acid	68

Part III. Impact of triclosan on proliferation of antibiotic resistance genes (ARGs) in previously unexposed and exposed methanogenic communities

This experiment was designed to test the hypothesis that triclosan would select for genes that aided or conferred antibiotic resistance. The integrase of class 1 integrons (*intI1*) was targeted as a marker of horizontal gene transfer and *mexB* was targeted as a marker of vertical gene transfer. Antibiotic resistance is often associated with lateral gene transfer of antibiotic resistance genes via the integrase of class 1 integrons (Rowe-Magnus and Mazel, 2002; Mazel, 2006). The *mexB* gene is a constituent of the MexAB-OprM multidrug efflux pump that confers resistance to triclosan as well as other antibiotics (Yoneda *et al.,* 2005). The impact of triclosan exposure on *intI1* and *mexB* quantities was investigated in samples taken over time from serum bottle reactors described above in Parts I and II. In addition, a set of anaerobic serum bottle reactors (160-mL) were set-up, fed, and operated as described in Part II, with the exception that

they were inoculated with 20 mL sludge from a bench-scale anaerobic digester (SRT of 45 days) processing cow manure, and triclosan was not detected in this seed sludge (Appendix A, Table A2). Triclosan was added to these reactors as described in Part II. The concentrations in these triplicate reactors were approximately 0, 5, 50, and 500 mg/kg to mimic the conditions in Part II (Table 3-6). The experiment lasted 17 days.

Table 3-6. Calculated Concentrations of Triclosan Normalized to Total Solids in Previously Unexposed Reactors in Part III

Day	5_A	5_B	5_C	50_A	50_B	50_C	500_A	500_B	500_C
3	7.0	7.7	4.8	48.5	54.8	72.5	716	621	733
9	6.5	6.4		65.3	68.8	62.0	613	629	623
17	3.8	3.8	3.7	30.1	31.8	26.8	364	359	375
Average	**5.8**	**6.0**	**4.3**	**48.0**	**51.8**	**53.8**	**565**	**536**	**577**

Molecular Methods

DNA Extraction. Reactor samples (1.5 mL) were centrifuged (13,200 *g*), the supernatant was discarded, and pellets were frozen at -20°C until extraction. Because of differences in the total solids present, samples from Part I were centrifuged for 1.5 min, and samples from Part II were centrifuged for 10 min. Lysis buffer (MP Biomedicals, Solon, OH) was added to pellets, and cells were lysed with 3 freeze-thaw cycles followed by incubation at 70°C for 90 minutes. The FastDNA Spin Kit (MP Biomedicals, Solon, OH) was used to extract the DNA, which was then stored at -20°C until use.

Automated Ribosomal Intergenic Spacer Analysis (ARISA). Community fingerprints were assessed by ARISA. Primers ITSF (5'-GTC GTA ACA AGG TAG CCG TA-3') and ITSReub (5'-GCC AAG GCA TCC ACC-3') were used to amplify the

intergenic spacer region of *Bacteria* (Cardinale *et al.,* 2004). Primers 1389F (5'-ACG GGC GGT GTG TGC AAG-3') and 71R (5'-TCG GYG CCG AGC CGA GCC ATC C-3') were used to amplify the intergenic spacer region of *Archaea* (Qu *et al.,* 2009). The forward primers were labeled with the phosphoramidite dye HEX. The PCR mixture (25 uL) contained 1x PCR buffer (Promega, Madison, WI), 20 nmol deoxynucleoside triphosphates, 25 pmol forward and reverse primers, 1.25 units of GoTaq DNA polymerase (Promega) and approximately 1 ng of genomic DNA. PCR was performed in a DNA Engine Thermal Cycler (Biorad, Hercules, CA). The PCR protocol for *Bacteria* was as follows: 3 minutes of initial denaturation at 94°C, 35 cycles of 94°C for 45 seconds, 55°C for 1 minute, and 72°C for 2 minutes, and a final extension of 7 minutes at 72°C. The PCR protocol for *Archaea* was as follows: 3 minutes of initial denaturation at 95°C, 35 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes, and a final extension of 7 minutes at 72°C. PCR products were separated by capillary electrophoresis with an ABI 3130xl capillary instrument (Applied Biosystems Inc, Foster City, CA). Peak areas were analyzed with PeakScanner Software v 1.0. *Bacteria* and *Archaea* fragment lengths less than 156 and 250 basepairs, respectively, were removed from subsequent analyses to eliminate primer dimers (Cardinale *et al.,* 2004; Qu *et al.,* 2009). Fragments greater than 1000 basepairs were also eliminated as the maximum size standard was 1000 basepairs. Only peaks that accounted for $\geq$ 0.5% of the total area in a sample were used for further analysis. Fragments were binned using the R code "interactive binner" (Ramette, 2009) for which the window size was set to two base pairs

and the shifting window was set to 0.1 basepairs. The percent contribution of one OTU to the total community (area of one OTU divided by area of all OTUs) was averaged across replicates, and this average percent contribution was used for statistical analysis.

Quantitative PCR (qPCR). The 16S rRNA, *intI1*, and *mexB* genes were enumerated via qPCR as described previously (LaPara *et al.,* 2011). Briefly, forward primer (5'-CCT ACG GGA GGC AGC AG-3') and reverse primer (5'-ATT ACC GCG GCT GCT GG-3') (Muyzer *et al.,* 1993), forward primer (5'-CCT CCC GCA CGA TGA TC-3') and reverse primer (5'-TCC ACG CAT CGT CAG GC-3') (Goldstein *et al.,* 2001), and forward primer (5'-GTG TTC GGC TCG CAG TAC TC-3') and reverse primer (5'-AAC CGT CGG GAT TGA CCT TG-3') (Yoneda *et al.,* 2005) were used to amplify the 16S rRNA gene, the *intI1* gene, and the *mexB* gene, respectively. The PCR mixture (25 μL) contained 12.5 μL SYBR green (Bio-Rad, Hercules, CA), 25 μg bovine serum albumin (Roche Applied Science, Indianapolis, IN), optimized forward and reverse primers, and 0.5 μL of template DNA. PCR reactions were performed in an Eppendorf Mastercycler ep *realplex* thermal cycler (Eppendorf, Westbury, NY) with the following sequence: 10 min at 95°C, 40 cycles of 95°C for 15 s followed by 60°C for 1 min. A final melting curve was run on every sample for quality assurance. The presence of inhibitory compounds in the DNA extract was tested via qPCR on serially diluted samples; inhibition was not an issue for these samples. The number of gene copies in a sample was determined from an external standard curve (9 standards) containing known quantities of template. All standard curves had $R^2 > 0.99$, and PCR amplification

efficiencies were between 0.902 and 1.105 (see appendix Table A5). Quantification limits for the 16S rRNA, *intI1*, and *mexB* genes were 8,600, 1,410, and 170 gene copies/µL DNA extract. All samples were run in triplicate.

Analytical Methods

Gas production was measured by injecting a wetted glass syringe into the serum bottle reactors and measuring the displacement volume. Methane was quantified on a gas chromatograph (GC) equipped with a thermal conductivity detector (TCD). The pH was measured on an Orion pH meter (8103BNUWP). Total solids were quantified gravimetrically by drying samples overnight in a 105°C oven; volatile solids were determined by quantifying the mass that volatilized upon heating the dried sample for two hours in a 550°C furnace.

Statistical Analysis

Nonmetric multidimensional scaling (nMDS) was used to analyze the ARISA data. The *vegan* package in R was used for nMDS analysis. Dissimilarity between samples corresponds to distance between samples (Turch *et al.,* 2007). ANOVA and Student's t-tests were performed using Graphpad Prism v. 5.04 (Graphpad Software, Inc.; La Jolla, CA). A p-value of ≤ 0.05 was considered significant.

3.3 Results

Part I. Impact of triclosan on structure and function in previously unexposed methanogenic communities

Community Structure Effects. Triclosan addition to previously unexposed communities changed the Bacterial community structure. Communities were similar at Day 0, but by Day 20 triclosan had shifted the exposed community away from the control (Figure 3-2). Interestingly, at Day 71 the communities reconverged, despite the increase in triclosan concentration to 40 mg/kg on Day 42; this reconvergence suggested that the impacts of triclosan on community structure were not permanent at concentrations ≤ 40 mg/kg (Figure 3-2). When triclosan concentrations increased again, however, to approximately 65 mg/kg on Day 196, the exposed and control communities again separated, and they remained separated for the remainder of the experiment through an additional increase in triclosan to 90 mg/kg (Figure 3-2). These results suggest that, although triclosan can alter the community structure of previously unexposed communities at low concentrations, communities appear to adapt, and their structure reconverges with that of unexposed communities up to triclosan concentrations of approximately 40 mg/kg. Nevertheless, when exposed to higher concentrations (65-90 mg/kg), community structures change again and are not able to reconverge with the control communities, at least over the period examined in this experiment. The impacts of triclosan on *Archaea* were less consistent (Figure 3-3). The Archaeal communities were initially different at Day 0 and Day 21. At Day 71 they overlapped, but by Day 196 they

were separate again. This separation observed at Day 196 could be the result of changes in Bacterial community structure that resulted in subsequent changes in Archaeal community structure; triclosan specifically targets *Bacteria* and is not known to target *Archaea* (McMurry *et al.,* 1998a).

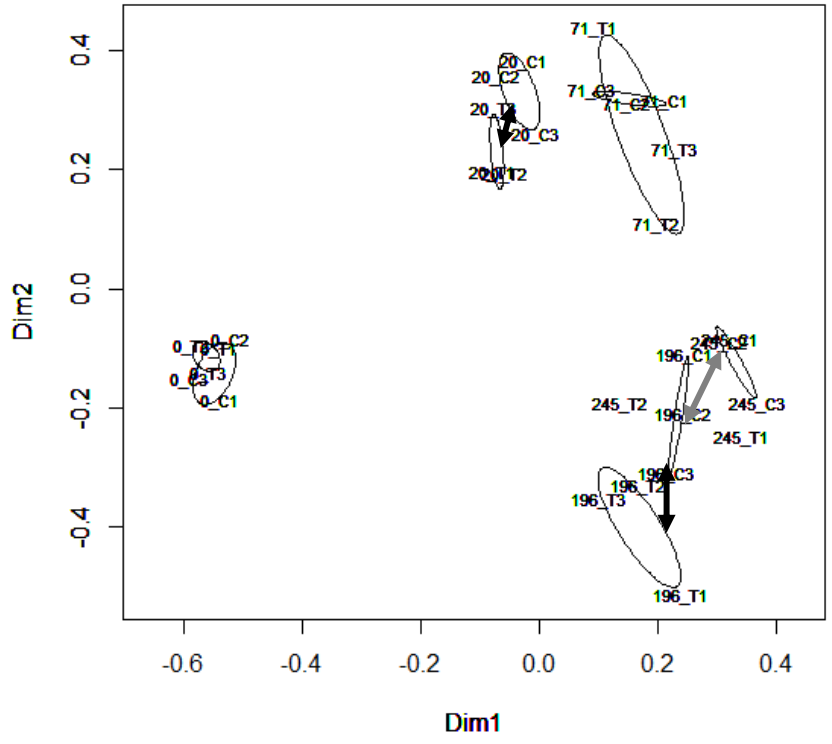


Figure 3-2. nMDS on Bacterial unperturbed communities. Community fingerprints at Day 0, 20, 71, 196, and 245 are shown; 'C' denotes control reactors and 'T' denotes triclosan-amended reactors. Differences between communities at a given time point are highlighted by black arrows. Gray arrow depicts difference at Day 245 between triplicate control communities and duplicate triclosan communities.

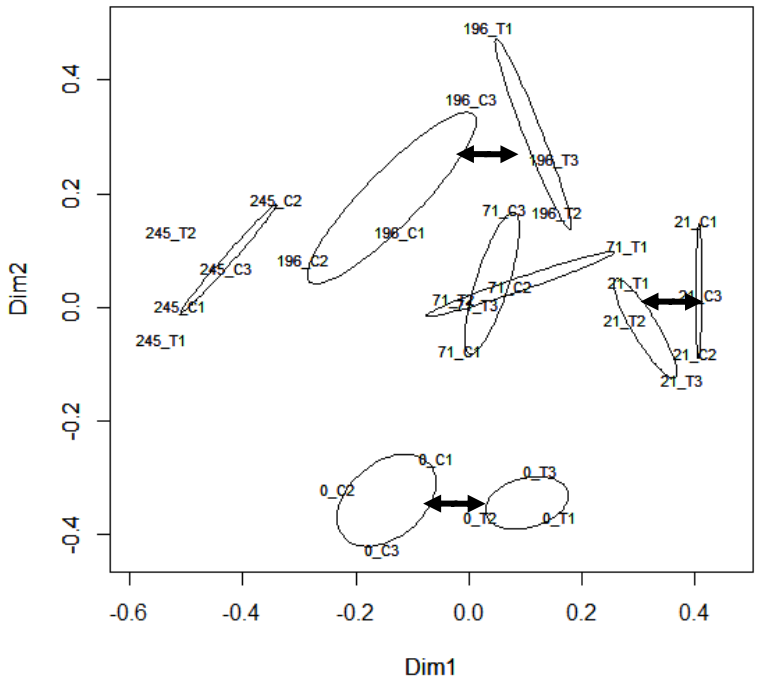


Figure 3-3. nMDS on Archaeal unperturbed communities. Community fingerprints at Day 0, 20, 71, 196, and 245 are shown; 'C' denotes control reactors and 'T' denotes triclosan-amended reactors. Differences between communities at a given time point are highlighted by black arrows.

Different patterns in Bacterial and Archaeal community structure were observed with respect to adaptation when exposed to triclosan and subsequently perturbed. In Bacterial communities exposure to triclosan did not seem to augment the impacts of perturbations (Appendix A, Figure A2). When exposed to triclosan, *Archaea* were more influenced by perturbations (Figure 3-4). These results suggest that triclosan augments the impacts of perturbations in *Archaea* and forces communities to change more drastically, but the perturbations themselves have a greater impact on Bacterial community structure.

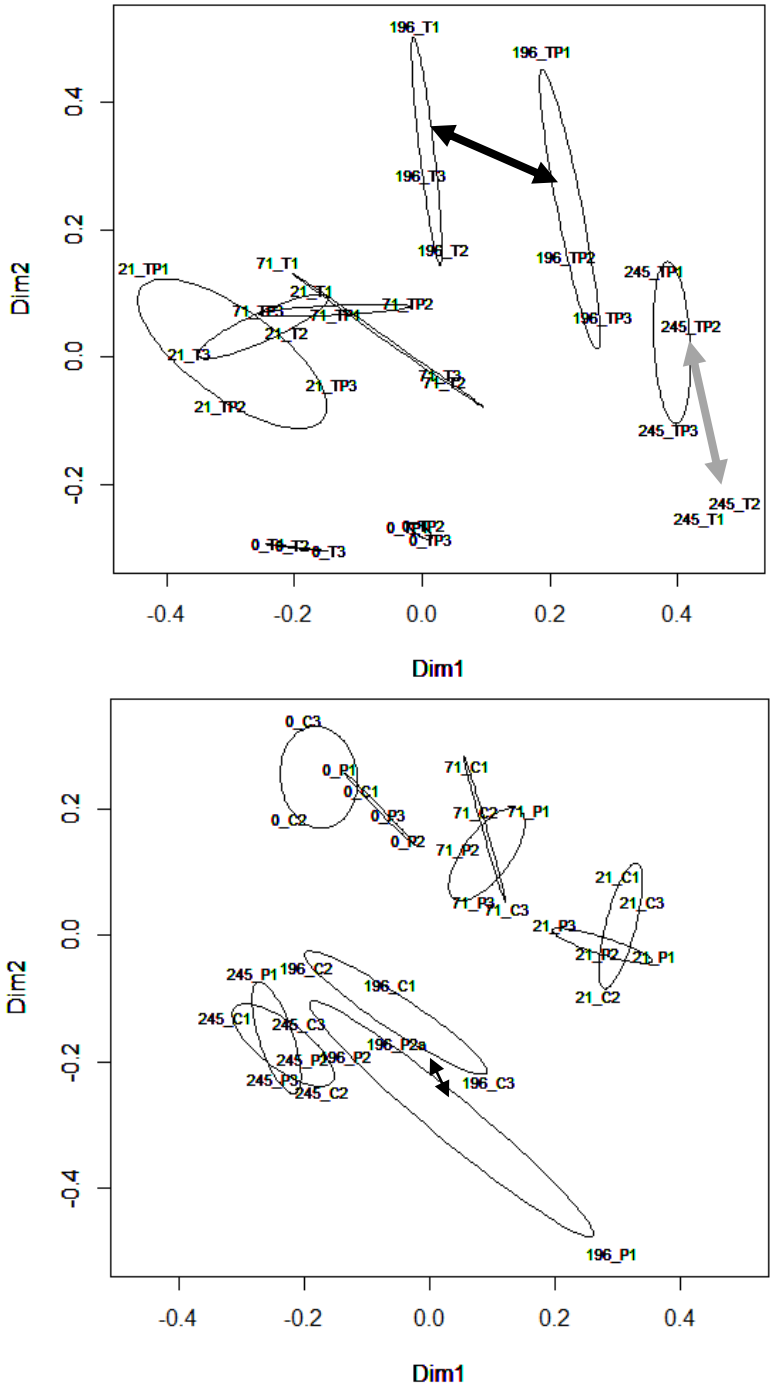


Figure 3-4. The impact of perturbations with triclosan (top) and without triclosan (bottom) on *Archaea*. Differences between communities at a given time point are highlighted by black arrows. Grey arrow highlights difference at Day 245 between triplicate triclosan-perturbed communities and duplicate triclosan communities.

Functional Effects. Overall triclosan, with or without perturbations, had no significant impact on methane production (Figure 3-5). Average methane production through initial 71 days was 47% greater in triclosan-amended reactors than in control reactors, though this difference was not significant (ANOVA *p*-value = 0.11; Appendix A, Figure A3). After approximately 71 days, methane production in triclosan amended reactors and in control reactors was similar; triclosan-amended reactors only produced 7% more methane on average; ANOVA *p*-value = 0.74). These functional data corroborated the community results and suggested that the community adapted to triclosan exposure, both in terms of structure and function. Although further *Bacterial* community shifts were observed from Day 127-245, the function of the total community did not mirror these changes and remained stable. This stable functional performance indicated that the anaerobic community was inherently resilient, both when exposed to triclosan and when exposed to triclosan with further perturbations.

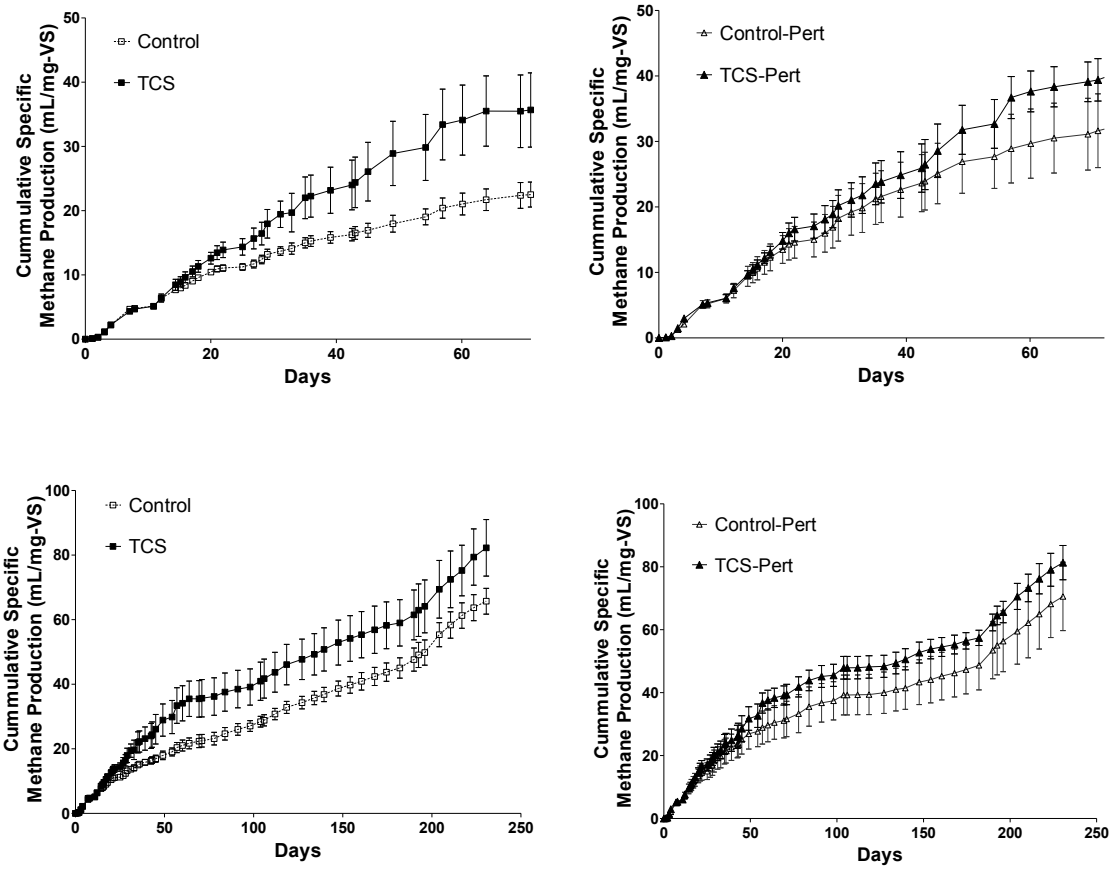
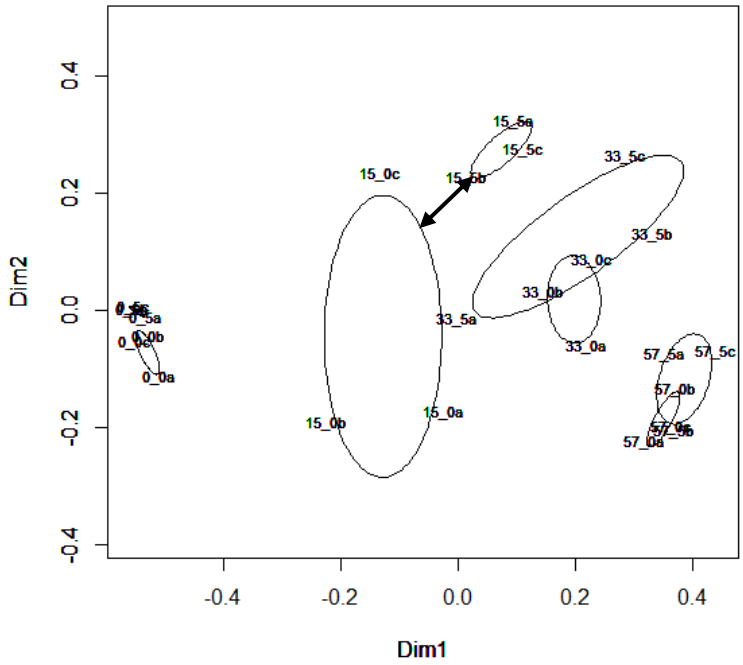


Figure 3-5. Impact of triclosan and perturbations on methane production (unperturbed communities on left and perturbed on right). Error bars represent standard error of the mean, n=3.

Part II. Impact of triclosan on structure and function in previously exposed methanogenic communities

Community Structure Effects. Most existing anaerobic communities are likely to have been exposed to triclosan and have therefore presumably adapted to it. Because of this previous exposure, these communities may adapt in a different manner to further increases in triclosan loading. Indeed, this was the case in these experiments. Triclosan addition to previously exposed communities altered both Bacterial and Archaeal

communities. Triclosan caused Bacterial community divergence, and for the lower two concentrations (5 and 50 mg/kg), reconvergence, as observed for the unexposed communities in Part I. Bacterial communities amended with higher levels of triclosan took longer to structurally reconverge with the control community; in the case of the highest concentration, 500 mg/kg, the community never did reconverge with the control community over the 57-day experiment (Figure 3-6). Triclosan caused all Archaeal communities to be different from the control at 57 days (Appendix A, Figure A9). Therefore, Bacterial communities did appear to be able to adapt (at least structurally) to environmentally relevant levels of triclosan, though Archaeal communities did not. At some concentration, 500 mg/kg in this case, communities may either not be able to adapt to triclosan exposure or may not adapt quickly enough to maintain function.



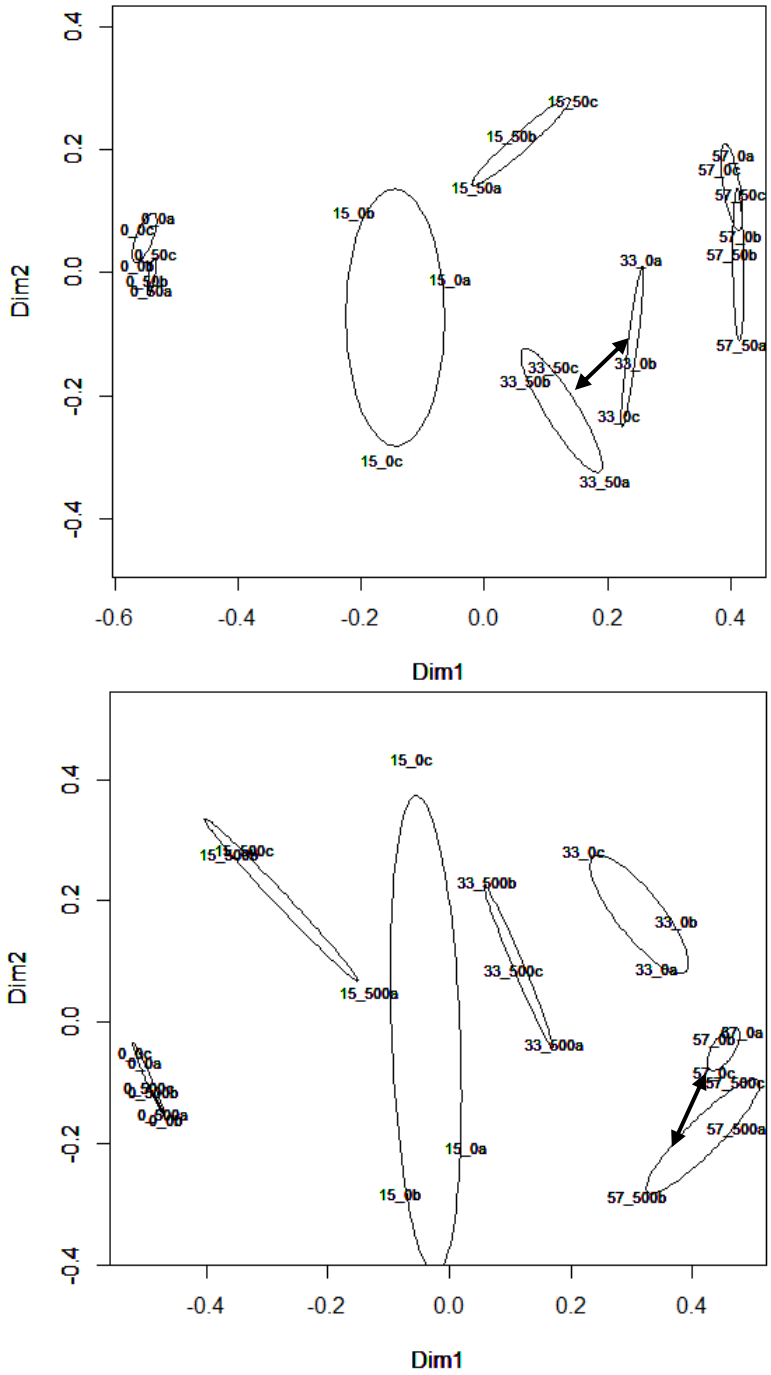


Figure 3-6. Impact of increased triclosan loading (5 mg/kg on top, 50 mg/kg in middle, 500 mg/kg on bottom) on Bacterial communities. Days 0, 15, 33, and 57 are shown. Arrow indicates latest time point that triclosan and control communities were different.

Functional Effects. Function with respect to methane production was also impacted by triclosan in the previously exposed communities (Figure 3-7). High concentrations of triclosan (500 mg/kg) caused a significant decline in methane production relative to the control (Student's t-test; *p*-value = 0.0017). In fact, the average methane produced in the triplicate 500 mg/kg reactors was only 56% of the average methane produced in triplicate control reactors. At 5 mg/kg, methane production increased slightly, though not significantly (Student's t-test; *p*-value = 0.054). Interestingly, at 50 mg/kg a large variability in methane production was observed as the relative standard deviation between triplicate reactors was 48% compared to a relative standard deviation of less than 10% in the triplicate reactors exposed to 0, 5 and 500 mg/kg. Exposure to 50 mg/kg triclosan may therefore represent a tipping point where increased concentrations could result in drastic functional declines in methanogenic communities.

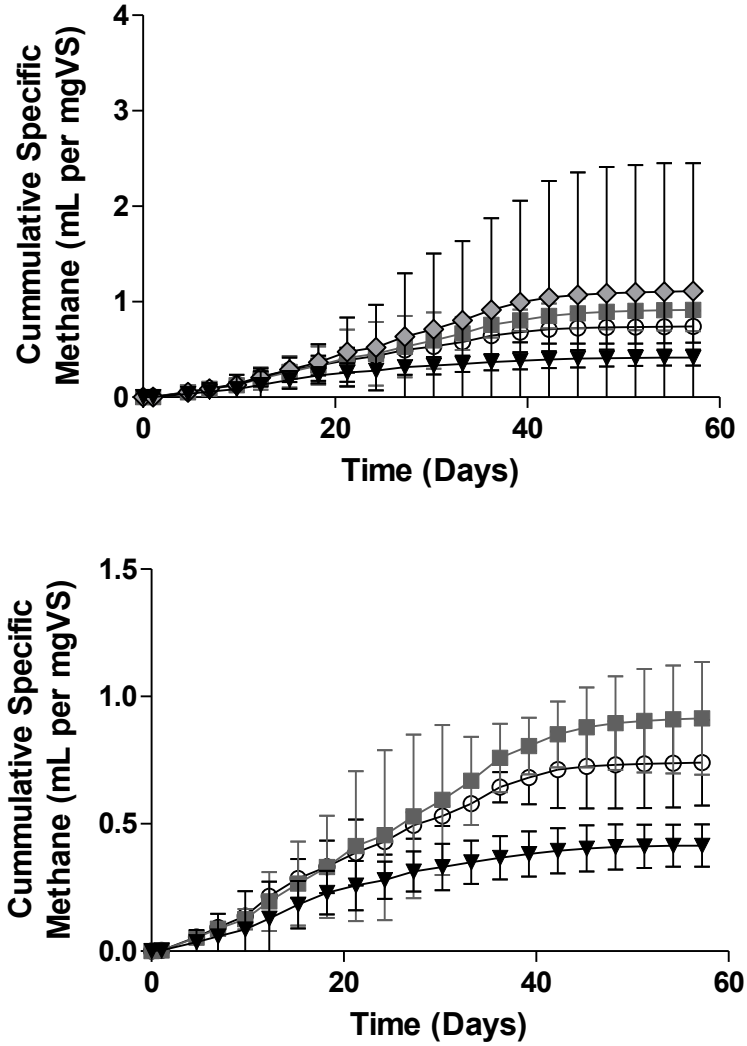


Figure 3-7. Impact of triclosan on specific methane production; 0, 5, 50, and 500 mg/kg are depicted by circles, squares, diamonds, and triangles, respectively. All experiments are shown in top plot, while 50 mg/kg is removed from bottom plot for clarity. Error bars represent 95% confidence intervals, n=3.

Part III. Impact of triclosan on proliferation of antibiotic resistance genes in previously unexposed and exposed methanogenic communities

Finally, in addition to the potential to cause functional collapse, triclosan exposure may also co-select for genes encoding resistance to other antibacterial compounds (Saleh *et al.,* 2010; Son *et al.,* 2010). In the previously unexposed communities described in Part 1, at Day 21 and Day 71 the abundances of *intI1* gene copies were statistically higher in the triclosan-perturbed reactors relative to the control-perturbed reactors (Figure 3-8), but this difference did not persist throughout the experiment. (Appendix A, Tables A8-A9). At Day 245 the abundance of *intI1* gene copies was significantly lower in the triclosan reactors relative to the control reactors. The abundance of *mexB* gene copies was significantly increased on Day 245 in the triclosan reactors relative to the control reactors, but it is noted that the samples from one of the triclosan triplicate reactors was lost and this result is based on duplicate reactors (Appendix A, Table A10-11).

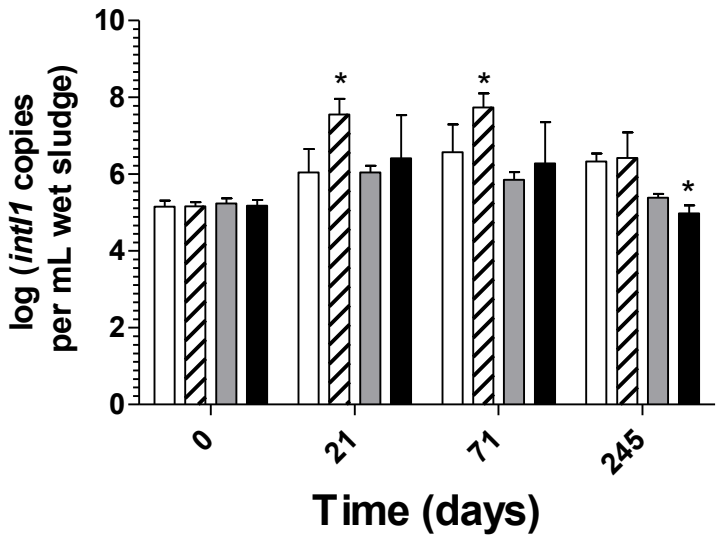


Figure 3-8 Impact of triclosan on abundance of *intI1* gene copies in unexposed communities in experiment from Part 1. Data were taken from Tables A8-9. Perturbed, triclosan-perturbed, control, and triclosan reactors are represented by white, slashed, grey, and black bars, respectively. Error bars depict standard deviation, n = 9 from triplicate PCR from triplicate reactors. At Day 245 n=6 for triclosan set. The (*) denotes statistical significance at $p < 0.05$.

In the previously exposed reactors described in Part II, neither *intI1* nor *mexB* increased as a result of triclosan exposure (Appendix A, Tables A14-17). Interestingly, the reactors exposed to 500 mg/kg triclosan had the lowest abundance of *intI1* by the end of the experiment at Day 57 (Figure 3-9). Nevertheless, in short-term experiments performed to test whether higher-than-current triclosan concentrations (500 mg/kg) could select for *mexB* or *intI1* in a previously unexposed mixed anaerobic community, *mexB* quantities increased in each of the triplicate reactors (Figure 3-10). The abundance of *intI1* was not altered by triclosan in these short-term triclosan exposure experiments (Figure 3-11).

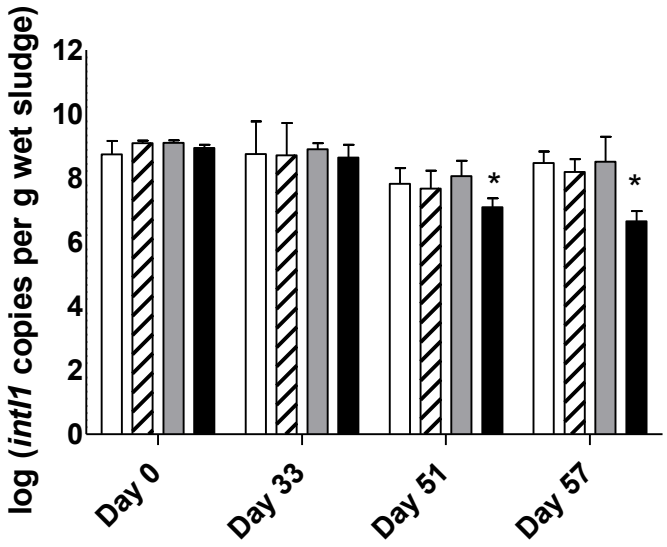


Figure 3-9. Impact of triclosan on abundance of *intI1* in previously exposed communities. 0, 5, 50, and 500 mg/kg are represented by white, slashed, grey, and black bars, respectively. Error bars represent standard deviation of the mean, n = 9 for triplicate extractions from triplicate reactors. Values from individual reactors are listed in Appendix A, Table A14-15. The (*) denotes statistical significance at $p < 0.05$ between a particular triclosan reactor set and the 0 mg/kg reactor set at a given time point.

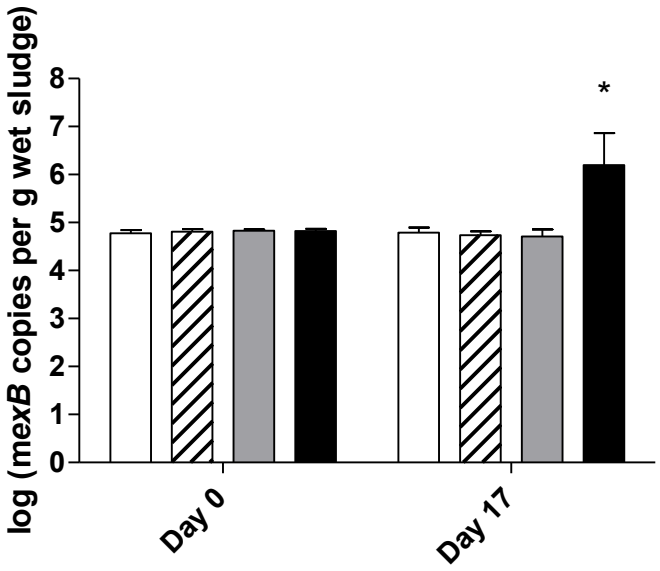


Figure 3-10. Impact of triclosan on abundance of *mexB* in previously exposed communities; values below quantification limit were plotted as ½ the quantification limit, and only 500 mg/kg at Day 17 yielded results above quantification limit. 0, 5, 50, and 500 mg/kg are represented by white, slashed, grey, and black bars, respectively. Error bars represent standard deviation of the mean, n = 9 for triplicate extractions from triplicate reactors. Values from individual reactors are listed in Appendix A, Table A21. The (*) denotes statistical significance at $p < 0.05$ between a particular triclosan reactor set and the 0 mg/kg reactor set at a given time point.

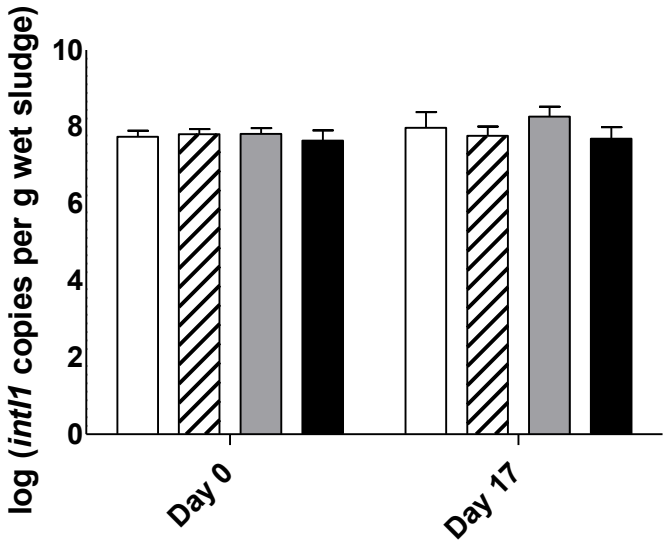


Figure 3-11. Impact of triclosan on abundance of *intI1* in previously exposed communities. 0, 5, 50, and 500 mg/kg are represented by white, slashed, grey, and black bars, respectively. Error bars represent standard deviation of the mean, n = 9 for triplicate extractions from triplicate reactors. Values from individual reactors are listed in Appendix A, Table A20. The (*) denotes statistical significance at $p < 0.05$ between a particular triclosan reactor set and the 0 mg/kg reactor set at a given time point.

3.4 Discussion

Anaerobic microbial communities are inherently dynamic over time, and in fact, the ability of a community to shift has been linked to functional stability (Fernandez *et al.,* 1999; Werner *et al.,* 2011; Lee *et al.,* 2012). In these experiments, the structure of previously unexposed communities shifted, and shifted in a unique manner, when exposed to triclosan. This shift did not correspond to a decline in function, and indeed, appeared to be part of the adaptation process of a previously unexposed community to

triclosan. In previously exposed communities, a unique shift in structure was also observed in response to triclosan exposure. When functionally healthy, however, these community structures reconverged with that of the control community over time. Although an increase in exposure may perturb these adapted communities, they reconverge with the control community structure. Interestingly, however, in the community that lost function when exposed to 500 mg/kg triclosan, the community structure did not reconverge with the structure of the control community over the course of the experiment. Thus, at least in the time frame of this experiment, 500 mg/kg is above the threshold for which the community can adapt and maintain function. Indeed, in the communities exposed to 50 mg/kg triclosan, methane production was highly variable, suggesting that the tipping point for which communities can adapt may be well under 500 mg/kg. There is a limit on the concentration of every pollutant for which communities can still adapt and maintain function (Blum and Speece, 1993), and we may be nearing that limit in some anaerobic systems currently exposed to triclosan at concentrations greater than or equal to 50 mg/kg. These results, showing triclosan impacting structure and function of microbial communities, corroborate other work that has seen shifts in community structure caused by exposure to biologically active micropollutants. Ketoprofen, naproxen, sulfamethoxazole, and erythromycin caused subtle community changes that corresponded to functional inhibition in activated sludge, nitrification, denitrification, and methanogenic systems, respectively (Wang *et al.,* 2008; Wang and Gunsch, 2011; Underwood *et al.,* 2011; Amin *et al.,* 2006). Even though long-term

impacts of biologically active micropollutants, like triclosan, on microbial communities are generally not included in risk assessments, these results indicate that micropollutants do indeed pose risks to the long-term health of microbial communities.

Triclosan changes community structure and may do so by elimination of weak members and perhaps by selection for community members with resistance to triclosan (Levy, 2000; Angenent *et al.,* 2008). McMurry *et al.,* (1998b) suggested that triclosan in the environment could preferentially select for *Bacteria* that are resistant to multiple antibiotics by selecting for multidrug efflux pumps, thus increasing multiple drug resistance. Indeed, studies on isolates of *Staphylococcus aureus* and *Psuedomonas aeruginosa* found that strains resistant to triclosan conferred cross resistance to ciprofloxacin (Tkachenko *et al.,* 2007; Chuanchen *et al.,* 2001). Additionally, in a study on batch activated sludge systems *tet(Q)* levels increased when amended with triclosan (Son *et al.,* 2010). The research in Part III demonstrated that, in previously unexposed communities, exposure to high concentrations of triclosan (500 mg/kg) increased *mexB* levels. This result furthers the argument that triclosan could lead to an increase in multiple drug resistance and suggests that we need to better understand the triclosan adaptation period and the effect that this adaptation may have on ARGs more broadly.

3.5 References

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Chapter 4 - Impact of Perfluorooctane Sulfonate, Exposure Time, and Chemical Mixtures on Methanogenic Community Structure and Function

4.1 Introduction

Perfluorooctane sulfonate (PFOS) is an organic surfactant that is ubiquitous in the environment, and has been found in the tissues of many animals, including bald eagles, polar bears, and marine animals (Giesy and Kannan, 2001). PFOS has been found at ng/L levels in surface waters (Nakayam *et al.,* 2010; Saito *et al.,* 2004) and was recently listed under the Stockholm Convention on Persistent Organic Pollutants (Lindstrom *et al.,* 2011). Wastewater treatment plants receive PFOS directly with influent sewage, and it also forms as other fluorinated surfactants degrade (Boulanger *et al.,* 2005). In US biosolids, PFOS is typically detected between 10 and 100 μg (PFOS)/kg (dry solids), with levels up to 2,610 μg/kg; in Switzerland, concentrations as high as 600 μg/kg have been detected (Higgins *et al.,* 2005; Sun *et al.,* 2011; Sinclair and Kannan, 2006; Sepulvado *et al.,* 2011).

The impacts of PFOS in biological systems are mixed. PFOS at very high concentrations, 500 mg/L, caused no detectable impacts on hydrogenotrophic methanogens in granular sludge when the exposure time was limited to 24 h (Hollingsworth *et al.,* 2005). Interestingly, an alcohol alkoxylate surfactant present at 200 mg/L inhibited methanogenic activity after 9 days but no inhibition was seen for the first 2 days of the experiment (Van Ginkle *et al.,* 2007). Therefore, exposure time could be an important factor in determining PFOS impact and thereby appropriating risk. In eukaryotes PFOS has been linked to detrimental effects, causing mitochondrial cell membranes to leak at 5 mg/L (Starkov and Wallace, 2002). At 30 mg/L PFOS both

increased and decreased the impacts of chlorinated compounds on algal cells (Liu *et al.,* 2009), suggesting that the impact of PFOS as a constituent of mixtures should also be further studied.

Because of the potential importance of exposure time and mixture effects, the objective of this research was to more fully explore the short-term and long-term impacts of PFOS, alone and in mixtures, on methanogenic communities. Because PFOS is found in biosolids along with other micropollutants, such as triclosan, anaerobic microorganisms are quite likely to be impacted by mixture effects (Higgins *et al.,* 2005; Kinney *et al.,* 2006; McClellan and Halden, 2010). Therefore, experiments were run with methanogenic communities amended with environmentally relevant levels of PFOS and in some cases, the ubiquitous antimicrobial triclosan. It was hypothesized that PFOS would have minimal impacts on communities alone, but would augment the impacts of triclosan. It was also hypothesized that effects would only be observed after PFOS exposure was long enough to allow penetration through flocs and into cells. This work will further our understanding of the impacts of this priority organic pollutant and shed light on how testing conditions (short term vs. long term) impact risk evaluation.

4.2 Methods

Long-term PFOS Exposure Tests

This experiment was designed to test the hypothesis that long-term exposure of methanogenic communities to PFOS would inhibit methane production, shift community

structure, and render communities more susceptible to the effects of triclosan. Experiments were run for 140 days at environmentally relevant low (60 μg/kg) and high (800 μg/kg) PFOS concentrations (Higgins *et al.,* 2005).

Methanogenic Source Reactors. Three bench-scale 3-L methanogenic source reactors were operated to provide innocula for daughter anaerobic serum bottle reactors. These 3-L source reactors were originally seeded with sludge from a full-scale manure-fed anaerobic digester (Haubenschild Farms, Princeton MN). These source reactors were fed a synthetic blend of organic acids, alcohols and glucose (Table 4-1) in minimal media (Shelton and Tiedje, 1984). The organic loading rate was 0.15 g COD/L-day and the HRT was 70 days.

Table 4-1. Synthetic Feed to Source Reactors

Organic Components	% COD
ethanol	40.5
potassium acetate	10.9
sodium acetate	9.4
propionic acid	11.4
glucose	10.8
butyric acid	8.5
valeric acid	2.8
2-propanol	4.0
methanol	1.6

Anaerobic Serum Bottle Reactors. Six sets of serum bottle reactors were operated in triplicate (Table 4-2). Two sets of reactors, called PFOS (60), were exposed to PFOS at approximately 60 μg/kg, two sets of reactors, called PFOS (800), were exposed to PFOS at 800 μg/kg, and two sets of control reactors were not exposed to PFOS. PFOS

(potassium salt of helptadecafluorooctane, Sigma Aldrich, 97%) was weighed gravimetrically and dissolved in methanol and the stock solution was diluted with methanol to achieve desired concentrations. The PFOS-methanol solutions were added to reactors with a gas tight syringe. The mass of PFOS in the reactor at any given time was calculated from Equation 3-1, and the concentration was determined from normalizing the mass of PFOS in the reactor to total solids. The calculated concentrations of PFOS are shown in Figure 4-1. One set of control reactors, one set of PFOS (60) reactors, and one set of PFOS (800) reactors were perturbed over the course of the experiment (Table 4-3) to study the effect of PFOS addition and stress on community structure and function. Perturbations included triclosan amendment to study the effect of PFOS when mixed with other common micropollutants. Triclosan (sold as Irgasan, Sigma Aldrich, ≥ 97% (HPLC)) was measured gravimetrically and dissolved in a methanol solution. The methanol solution was loaded into the serum bottle reactors with a gas tight syringe.

Reactors were constructed from 160-mL serum bottles with 50 mL of active volume. Reactors were originally filled with 20 mL of sludge (12 g VS/L) from the 3-L source reactors and 30 mL of minimal media (Shelton and Tiedje, 1984). Once a week 7 mL of the reactor contents were removed via pipetting with a 1-mL pipet tip that had been severed with a scissors to create a wider opening, and 7 mL minimal media were added along with 0.2 mL of feed; the HRT was 50 days. Each bottle was fed a blend of acetate and glucose (3:2 based on COD) and 0.03 mL of methanol on PFOS feeding days. For control reactors the methanol did not contain PFOS and for exposed reactors the

methanol contained PFOS. The average organic loading rate was 0.19 g COD per L-day. The bottles were purged with N_2 gas on the benchtop, capped with Teflon-lined septa, and crimp-capped with aluminum seals. Bottles were incubated at 37°C resting upright in the dark. A 1.5-mL aliquot of sludge was periodically taken from each reactor for DNA extraction and subsequent microbial analysis.

Table 4-2. Experimental Reactor Sets for Long-Term Exposure Tests

Set	Name	Abbreviation	PFOS
1	Control	C	No
2	Perturbed	CP	No
3	PFOS (60)	PFOS-L	Yes
4	PFOS (60)+Perturbation	PFOS-LP	Yes
5	PFOS (800)	PFOS-H	Yes
6	PFOS (800)+Perturbation	PFOS-HP	Yes

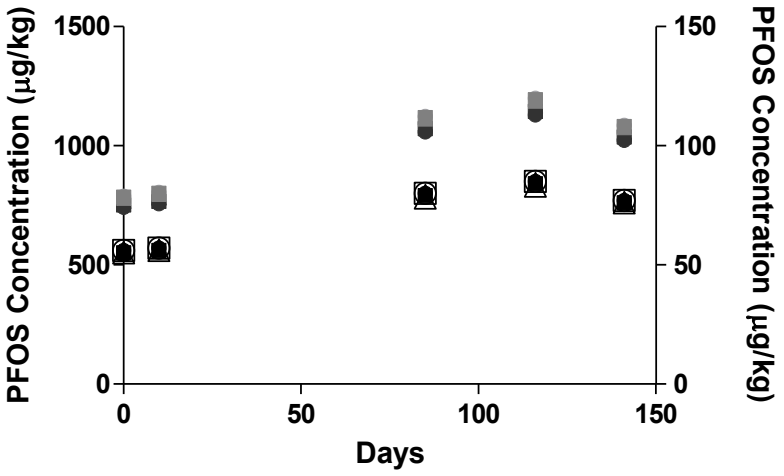


Figure 4-1. Calculated concentrations of PFOS during long-term PFOS exposure tests. The clear open circles, squares and triangles correspond to concentrations in PFOS (60) reactors; the black circles, squares and triangles correspond to concentrations in PFOS (60)+perturbation reactors, and these two sets are plotted on the right axis. The dark grey circles, squares and triangles correspond to concentrations in PFOS (800) reactors; the light grey circles, squares and triangles correspond to concentrations in PFOS (800)+perturbation reactors, and these two sets are plotted on the left axis.

Table 4-3. Perturbations Imposed on Control-Perturbed, PFOS (60)-Perturbed, and PFOS (800)-Perturbed Reactor Sets

Day	Perturbation Name	Perturbation Details
3	Mixing	125 r.p.m., 1 week
10	Ammonia	NH_4Cl (1 g/L as NH_4)
20	Cold Shock	4°C, 72 h
115	Triclosan	20 mg/kg
121	Triclosan	60 mg/kg
131	Triclosan	225 mg/kg

Short-term PFOS Exposure Tests

This experiment was designed to test the hypothesis that short-term exposure to PFOS, even at high concentrations, would not impact methane production or alter the

impacts of triclosan in a PFOS mixture. PFOS concentrations were set to 5x environmental levels in this short-term experiment to test a worst-case environmental scenario. If short-term effects were not observed at these concentrations then it would be assumed that current environmental levels do not have short-term effects. Anaerobic serum bottle batch reactors (160-mL) were initially set-up and fed as described for the long-term exposure tests. After seven days, the bottles were re-fed acetate and glucose. The bottles were amended with micropollutants on Day 0. Micropollutants were weighed gravimentrically and dissolved in methanol. The micropollutant solutions were added with a gas-tight syringe. The concentrations of micropollutants in the reactor were calculated from the mass of micropollutant added divded by the total solids present (Table 4-4). The experiment lasted for 14 days.

Table 4-4. Concentration of Micropollutants in Short-Term Exposure Test

Name	Concentration
Control	NA
Triclosan	230 mg/kg
PFOS	14,000 µg/kg
Triclosan + PFOS	230 mg/kg; 14,000 µg/kg

Impacts of Micropollutants on ATP

These experiments were designed to test the hypothesis that PFOS and triclosan behave as metabolic uncouplers. It was assumed that PFOS and triclosan would be able to penetrate the cell membrane and dissipate the proton motive force, thereby decreasing ATP production.

Nine-day ATP test. Serum bottles (160-mL) were inoculated with 2 mL of manure-digesting biosolids and 50 mL of anaerobic minimal media (Shelton and Teidje, 1984). Bottles were fed 0.67 g COD/L of acetate and 1.33 g COD/L of ethanol so that the total COD loading was 2 g COD/L. The bottles were purged with N_2 gas on the benchtop, capped with Teflon-lined septa, and crimp-capped with aluminum seals. Micropollutants were weighed gravimentrically and dissolved in ethanol. The micropollutant solutions were added with a gas-tight syringe. The concentration of micropollutants in the reactor was calculated from the mass of micropollutant added divded by the total solids present (Table 4-5). Bottles were incubated at 37°C in the dark in a shaking incubator (125 rpm).

Two mL of sample were periodically removed for ATP analysis while the bottle was purged with nitrogen. The aliquot was processed as described in Chung and Neethling (1988). Briefly, the 2 mL aliquot was loaded into 10 mL of boiling 2 mM EDTA in 20 mM Tris buffer for 90 s. Samples were put on ice until cooled and then filtered through a 0.2 μm filter. Samples were frozen until ATP quantification; ATP was quantified with the ATP Bioluminescent Assay Kit (Sigma-Aldrich) as per manufacturer's instructions.

Table 4-5. Micropollutant Concentrations in ATP Experiments

Name	Concentration
Control	40 μL ethanol
Triclosan	50 mg/kg
PFOS	45 μg/kg
Triclosan + PFOS	50 mg/kg + 45 μg/kg
2,4-DNP	8 g/kg (10 mg/L)

Two-hour batch ATP test. Shorter ATP batch experiments were performed (2 hr) to verify that micropollutants did not immediately impact ATP concentrations. These tests were operated in a similar manner as the nine-day ATP experiment, except the feed was limited to ethanol (no glucose added).

Molecular Methods

DNA Extraction. Reactor samples (1.5 mL) were centrifuged (13,200 *g*) for 1.5 min, the supernatant was discarded, and pellets were frozen at -20°C until extraction. Lysis buffer (MP Biomedicals) was added to pellets and cells were lysed with 3 freeze-thaw cycles followed by incubation at 70°C for 90 minutes. The FastDNA Spin Kit (MP Biomedicals, Solon, OH) was used to extract the DNA, which was then stored at -20°C until use.

Automated Ribosomal Intergenic Spacer Analysis (ARISA). Community fingerprints were assessed by ARISA. Primers ITSF (5'-GTC GTA ACA AGG TAG CCG TA-3') and ITSReub (5'-GCC AAG GCA TCC ACC-3') were used to amplify the intergenic spacer region of *Bacteria* (Cardinale *et al.,* 2004). The forward primers were labeled with the phosphoramidite dye HEX. The PCR mixture (25 uL) contained 1x PCR buffer (Promega, Madison, WI), 20 nmol deoxynucleoside triphosphates, 25 pmol forward and reverse primers, 1.25 units of GoTaq DNA polymerase (Promega) and approximately 1 ng of genomic DNA. PCR was performed in a DNA Engine Thermal Cycler (Biorad, Hercules, CA). The PCR protocol was as follows: 3 minutes of initial denaturation at 94°C, 35 cycles of 94°C for 45 seconds, 55°C for 1 minute, and 72°C for

2 minutes, and a final extension of 7 minutes at 72°C. PCR products were separated by capillary electrophoresis with an ABI 3130xl capillary instrument (Applied Biosystems Inc, Foster City, CA). Peak areas were analyzed with PeakScanner Software v 1.0. Fragment lengths less than 156 basepairs were removed from subsequent analyses to eliminate primer dimers (Cardinale *et al.,* 2004). Fragments greater than 1000 basepairs were also eliminated because the maximum size standard was 1000 basepairs. Only peaks that accounted for ≥ 0.5% of the total area in a sample were used for further analysis. Fragments were binned using the R code "interactive binner" (Ramette, 2009). The window size was two base pairs and the shifting window was 0.1 basepairs. The percent contribution of one OTU to the total community (area of one OTU divided by area of all OTUs) was averaged across replicates, and this average percent contribution was used for statistical analysis.

Analytical Methods

Gas production was measured by injecting a wetted glass syringe into the serum bottle reactors and measuring the displacement volume. Methane was quantified on a gas chromatograph (GC) equipped with a thermal conductivity detector (TCD). The pH was measured on an Orion pH meter (8103BNUWP). Total solids were quantified gravimetrically by drying samples overnight in a 105°C oven; volatile solids were determined by quantifying the mass that volatilized upon heating the dried sample for two hours in a 550°C furnace. Total and volatile suspended solids were quantified by passing a known volume of sample through a glass fiber filter (Millipore AP4004700)

and drying the filter in a 105°C oven (total suspended solids) and then in a 550°C furnace (volatile suspended solids).

Statistical Analysis

Nonmetric multidimensional scaling (nMDS) was used to analyze the ARISA data. The *vegan* package in R was used for nMDS analysis. Dissimilarity between samples corresponds to distance between samples. ANOVA and Student's t-tests were performed using Graphpad Prism v. 5.04 (Graphpad Software, Inc.; La Jolla, CA). A *p*-value of ≤ 0.05 was considered significant.

4.3 Results and Discussion

Long-term PFOS Exposure Tests

At 800 μg/kg PFOS correlated to increased methane production and shifted community structure during the long-term (140 day) exposure experiment when reactors were also perturbed as described in Table 4-3. At 800 μg/kg PFOS concentrations, PFOS increased methane production in perturbed reactors by 51% relative to control reactors (Figure 4-2; t-test on control vs high-PFOS $p = 0.0207$; t-test on control vs low-PFOS $p = 0.9360$; t-test on low-PFOS vs high-PFOS $p = 0.0353$). At these higher, but still environmentally relevant concentrations (Higgins *et al.,* 2005), PFOS could have been exerting subtle uncoupling impacts, as observed previously with fluorinated surfactants (Starkov and Wallace, 2002). The mixing perturbation occurred at Day 3 which could have increased mass transfer of PFOS into sludge flocs and cells and exacerbated

uncoupling impacts. The ARISA profiles revealed that PFOS had no impact on Bacterial community structure through the first 50 days, but by Day 115 the higher levels of PFOS had shifted the community profile away from the control, and this shift persisted through Day 140 at the end of the experiment (Figure 4-3).

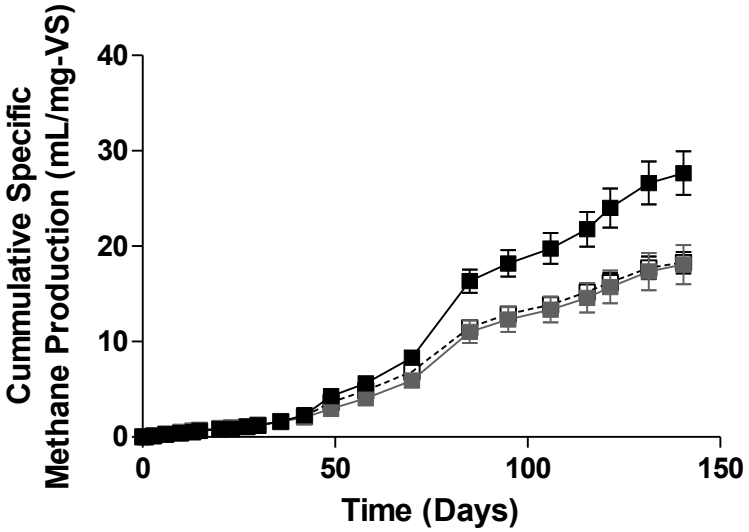


Figure 4-2. Impact of PFOS on methane production in perturbed reactors in long-term 140 day exposure experiment. Error bars represent standard error of the mean, n = 3. Control, PFOS(60), and PFOS(800) reactors are depicted by open, grey, and black squares, respectively. Data prior to Day 84 were normalized to total volatile solids; data after Day 84 were normalized to total volatile suspended solids.

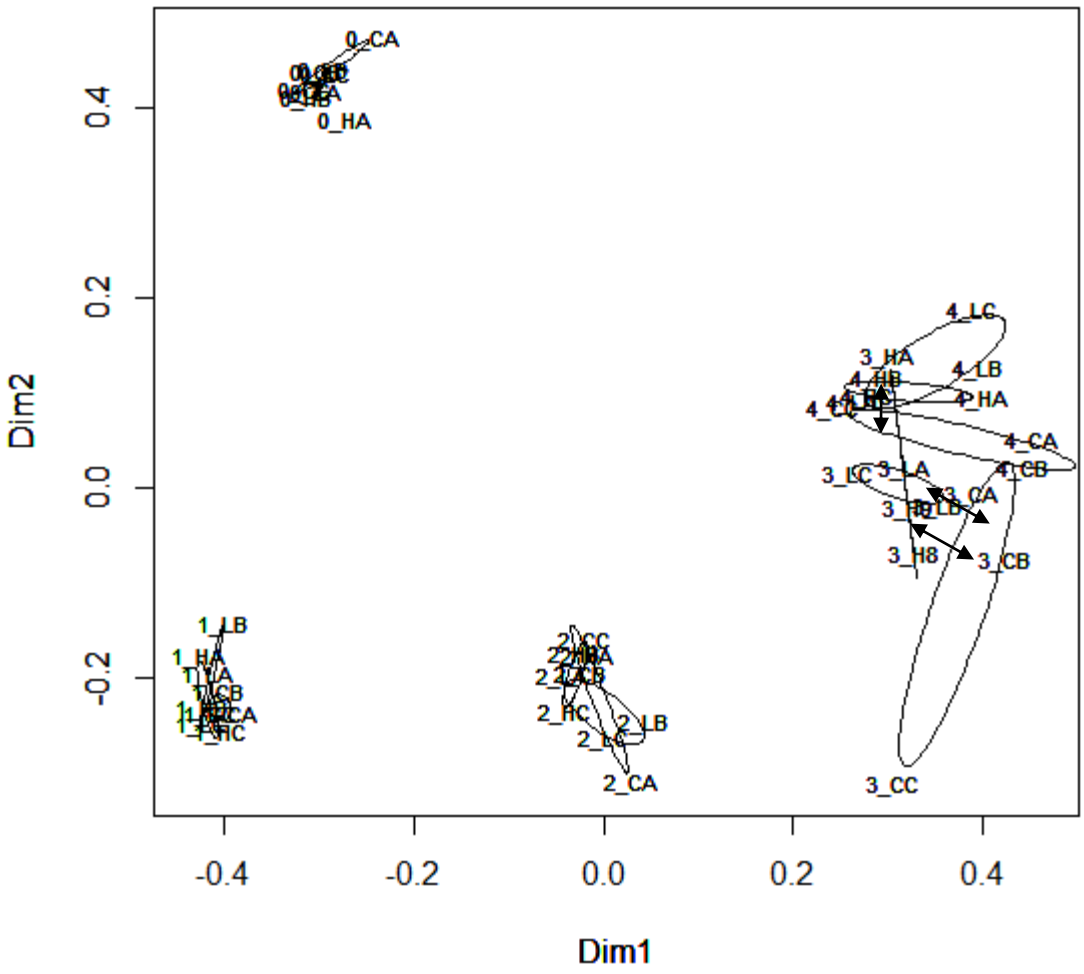


Figure 4-3. nMDS on *Bacteria* in perturbed reactors. C = control perturbed; L = PFOS (60)-perturbed; H = PFOS (800)-perturbed. 0 = Day 0, 1 = Day 15, 2= Day 50, 3 = Day 115, 4 = Day 140. Differences between communities at a given time point are highlighted by black arrows.

Surfactants exhibit subtle, broad-spectrum inhibitory effects on *Bacteria*. Effects of the surfactant nonylphenol on *Bacteria* are more widely studied than effects of PFOS, and nonylphenol has been shown to inhibit *E. coli, S. aereus*, and nitrifiers (Okai *et al.,* 2000; Okai *et al.,* 2004; Stasinakis *et al.,* 2008). Both compounds, however, have been

linked to increases in membrane permeability (Wu and Kuzmenko, 2003; Starkov and Wallace, 2002). An increase in membrane permeability would diminish the proton motive force (a.k.a. increasing the uncoupling effect), and force microorganisms to consume more substrate to maintain ATP levels. The increase in methane production observed herein indicates that PFOS may be acting as an uncoupler to *Bacteria*, causing leakage in membranes and faster turnover of substrate. These results are different than those of Hollingsworth *et al.,* (2005) who found that PFOS had no impact on hydrogenotrophic methanogens. Other than exposure time, another difference between the two studies is that Hollingsworth and coworkers fed only H_2/CO_2, and thus the impacts of PFOS on *Bacteria* and acetoclastic methanogens were not tested. Methanogenesis is a syntrophic, sequential process. It is feasible that PFOS uncouples *Bacteria,* causing faster turnover of substrate and increased methane production. Alternatively, acetoclasitic methanogens, which are thought to account for approximately two thirds of methane production during anaerobic digestion (Tchobanoglous *et al.,* 2003), may be impacted by PFOS. As opposed to Hollingsworth and coworkers' results, the results presented herein demonstrate that over exposure times >50 days PFOS can alter the function and structure of a methanogenic community at environmentally relevant levels if other perturbations, which are common in both engineered and natural systems, occur.

PFOS also appeared to exacerbate the impacts of triclosan on methane production and community structure. When communities were amended with PFOS, triclosan

addition had greater impacts on the methane production rate relative to control communities not amended with PFOS (Figure 4-4). Following triclosan amendment, methane production rates increased more drastically in reactors also amended with PFOS (Table 4-3). Subsequently, methane production declined more rapidly in reactors amended with both PFOS and triclosan. These results suggest that PFOS augmented the potential uncoupling effects of triclosan (Newton *et al.,* 2005). These results are especially important because PFOS and triclosan are both found in biosolids (Higgins *et al.,* 2005; McClellan and Halden, 2010). Nevertheless, because of the limited number of data points, more work is needed to corroborate these results. In addition to functional impacts, triclosan also had bigger impacts on communities amended with PFOS, *i.e.,* more distance existed between triclosan and non-triclosan communities when PFOS was present (Figure 4-5).

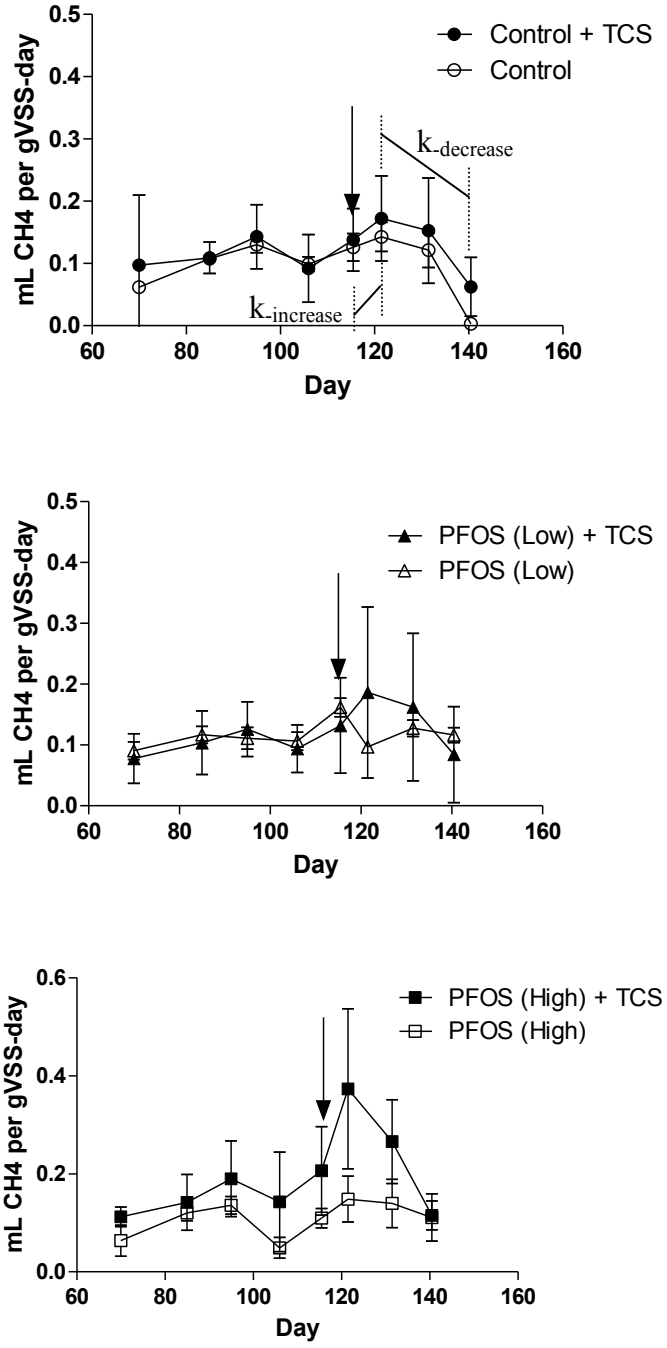
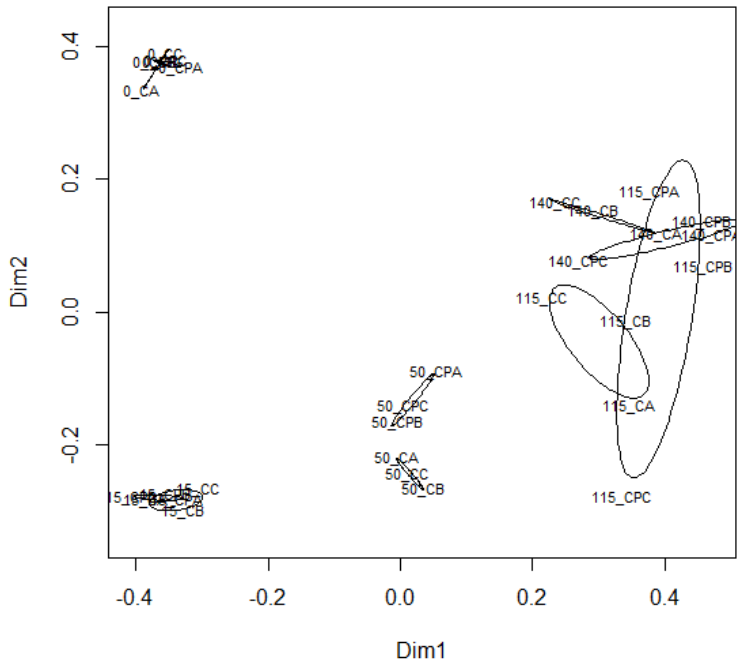


Figure 4-4. Impact of triclosan on methane production rate. Arrows denote time of triclosan addition. Error bars are 95% confidence intervals, n = 3.

Table 4-6. Average k-values from Triplicate Reactors for Increase and Decrease in Methane Rates Following Triclosan Amendment. P-value is Result of Student's t-Test between Perturbed and Unperturbed Reactor Sets for Each PFOS Amendment Level (Control, 60, 800).

	Control Pert*	Control	p-value	PFOS (Low)Pert	PFOS (Low)	p-value	PFOS (High)Pert	PFOS (High)	p-value
$k_{increase}$	0.006	0.003	0.236	0.009	-0.011	**0.010**	0.028	0.006	**0.016**
$k_{decrease}$	-0.006	-0.007	0.442	-0.005	0.001	**0.028**	-0.014	-0.002	**0.003**

*perturbation



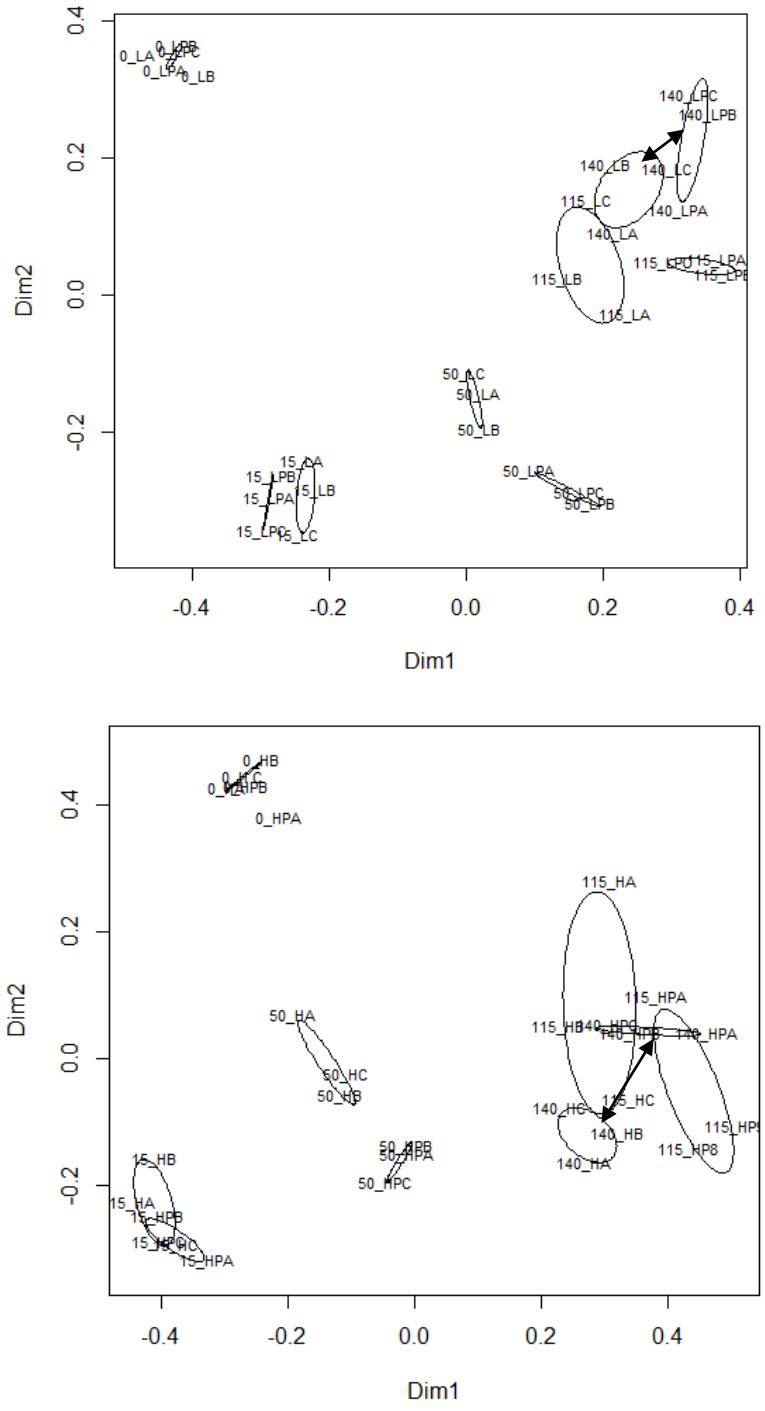


Figure 4-5 C vs CP (top), L vs LP (middle), H vs HP (bottom). Black arrows indicate dissimilarity between triplicate reactors at Day 140. C = 0 µg/kg, L = 60 µg/kg, H = 800 µg/kg.

Interestingly, other studies have also shown that mixtures containing PFOS alter, both synergistically and antagonistically, the effects of other micropollutants. PFOS exposure at 30 mg/L for 72 h decreased the inhibition of atrazine and diuron to algae. In contrast, it increased the inhibitory effects of pentachlorophenol on algae (Liu *et al.,* 2009). When PFOS was mixed with either triclosan or trichlorophenol, the inhibition of algae decreased, but when all three compounds were combined, inhibition increased (Boltes *et al.,* 2012). Thus, evaluating toxicity by adding the effects of individual compounds could substantially underestimate or overestimate the toxicity of mixtures as seen in the environment. Indeed, the results presented herein demonstrated that PFOS at 0.02 mg/L (an environmentally relevant concentration in biosolids, equivalent to 800 μg/kg) following 115 days of exposure augmented the impacts of triclosan by generating faster methane production rates and causing larger shifts in community structure. Therefore, not only does PFOS directly impact methanogenic communities (Figures 4-2 and 4-3), but the role PFOS plays in mixtures may be equally important for appropriating risk.

Short-term PFOS Exposure Tests

While PFOS had functional and structural effects in long-term exposure experiments, no effects were observed in the short-term 14-day exposure experiments, even with much higher levels of PFOS added. PFOS did not alter methane production during the 14-day experiment (Figure 4-6; ANOVA $p = 0.1967$). Similarly, PFOS did not

alter methane production during the first 14 days of the long-term 140-day exposure experiment (Appendix B, Figure B5; ANOVA $p = 0.1233$). Neither did PFOS augment the impacts of triclosan in these short-term experiments, again indicating that short-term impacts of PFOS are different than long-term impacts. PFOS also did not impact ATP production during 2 h and 9 day experiments, which corroborates the null impacts observed during the short-term experiment (Appendix B, Figures B9-10). These results indicate that more time is likely required for PFOS to transport into flocs or cells where it can have an impact on microorganisms. Similar to the trends reported in this chapter, an alcohol alkoxylate surfactant at 200 mg/L had no effect on methane production after 2 days, but after 9 days the compound was inhibitory (Van Ginkle *et al.,* 2007). Thus, in the 24 h exposure study by Hollingsworth *et al*. (2005)*,* different results may have been observed had their experiments been longer-term.

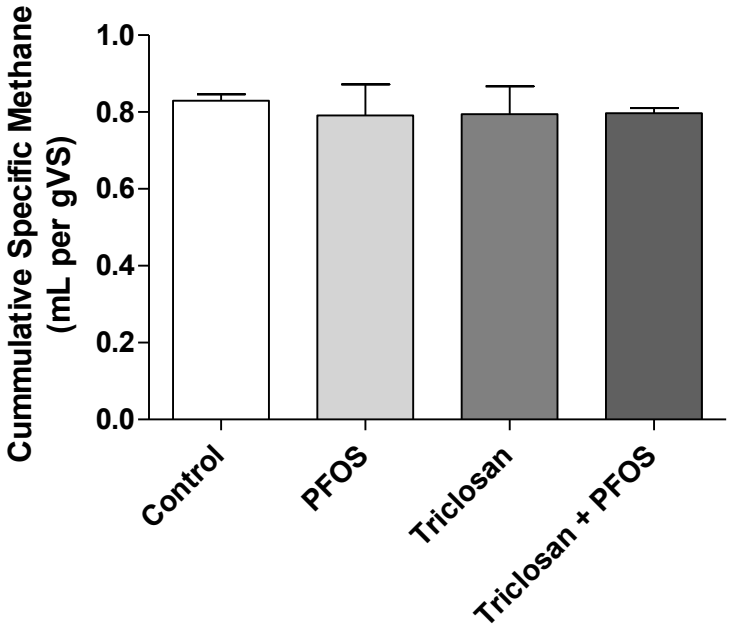


Figure 4-6 Impact of PFOS, triclosan, and PFOS-triclosan mixture on methane production during short-term 14-day exposure experiment. Error bars are 95% confidence intervals, n=3.

4.4 Conclusions

In summary, exposure time, in addition to concentration and whether co-contaminants are present, matter when assessing the risk and impacts of PFOS. Overall, the work in this chapter demonstrated that PFOS has subtle but definitive impacts on methanogenic community function and structure. Exposure time appears to greatly influence the impacts of PFOS, as it does with other surfactants (Van Ginkle *et al.,* 2008) and chlorinated chemicals (Patel *et al.,* 1991). Exposure time also influences the impacts of mixtures. When triclosan was added after 115 days of PFOS exposure, methane production and community structure were impacted, but function was not altered when

triclosan and PFOS were added simultaneously in short-term (14 day) experiments. Other research has demonstrated that PFOS can alter the toxicity of mixtures in algal cultures (Liu *et al.,* 2009; Boltes *et al.,* 2012). Tests designed to evaluate environmental risk must therefore account for mixtures and incorporate an element of exposure time if they are to appropriately evaluate chemical risk. While it is impossible to test all chemicals in every possible mixture for prolonged periods of time, it is clear that testing the effects of individual compounds on one particular microorganism, *e.g., E. coli*, does little to establish risk. Additionally, the duration of the test should reflect the predicted fate of a chemical, in addition to the time that a given chemical will reside in a particular environment. For example, effects from a hydrophobic chemical that will reside in sediments should be assessed after a much greater exposure time than those effects from a hydrophilic chemical that will degrade quickly in river water. Without a proper understanding of the long-term effects of micropollutants, alone and in mixtures, we could be stressing environments far beyond what has been predicted in laboratories. If proper testing protocols are not developed, then new, potentially harmful chemicals will be released without appropriate risk assessment.

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Chapter 5 - Summary

The research presented in this dissertation is summarized below. Implications of this research and recommendations for future research are also included.

- The results in Chapter 2 revealed that an emerging solids wastewater treatment process, thermal-hydrolysis coupled to mesophilic anaerobic digestion (TH-MAD), actually hampered the biodegradation of nonylphenol ethoxylates compared to conventional MAD. This result could have occurred from a decrease in bioavailability. In future research, the fate of nonylphenol ethoxylates in the solid, colloidal, and dissolved phases, before and after thermal hydrolysis, should be quantified to determine precisely how thermal hydrolysis influences bioavailability of nonylphenol ethoxylates. More broadly, this research demonstrated that anaerobic treatment does not readily remove the sum of nonylphenol ethoxylates + nonylphenol, regardless of pretreatment, and more costly aerobic treatment is required to reduce the sum of nonylphenol ethoxylates + nonylphenol. These results implicate that source control is more efficient at removing micropollutants than expensive treatment options, and source control may be necessary to protect environmental health.

- In Chapter 3, research was performed to understand how micropollutants that are specifically designed to inhibit *Bacteria*, specifically triclosan, impact methanogenic microbial communities. Communities that were not previously

exposed to triclosan were able to adapt and maintain function when amended with environmental levels of triclosan. When communities that were previously exposed to triclosan were amended with triclosan at 4x current environmental levels the community structure shifted and methane production was inhibited by 50%. While this work showed that triclosan does indeed impact methanogenic communities, an extended experiment at these 4x levels should be performed to determine if function is able to rebound back to 100%, if it is perpetually hindered at 50%, or if complete failure eventually occurs. Future research should also include an experiment that monitors functional and molecular responses to track adaptation during an increase in triclosan loading from current levels to 4x levels. Illumina sequencing could be performed to understand which community members are lost, and in particular, if crucial syntrophic members are lost. This research showed that one prevalent biologically active micropollutant impacts methanogenic communities. Real-world systems, however, are chronically exposed to mixtures of micropollutants. *Bacteria* will adapt to low levels of stress caused by these micropollutants, but as evidenced by these results, a tipping point exists where *Bacteria* cannot adapt quickly enough to maintain function. Indeed, several micropollutants are designed to attack *Bacteria*. Therefore micropollutants will either cause systems to collapse or force *Bacteria* to develop resistance to an array of chemicals, including pharmaceuticals and antimicrobials.

- The work in Chapter 3 also demonstrated that triclosan can enrich for a multidrug efflux pump (MexAB-OprM as quantified by the *mexB* gene) in a mixed-culture anaerobic community. It is possible that prevalent usage of triclosan could be exerting pressure to proliferate the spread of antibiotic resistance, and thus more research is required to quantify this risk. Specifically, the rate at which *mexB* increases and the lifespan, *i.e.,* how long *mexB* is sustained in systems, should be quantified. The response of *mexB* to triclosan loading should be quantified to understand if continuous loading of triclosan is required for permanent effects, or if high pulse events alone result in sustained increases in *mexB.* Also, the microbial communities, *e.g.,* from municipal digesters, agricultural digesters, or sediments, that sustain *mexB* need to be identified. Lastly, the impact of triclosan on antibiotic resistance in humans following exposure through oral products such as toothpaste should be researched. Though triclosan is not a prescription drug, it should be viewed with similar caution when evaluating its risk in the environment as it induces biological responses in microbial communities.

- The work in Chapter 4 demonstrated that perfluorooctane sulfonate (PFOS) directly impacts function and structure of methanogenic communities. These impacts were only evident in long-term 140 day experiments, however, and not in 14 day experiments. PFOS also augmented the impacts of triclosan, but again only in long-term experiments. Follow-up research should be performed to study

how PFOS impacts triclosan, *i.e.* determine if PFOS influences bioavailability and uptake of triclosan. Moving forward, the relevance of exposure time must also be taken into account when appropriating risk of micropollutants. A central database where researchers can upload exposure results would be beneficial so that we can build models and predict how chemicals alone and in mixtures will chronically impact biological systems.

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Appendix A - Supporting Information for Chapter 3

Background triclosan levels

The effluent from the manure digester at Haubenschild's Farm was used to seed lab-scale anaerobic digesters in Part 1 and served as the initial unexposed source of triclosan (Table A1). Concentrations in Table A1 were detected with LC-MS.

Table A1. Background Triclosan Levels in Manure Digester Effluent from Farm

Sample	TCS in aliquot (g/L)	mg/kg	TCS Recovery
FarmDig-A	0.0008	0.0048	na
FarmDig-B	0.0011	0.0054	na
FarmDig-Spike(low)	0.0174	0.1145	1.73%
FarmDig-Spike(high)	2.2008	13.208	29.1%
Blank	0.0009	0.0051	na

Triclosan levels in the unexposed manure sludge taken from a lab-scale manure digester (part three) were detected below the limit of quantification (2.18×10^{-5} g/L in final sample extract) and below the levels found in the blank (Table A2). This manure source is considered to be unexposed. The previously-exposed source sludge taken from lab-scale municipal-sludge digesting reactor (part two) had an average triclosan concentration of 3.96 (±0.83) mg/kg. Triclosan concentrations were detected on the LC-MS-MS described in the methods of Chapter 2.

Table A2. Background triclosan levels in unexposed and previously-exposed sources

Sample	TCS in aliquot g/L	mg/kg	13C-Recovery	RSD	AVG (mg/kg)
unexposed-A	1.81E-06	0.02	28%	26%	**0.02**
unexposed-B	3.03E-06	0.03	26%		
unexposed-C	1.66E-06	0.02	37%		
exposed-A	3.90E-04	3.31	21%	21%	**3.96**
exposed-B	4.39E-04	3.68	23%		
exposed-C	4.70E-04	4.89	30%		
sand blank	4.17E-06	0.0004	19%		
LOQ	**2.18E-05**	NA	NA		

Part I Supplemental Results

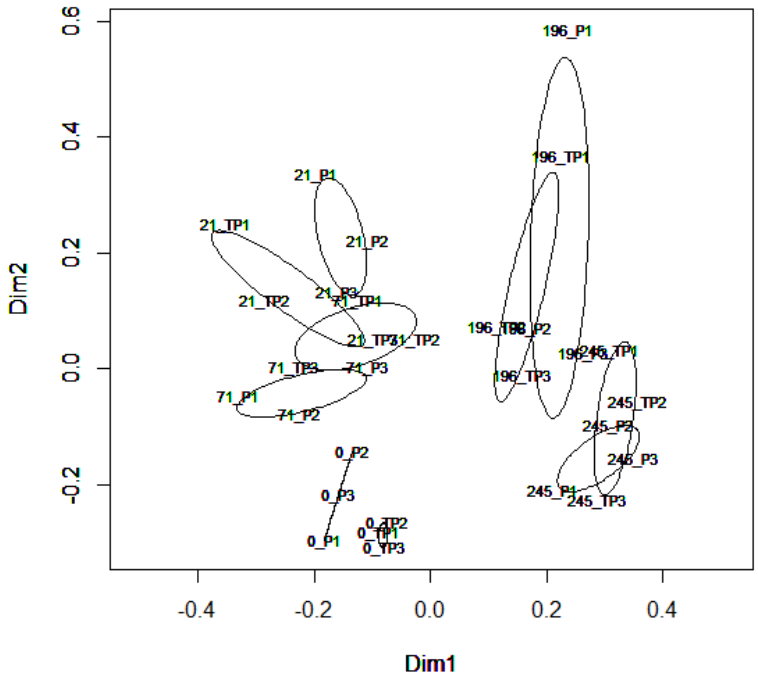
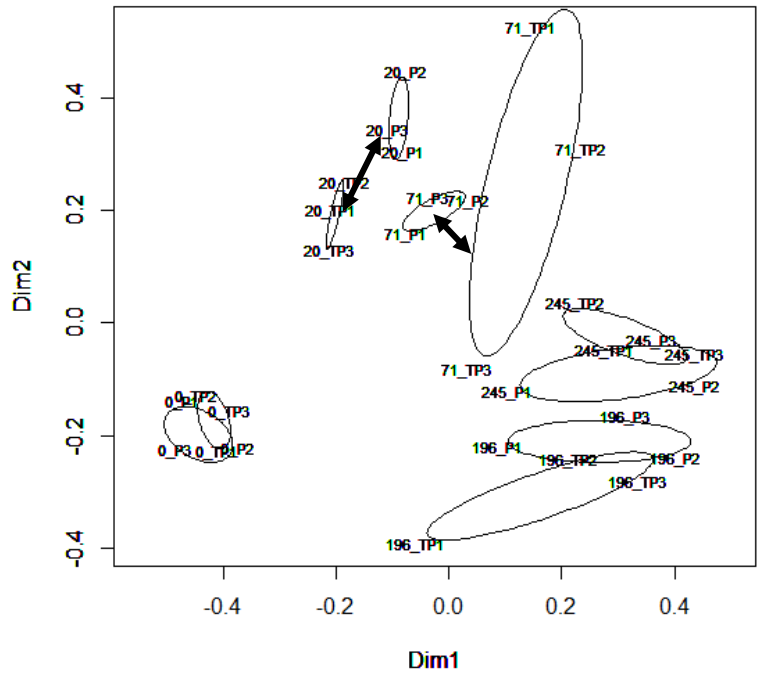


Figure A1. nMDS on Archaeal communities exposed to environmentally relevant levels of triclosan in unexposed perturbed communities.



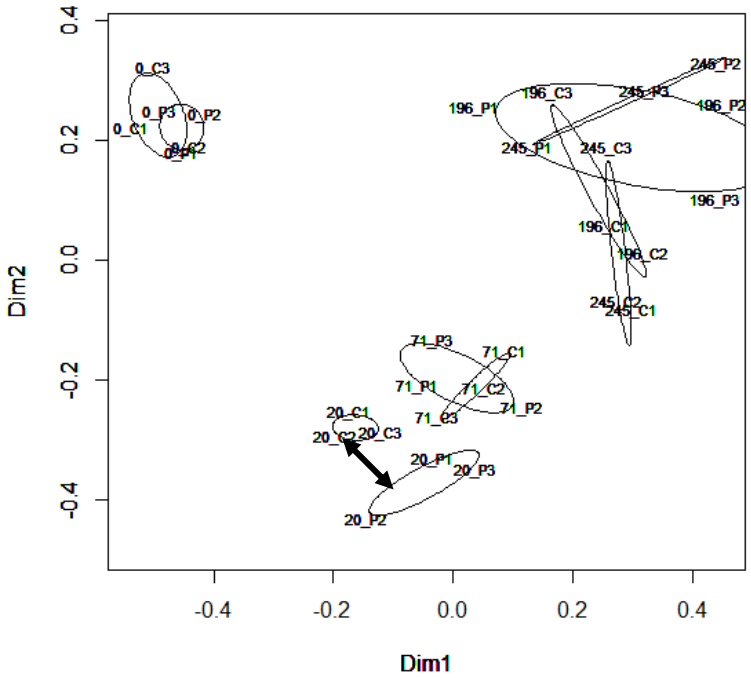
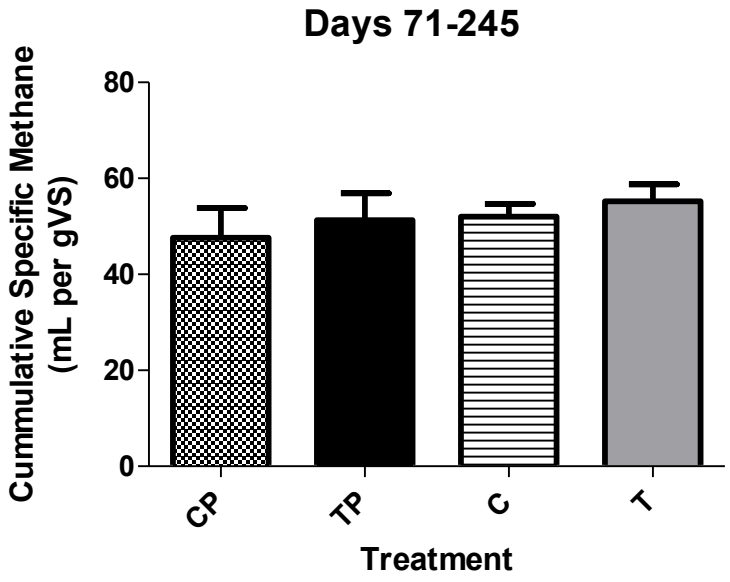


Figure A2. The impact of perturbations with and without triclosan on *Bacteria*. T vs TP (top) and C vs CP (bottom). Differences between communities at a given time point are highlighted by black arrows.



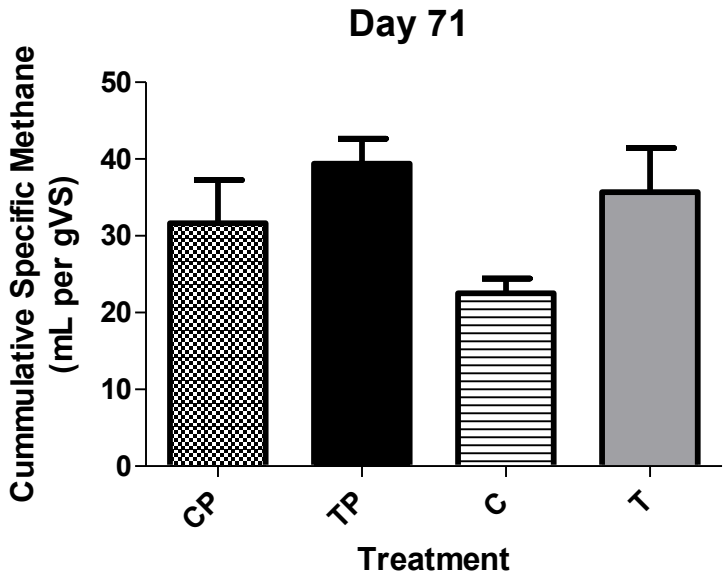
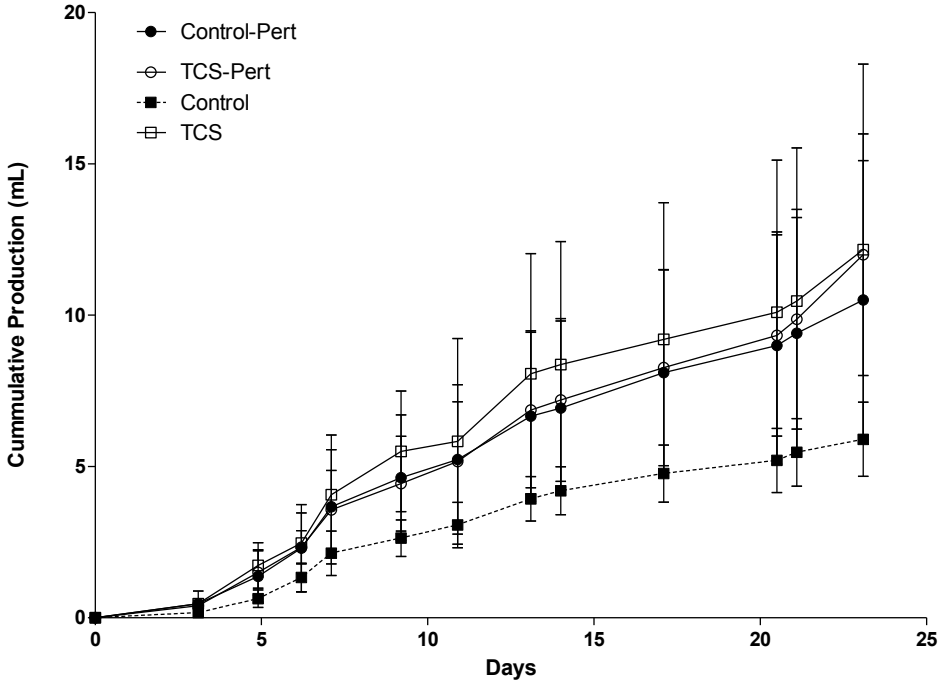
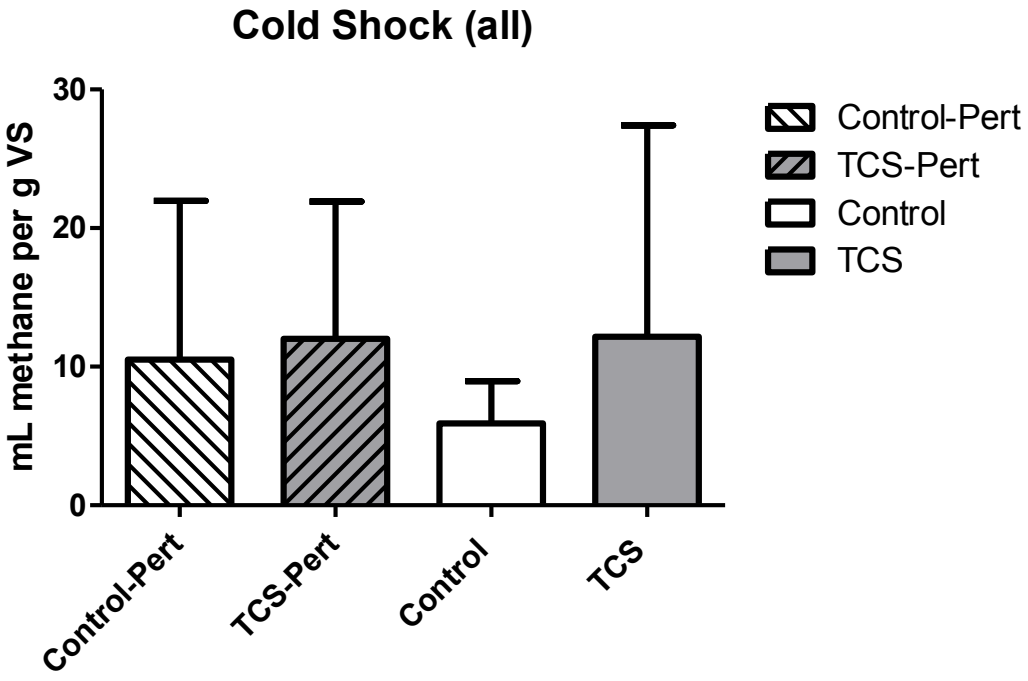
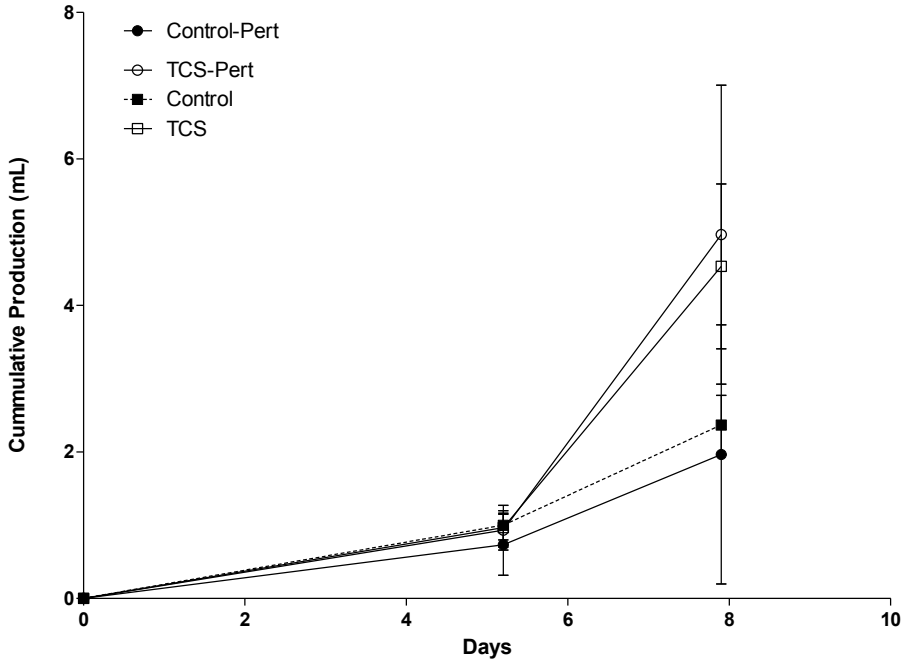
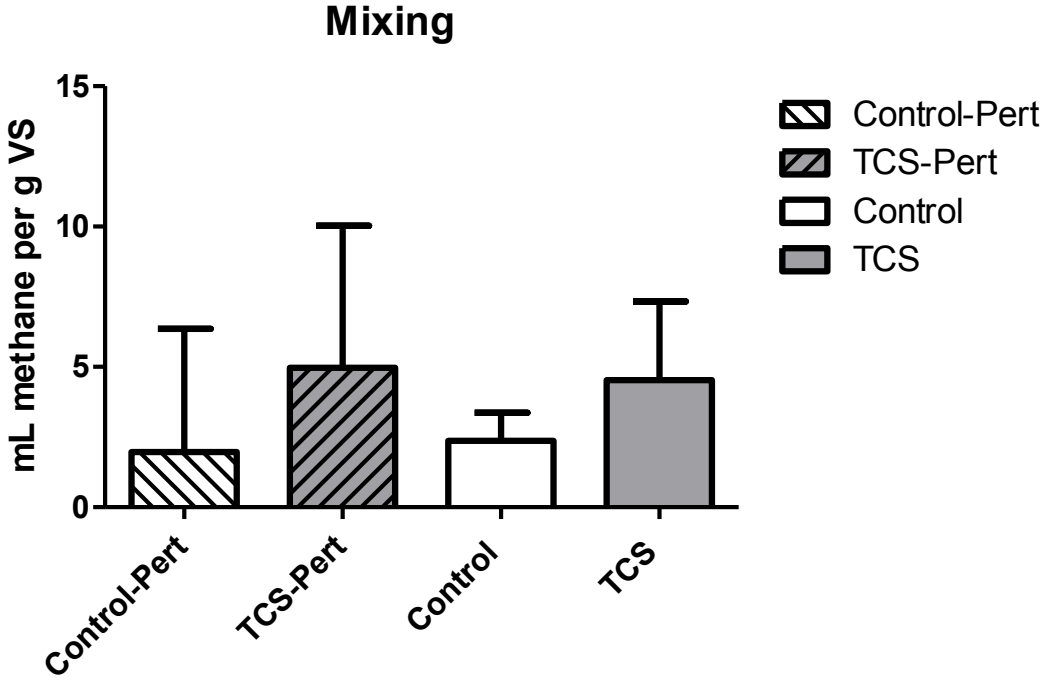


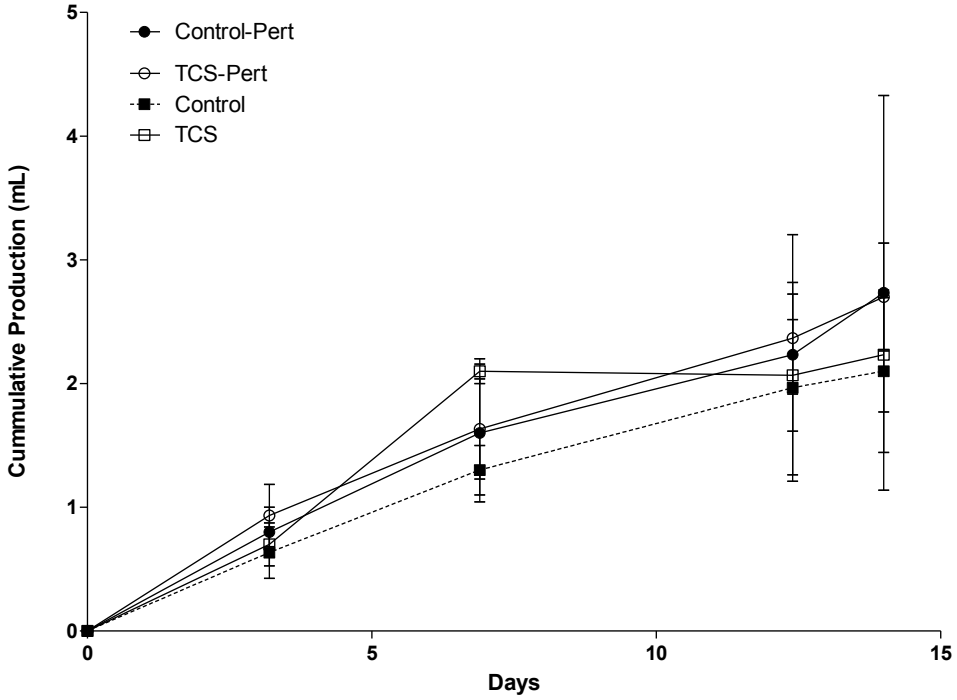
Figure A3. Impact of triclosan on cumulative specific methane production. Error bars are SEM, n = 3. ANOVA p-value = 0.1196 (Day 71); ANOVA p-value = 0.7366 (Day 71-245).

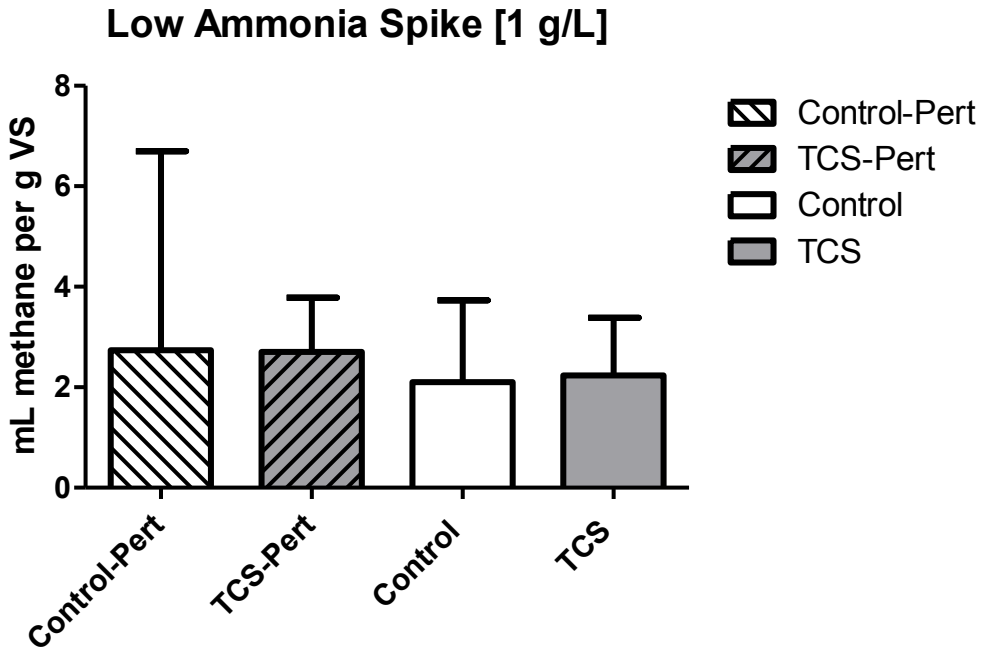

Control-Pert
TCS-Pert
Control
TCS
Cummulative Production (mL)
Days
0
5
10
15
20
25

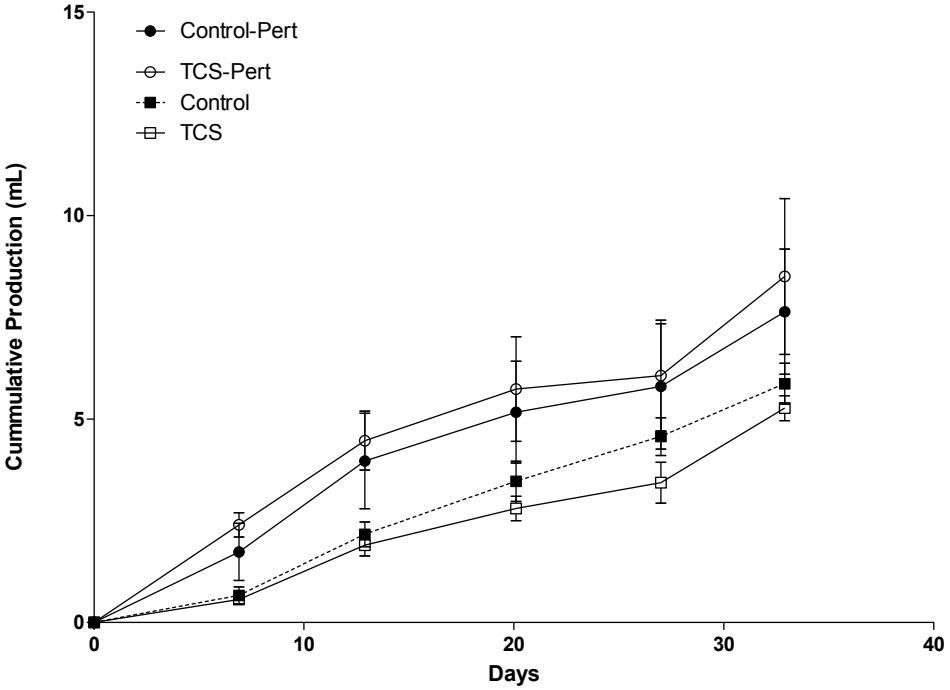

Cold Shock (all)
mL methane per g VS
0
10
20
30
Control-Pert
TCS-Pert
Control
TCS

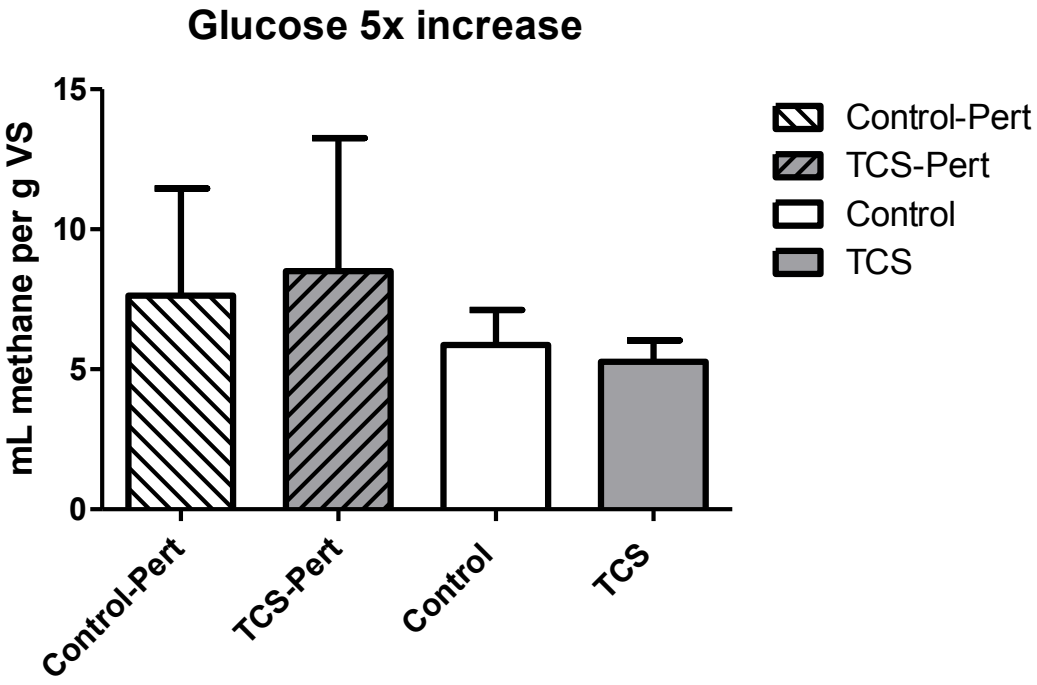

Control-Pert
TCS-Pert
Control
TCS
Cummulative Production (mL)
Days
0
2
4
6
8
10

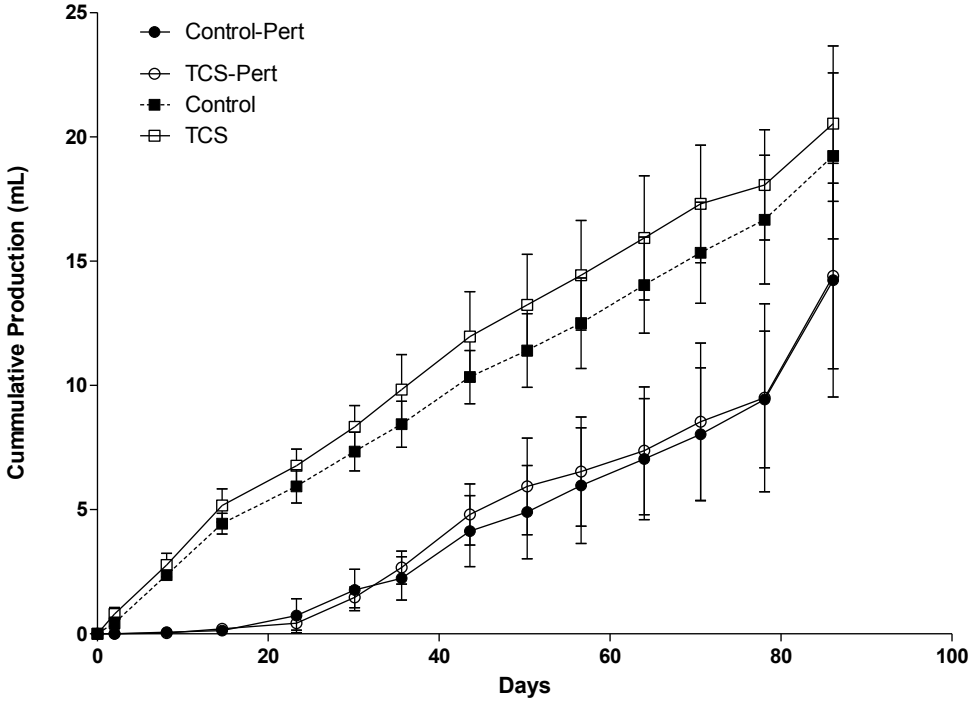

Mixing
mL methane per g VS
0
5
10
15
Control-Pert
TCS-Pert
Control
TCS

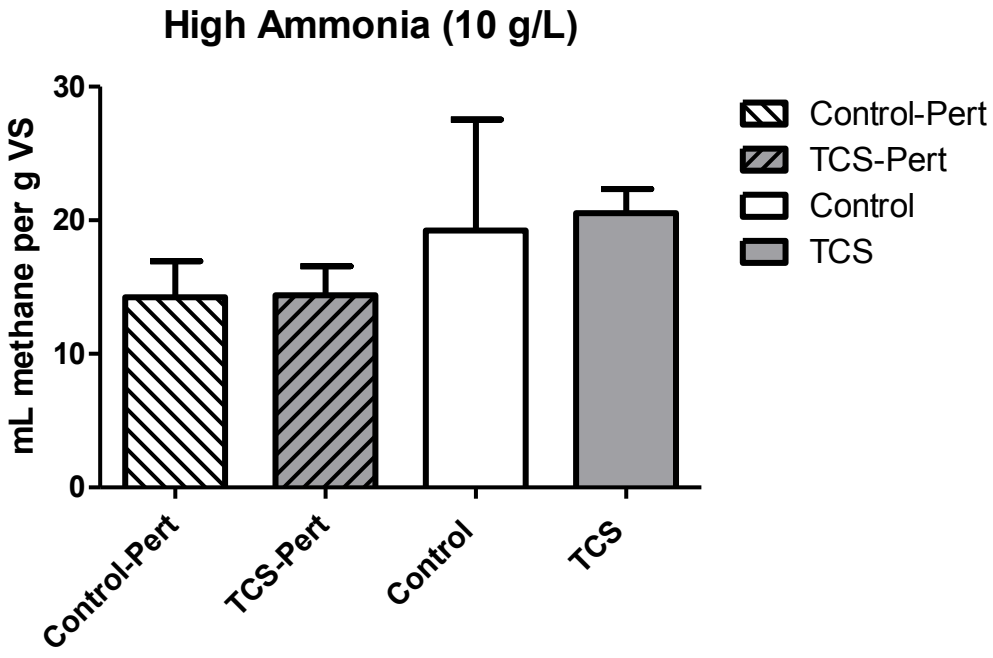

Control-Pert
TCS-Pert
Control
TCS
Cummulative Production (mL)
Days
0
1
2
3
4
5
0
5
10
15

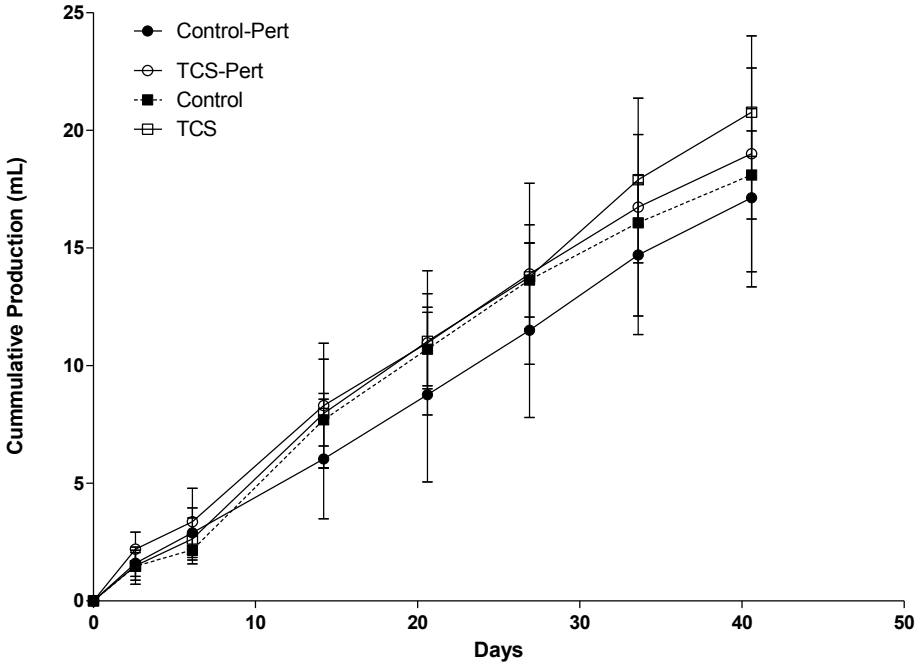

Low Ammonia Spike [1 g/L]
mL methane per g VS
0
2
4
6
8
Control-Pert
TCS-Pert
Control
TCS
Control-Pert
TCS-Pert
Control
TCS


Control-Pert
TCS-Pert
Control
TCS
Cummulative Production (mL)
Days
0
5
10
15
0
10
20
30
40

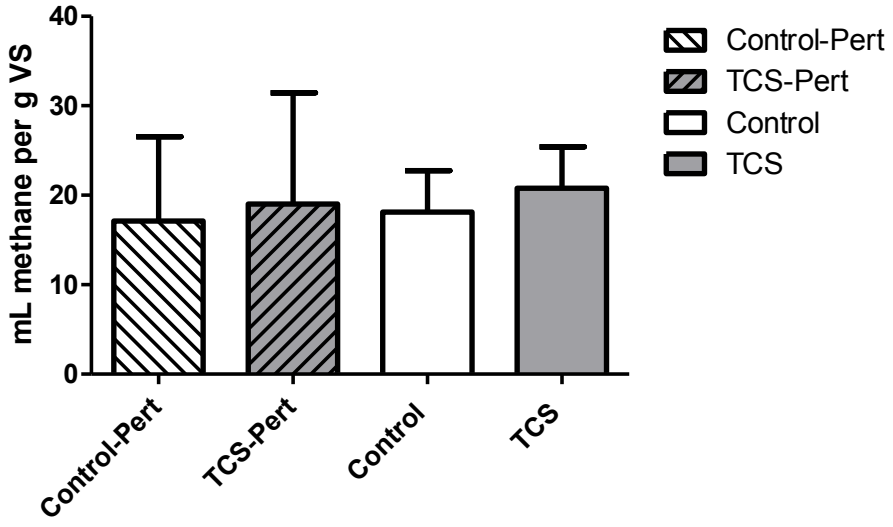

Glucose 5x increase
mL methane per g VS
Control-Pert
TCS-Pert
Control
TCS
0
5
10
15


Control-Pert
TCS-Pert
Control
TCS
Cummulative Production (mL)
Days
0
5
10
15
20
25
0
20
40
60
80
100


High Ammonia (10 g/L)
mL methane per g VS
0
10
20
30
Control-Pert
TCS-Pert
Control
TCS



Mixed Acid (Unperturbed); HOCl (Perturbed)



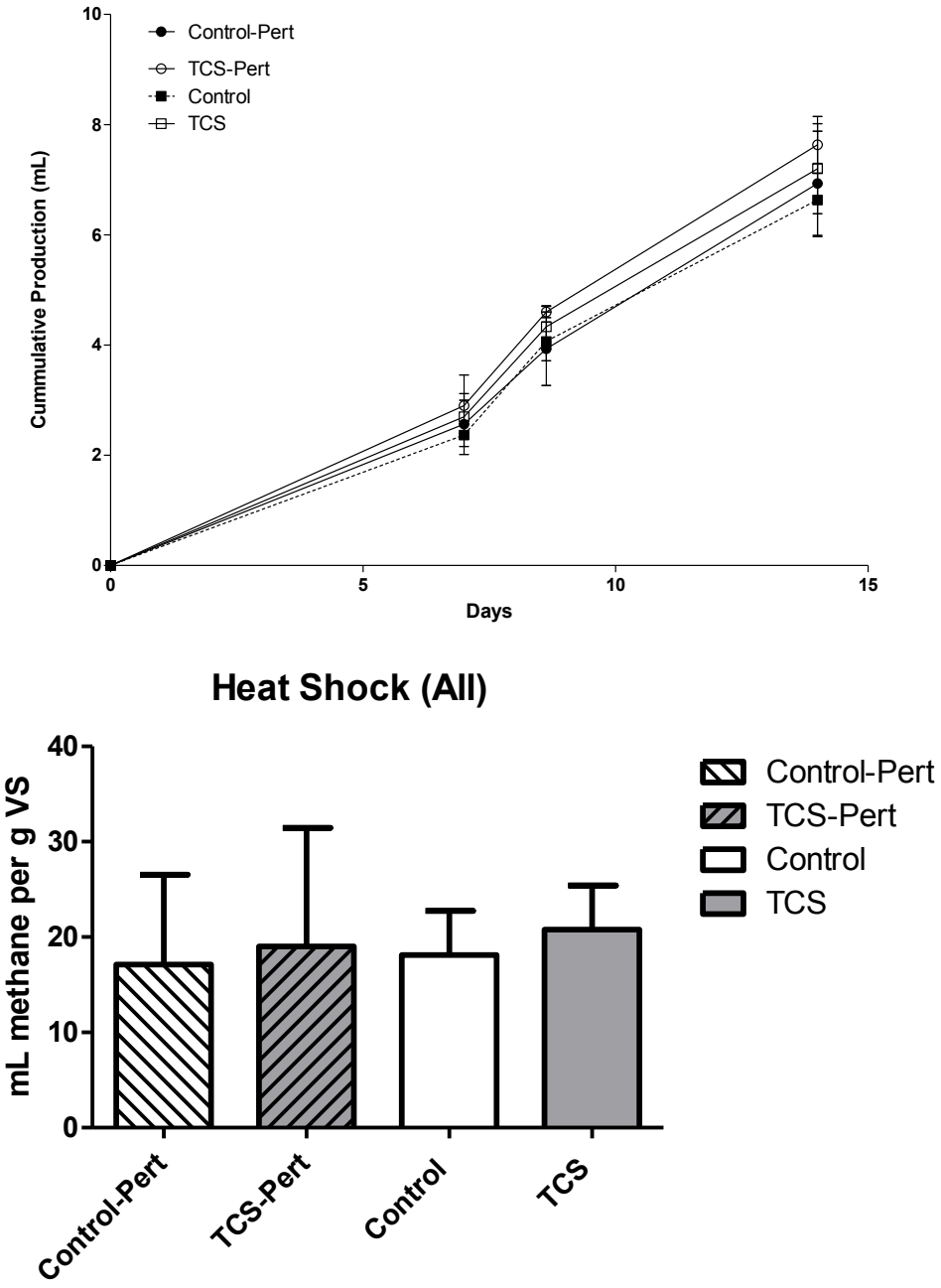


Figure A4. Impact of perturbations on methane production. Methane production over time following perturbation are shown on top; error bars are standard deviation, n=3. Cumulative methane production is shown in bar charts below; error bars are 95% confidence intervals, n=3.

Table A3. Mixed Acid Feed for Testing Performance of Unperturbed Reactors; 2 gCOD/L Added

Organic Components	gCOD/L	% COD influent
propionic acid	811	50
butyric acid	289	18
isobutyric acid	292	18
valeric acid	98	6
isovaleric acid	99	6
caproic acid	28	2

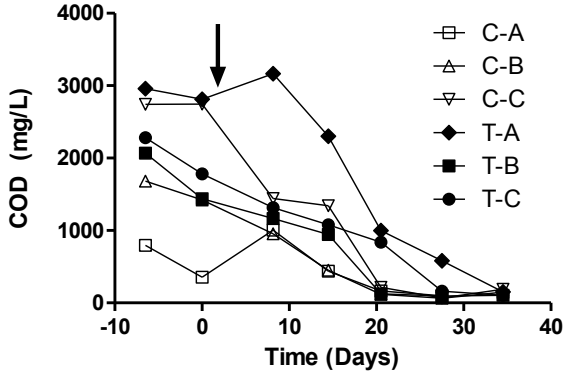


Figure A5. Soluble COD in unpertubed reactors following a spiked of mixed organic acids after sampling at time = 0 days.

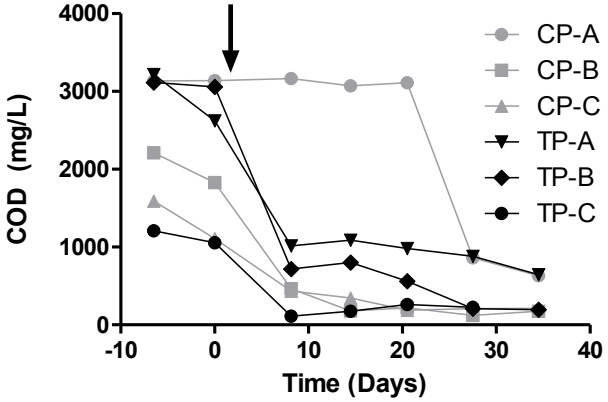


Figure A6. Soluble COD in perturbed reactors following a spike in HOCl after sampling at time = 0 days. COD was quantified in filtered (0.45 μm) supernatant from centrifuged samples using High Range Hach Vials (2125925) according to the manufacturer's instructions.

Table A4. pH in Previously Unexposed Serum Bottle Reactors

Day	CP-A	CP-B	CP-C	TP-A	TP-B	TP-C	C-A	C-B	C-C	T-A	T-B	T-C
7	6.7	6.81	6.77	6.85	6.72	6.7	6.72	6.75	6.65	6.84	6.72	6.83
14	7.03	7.3	7.19	7.29	7.04	7.06	7.05	7.08	6.97	7.04	7.07	6.94
29	7.18	7.39	7.36	7.71	7.32	7.33	7.23	7.2	7.19	7.17	7.36	7.4
35	7.18	7.64	7.75	8.04	7.49	7.69	7.35	7.36	7.08	7.13	7.55	7.55
42	7.29	7.88	8.45	8.67	8.34	8.44	7.72	7.81	7.1	7.29	8.2	7.98
64	7.79	8.02	8.15	8.07	7.49	7.97	7.78	7.76	7.26	7.8	8.11	
69	7.94	8.14	8.11	8.03	8.03	8.03	8.02	7.99	7.46	7.82	8.18	8.23
84	7.5	7.71	7.78	7.76	7.82	7.69	7.79	7.75	7.69	7.72	7.85	7.92
104	7.78	7.67	7.74	7.9	7.85	7.74	7.84	7.85	7.85	7.72	7.85	7.74
112	7.51	7.47	7.53	7.43	7.51	7.48	7.86	7.81	7.78	7.69	7.74	8.1
127	7.49	7.59	7.62	7.56	7.73	7.69	8.2	8.03	8.12	7.97	8.13	8.05
161	7.1	7.77	7.65	6.99	7.68	7.87	8.35	8.21	8.15	8.05	8.1	7.92
190	6.51	7.7	7.87	7.2	6.95	7.79	7.84	7.87	7.59	7.44	7.56	7.49
196	7.19	7.85	7.95	7.61	7.53	8.02	8.11	8.03	7.67	7.55	7.79	7.74
204	7.68	7.68	7.77	7.47	7.47	7.69	7.48	7.53	7.51	7.22	7.54	7.25
210	7.72	7.47	7.42	7.35	7.57	7.55	7.37	7.5	7.36	7.18	7.42	7.41
217	7.16	7.6	7.42	7.5	7.49	7.56	7.47	7.7	7.58	7.49	7.56	7.61
224	7.94	7.96	7.95	7.87	7.92	7.91	8.01	7.91	8.03	8	7.99	8.03
224	7.45	7.33	7.27	7.34	7.2	7.44	7.34	7.43	7.28	7.24	7.25	7.21
231	8.01	7.88	7.89	7.87	7.82	7.89	8.05	7.95	7.94	7.85	7.89	7.96
231	7.62	7.52	7.21	7.18	7.12	7.35	7.21	7.31	7.25	7.29	7.23	7.19
238	7.77	7.68	7.53		7.61	7.68	7.78	7.71	7.75	7.72	7.68	7.72
238	7.33	7.31	6.96	7.53	7.38	7.35	7.19	7.3	7.51	7.45	7.34	7.39
245	7.67	7.52	7.35	7.34	7.44	7.6	7.52	7.58	7.56	7.56	7.57	7.53

Table A5. qPCR R^2 and Efficiency Values.

Set	Target	Days	R^2	Efficiency
Part 1	16S rRNA	0, 21	0.999	1.067
	16S rRNA	75, 245	0.999	1.105
	intI1	0, 21	0.994	0.972
	intI1	75,245	0.998	1.006
	mexB	0, 21	0.994	0.999
	mexB	71, 245	0.992	0.979
Part 2	16S rRNA	0, 33	0.991	0.953
	16S rRNA	51, 57	0.999	1.023
	16S rRNA	17	0.999	1.016
	intI1	0, 33	0.997	0.934
	intI1	51, 57	0.992	1.080
	intI1	17	0.997	1.054
	mexB	0, 57	0.999	1.078
	mexB	17	0.995	0.987
Part 3	16S rRNA	0	0.995	0.902
	16S rRNA	17	0.999	1.016
	intI1	0	0.996	0.988
	intI1	17	0.997	1.054
	mex B	0, 17	0.996	1.033

Table A6. 16S rRNA gene copies in Part 1, Day 0 & Day 21

Sample	16S rRNA (gene copies per mL wet sludge)		Log transformed		Log transformed average		*p*-value control vs tric	*p*-value control vs tric
	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21
1a	1.77E+09	7.32E+09	9.25	9.86	9.26	9.76		
1b	2.07E+09	7.42E+09	9.32	9.87				
1c	2.04E+09	8.16E+09	9.31	9.91				
2a	1.56E+09	8.22E+09	9.19	9.91				
2b	1.48E+09	8.90E+09	9.17					
2c	1.76E+09	9.85E+09	9.25	9.99				
3a	2.02E+09	2.87E+09	9.31	9.46				
3b	1.69E+09	3.46E+09	9.23	9.54				
3c	1.98E+09	3.59E+09	9.30	9.56				
4a	1.26E+09	6.61E+09	9.10	9.82	9.15	10.00	0.00	0.05
4b	1.39E+09	5.88E+09	9.14	9.77				
4c	1.47E+09	5.43E+09	9.17	9.73				
5a	1.26E+09	1.92E+10	9.10	10.28				
5b	1.56E+09	2.22E+10	9.19	10.35				
5c	1.25E+09	2.07E+10	9.10	10.32				
6a	1.37E+09	7.70E+09	9.14	9.89				
6b	1.70E+09	8.97E+09	9.23	9.95				
6c	1.63E+09	8.28E+09	9.21	9.92				
7a	1.45E+09	1.52E+10	9.16	10.18	9.15	10.03		
7b	1.37E+09	1.52E+10	9.14	10.18				
7c	1.53E+09	1.45E+10	9.19	10.16				
8q	1.20E+09	5.80E+09	9.08	9.76				
8b	1.31E+09	5.71E+09	9.12	9.76				
8c	1.11E+09	6.06E+09	9.05	9.78				
9a	1.35E+09	1.31E+10	9.13	10.12				
9b	1.89E+09	1.39E+10	9.28	10.14				
9c	1.74E+09	1.58E+10	9.24	10.20				
10a	1.88E+09	1.14E+10	9.27	10.06	9.19	10.10	0.61	0.31
10b	2.02E+09	1.19E+10	9.31	10.08				
10c	1.95E+09	1.21E+10	9.29	10.08				
11a	2.50E+09	1.64E+10	9.40	10.21				
11b	2.39E+09	1.50E+10	9.38	10.18				
11c	2.24E+09	1.50E+10	9.35	10.18				
12a	8.52E+08	1.11E+10	8.93	10.04				
12b	7.37E+08	1.20E+10	8.87	10.08				
12c	8.52E+08	1.09E+10	8.93	10.04				

1,2,3 = control-perturbed

4,5,6 = triclosan-perturbed

7,8,9 = control

10,11,12 = triclosan

Table A7. 16S rRNA Gene Copies in Part 1, Day 71 & Day 245

Sample	16S rRNA (gene copies per mL wet sludge)		Log transformed		Log transformed average		*p*-value control vs tric	*p*-value control vs tric
	Day 71	Day 245	Day 71	Day 245	Day 71	Day 245	Day 71	Day 245
1a	1.71E+10	3.83E+10	10.23	10.58	10.50	10.32		
1b	1.61E+10	3.58E+10	10.21	10.55				
1c	1.63E+10	4.37E+10	10.21	10.64				
2a	3.63E+10	1.38E+10	10.56	10.14				
2b	3.27E+10	1.48E+10	10.51					
2c	3.37E+10	1.70E+10	10.53	10.23				
3a	6.30E+10	1.48E+10	10.80	10.17				
3b	5.35E+10	1.37E+10	10.73	10.14				
3c	4.75E+10	1.34E+10	10.68	10.13				
4a	4.71E+10	4.15E+10	10.67	10.62	10.62	10.30	0.12	0.85
4b	4.51E+10	2.80E+10	10.65	10.45				
4c	3.83E+10	3.04E+10	10.58	10.48				
5a	4.44E+10	9.30E+09	10.65	9.97				
5b	4.93E+10	1.04E+10	10.69	10.02				
5c	4.47E+10	1.22E+10	10.65	10.08				
6a	3.55E+10	2.31E+10	10.55	10.36				
6b	3.83E+10	2.34E+10	10.58	10.37				
6c	3.85E+10	2.29E+10	10.59	10.36				
7a	1.29E+10	4.34E+10	10.11	10.64	10.23	10.64		
7b	1.35E+10	4.12E+10	10.13	10.62				
7c	1.38E+10	4.15E+10	10.14	10.62				
8q	2.64E+10	4.85E+10	10.42	10.69				
8b	1.93E+10	4.25E+10	10.29	10.63				
8c	1.74E+10	3.94E+10	10.24	10.60				
9a	1.65E+10	4.64E+10	10.22	10.67				
9b	1.96E+10	5.39E+10	10.29	10.73				
9c	1.73E+10	4.03E+10	10.24	10.61				
10a	2.66E+10	1.93E+10	10.42	10.29	10.50	10.37	0.00	0.00
10b	2.88E+10	1.82E+10	10.46	10.26				
10c	3.01E+10	2.22E+10	10.48	10.35				
11a	3.35E+10	3.01E+10	10.52	10.48				
11b	3.01E+10	2.70E+10	10.48	10.43				
11c	2.82E+10	2.62E+10	10.45	10.42				
12a	3.77E+10	3.77E+10	10.58					
12b	3.22E+10	2.60E+10	10.51					
12c	3.66E+10	2.62E+10	10.56					

1,2,3 = control-perturbed

4,5,6 = triclosan-perturbed

7,8,9 = control

10,11,12 = triclosan

Table A8. *intI1* gene copies in Part 1, Day 0 & Day 21

Sample	*intI1* rRNA (gene copies per mL wet sludge)		Log transformed		Log transformed average		*p*-value control vs tric	*p*-value control vs tric
	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21
1a	2.05E+05	1.98E+05	5.31	5.30	5.15	6.04		
1b	2.51E+05	2.13E+05	5.40	5.33				
1c	2.30E+05	2.02E+05	5.36	5.31				
2a	1.16E+05	3.31E+06	5.07	6.52				
2b	1.22E+05	3.57E+06	5.09					
2c	1.00E+05	3.69E+06	5.00	6.57				
3a	1.13E+05	2.74E+06	5.05	6.44				
3b	1.12E+05	2.61E+06	5.05	6.42				
3c	1.08E+05	2.85E+06	5.03	6.46				
4a	1.14E+05	1.38E+07	5.06	7.14	5.16	7.15	0.88	0.00
4b	1.46E+05	1.45E+07	5.16	7.16				
4c	1.33E+05	1.41E+07	5.12	7.15				
5a	1.35E+05	1.29E+08	5.13	8.11				
5b	1.04E+05	1.13E+08	5.02	8.05				
5c	1.26E+05	1.10E+08	5.10	8.04				
6a	2.02E+05	2.70E+07	5.31	7.43				
6b	1.54E+05	2.93E+07	5.19	7.47				
6c	2.31E+05	2.77E+07	5.36	7.44				
7a	1.53E+05	1.52E+06	5.18	6.18	5.24	6.04		
7b	1.20E+05	1.57E+06	5.08	6.20				
7c	1.39E+05	1.35E+06	5.14	6.13				
8q	1.39E+05	6.49E+05	5.14	5.81				
8b	1.85E+05	6.36E+05	5.27	5.80				
8c	1.39E+05	6.62E+05	5.14	5.82				
9a	1.82E+05	1.51E+06	5.26	6.18				
9b	3.08E+05	1.44E+06	5.49	6.16				
9c	2.60E+05	1.27E+06	5.41	6.10				
10a	1.79E+05	2.07E+06	5.25	6.32	5.18	6.41	0.45	0.34
10b	1.99E+05	2.03E+06	5.30	6.31				
10c	1.73E+05	2.12E+06	5.24	6.33				
11a	1.75E+05	5.69E+07	5.24	7.76				
11b	1.80E+05	5.54E+07	5.26	7.74				
11c	2.06E+05	5.89E+07	5.31	7.77				
12a	1.39E+05	1.51E+05	5.14	5.18				
12b	1.10E+05	1.48E+05	5.04	5.17				
12c	7.49E+04	1.39E+05	4.87	5.14				

1,2,3 = control-perturbed

4,5,6 = triclosan-perturbed

7,8,9 = control

10,11,12 = triclosan

Table A9. *intI1* gene copies in Part 1, Day 71 & Day 245

Sample	*intI1* rRNA (gene copies per mL wet sludge)		Log transformed		Log transformed average		*p*-value control vs tric	*p*-value control vs tric
	Day 71	Day 245	Day 71	Day 245	Day 71	Day 245	Day 71	Day 245
1a	4.65E+05	2.09E+06	5.67	6.32	6.57	6.33		
1b	4.52E+05	2.20E+06	5.66	6.34				
1c	4.23E+05	2.44E+06	5.63	6.39				
2a	6.08E+06	4.05E+06	6.78	6.61				
2b	5.72E+06	4.02E+06	6.76					
2c	6.12E+06	4.31E+06	6.79	6.63				
3a	1.88E+07	1.22E+06	7.27	6.09				
3b	1.97E+07	1.38E+06	7.30	6.14				
3c	1.96E+07	1.30E+06	7.29	6.11				
4a	2.20E+07	7.12E+05	7.34	5.85	7.74	6.42	0.00	0.71
4b	2.23E+07	5.04E+05	7.35	5.70				
4c	2.10E+07	6.70E+05	7.32	5.83				
5a	1.48E+08	1.64E+06	8.17	6.21				
5b	1.45E+08	1.46E+06	8.16	6.16				
5c	1.46E+08	1.54E+06	8.17	6.19				
6a	4.86E+07	1.88E+07	7.69	7.27				
6b	5.20E+07	1.83E+07	7.72	7.26				
6c	5.56E+07	2.07E+07	7.75	7.32				
7a	3.69E+05	2.03E+05	5.57	5.31	5.86	5.38		
7b	5.32E+05	2.01E+05	5.73	5.30				
7c	5.04E+05	2.12E+05	5.70	5.33				
8q	1.27E+06	3.20E+05	6.10	5.51				
8b	1.25E+06	3.22E+05	6.10	5.51				
8c	1.21E+06	3.45E+05	6.08	5.54				
9a	6.43E+05	2.16E+05	5.81	5.33				
9b	6.52E+05	2.16E+05	5.81	5.33				
9c	6.61E+05	2.02E+05	5.82	5.31				
10a	2.39E+06	6.84E+04	6.38	4.84	6.28	4.98	0.27	0.00
10b	2.12E+06	6.27E+04	6.33	4.80				
10c	2.52E+06	5.55E+04	6.40	4.74				
11a	3.15E+07	1.29E+05	7.50	5.11				
11b	2.85E+07	1.61E+05	7.45	5.21				
11c	2.83E+07	1.47E+05	7.45	5.17				
12a	9.66E+04		4.99					
12b	8.50E+04		4.93					
12c	1.14E+05		5.06					

1,2,3 = control-perturbed

4,5,6 = triclosan-perturbed

7,8,9 = control

10,11,12 = triclosan

Table A10. *mexB* gene copies in Part 1, Day 0 & Day 21

Sample	*mexB* (gene copies per mL wet sludge)		Log transformed		Log transformed average		*p*-value control vs tric	*p*-value control vs tric
	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21	Day 0	Day 21
1a	8.40E+04	3.00E+02	4.92	2.48	4.82	3.05		
1b	1.03E+05	8.06E+02	5.01	2.91				
1c	7.68E+04	9.79E+02	4.89	2.99				
2a	1.24E+05	2.69E+03	5.09	3.43				
2b	5.82E+04	2.14E+03	4.76					
2c	3.98E+04	3.57E+03	4.60	3.55				
3a	4.23E+04	2.08E+03	4.63	3.32				
3b	4.79E+04	1.85E+03	4.68	3.27				
3c	5.98E+04	3.00E+02	4.78	2.48				
4a	4.38E+04	2.05E+03	4.64	3.31	4.78	2.80	0.64	0.26
4b	3.74E+04	2.33E+03	4.57	3.37				
4c	4.38E+04	6.20E+02	4.64	2.79				
5a	4.76E+04	1.15E+03	4.68	3.06				
5b	4.70E+04	1.09E+03	4.67	3.04				
5c	7.52E+04	3.00E+02	4.88	2.48				
6a	1.34E+05	5.82E+02	5.13	2.77				
6b	8.82E+04	3.00E+02	4.95	2.48				
6c	7.11E+04	7.81E+01	4.85	1.89				
7a	5.25E+04	8.67E+01	4.72	1.94	4.61	2.63		
7b	5.51E+04	3.00E+02	4.74	2.48				
7c	5.07E+04	1.33E+03	4.70	3.12				
8q	2.91E+04	3.00E+02	4.46	2.48				
8b	3.06E+04	3.00E+02	4.49	2.48				
8c	2.19E+04	1.17E+03	4.34	3.07				
9a	4.35E+04	3.00E+02	4.64	2.48				
9b	4.66E+04	6.33E+02	4.67	2.80				
9c	5.07E+04	6.24E+02	4.70	2.80				
10a	7.89E+04	8.17E+02	4.90	2.91	4.75	2.35	0.04	0.35
10b	5.90E+04	3.00E+02	4.77	2.48				
10c	7.21E+04	1.42E+03	4.86	3.15				
11a	6.41E+04	2.75E+03	4.81	3.44				
11b	5.47E+04	3.00E+02	4.74	2.48				
11c	8.64E+04	2.05E+01	4.94	1.31				
12a	3.74E+04	3.00E+02	4.57	2.48				
12b	3.76E+04	4.19E+01	4.58	1.62				
12c	4.17E+04	1.73E+01	4.62	1.24				

1,2,3 = control-perturbed

4,5,6 = triclosan-perturbed

7,8,9 = control

10,11,12 = triclosan

Table A11. *mexB* gene copies in Part 1, Day 71 & Day 245

Sample	*intI1* rRNA (gene copies per mL wet sludge)		Log transformed		Log transformed average		*p*-value control vs tric	*p*-value control vs tric
	Day 71	Day 245	Day 71	Day 245	Day 71	Day 245	Day 71	Day 245
1a	2.83E+03	1.12E+06	3.45	6.05	3.54	5.82		
1b	6.67E+03	1.10E+06	3.82	6.04				
1c	7.91E+03	1.28E+06	3.90	6.11				
2a	2.83E+03	1.04E+06	3.45	6.02				
2b	2.83E+03	8.05E+05	3.45					
2c	2.83E+03	1.30E+06	3.45	6.11				
3a	2.83E+03	2.76E+05	3.45	5.44				
3b	2.83E+03	2.07E+05	3.45	5.32				
3c	2.83E+03	2.91E+05	3.45	5.46				
4a	2.83E+03	4.69E+06	3.45	6.67	3.45	5.30	0.15	0.19
4b	2.83E+03	4.53E+06	3.45	6.66				
4c	2.83E+03	4.98E+06	3.45	6.70				
5a	2.83E+03	4.24E+04	3.45	4.63				
5b	2.83E+03	4.27E+04	3.45	4.63				
5c	2.83E+03	4.36E+04	3.45	4.64				
6a	2.83E+03	3.99E+04	3.45	4.60				
6b	2.83E+03	3.70E+04	3.45	4.57				
6c	2.83E+03	3.75E+04	3.45	4.57				
7a	2.83E+03	1.82E+05	3.45	5.26	3.45	4.70		
7b	2.83E+03	1.85E+05	3.45	5.27				
7c	2.83E+03	2.54E+05	3.45	5.40				
8q	2.83E+03	6.86E+03	3.45	3.84				
8b	2.83E+03	8.83E+03	3.45	3.95				
8c	2.83E+03	1.10E+04	3.45	4.04				
9a	2.83E+03	6.48E+04	3.45	4.81				
9b	2.83E+03	7.03E+04	3.45	4.85				
9c	2.83E+03	7.03E+04	3.45	4.85				
10a	2.83E+03	6.37E+06	3.45	6.80	3.49	5.75	0.33	0.03
10b	2.83E+03	6.24E+06	3.45	6.80				
10c	6.86E+03	5.19E+06	3.84	6.72				
11a	2.83E+03	5.14E+04	3.45	4.71				
11b	2.83E+03	4.24E+04	3.45	4.63				
11c	2.83E+03	6.61E+04	3.45	4.82				
12a	2.83E+03		3.45					
12b	2.83E+03		3.45					
12c	2.83E+03		3.45					

1,2,3 = control-perturbed

4,5,6 = triclosan-perturbed

7,8,9 = control

10,11,12 = triclosan

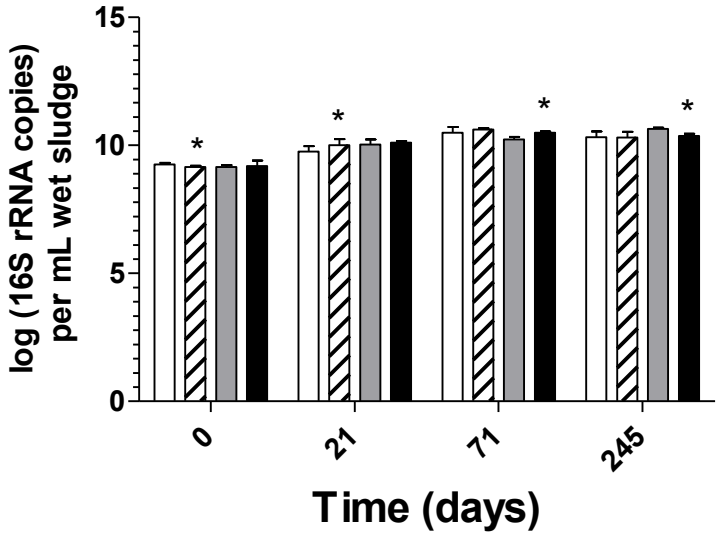


Figure A7. Impact of triclosan on abundance of 16S rRNA gene copies in unexposed communities in experiment from Part 1. Data taken from Tables A6-A7. Perturbed, triclosan-perturbed, control, and triclosan reactors are represented by white, slashed, grey, and black bars, respectively. Error bars depict standard deviation, n = 9 from triplicate PCR from triplicate reactors. At Day 245 n=6 for triclosan set. The (*) denotes statistical significance at $p < 0.05$.

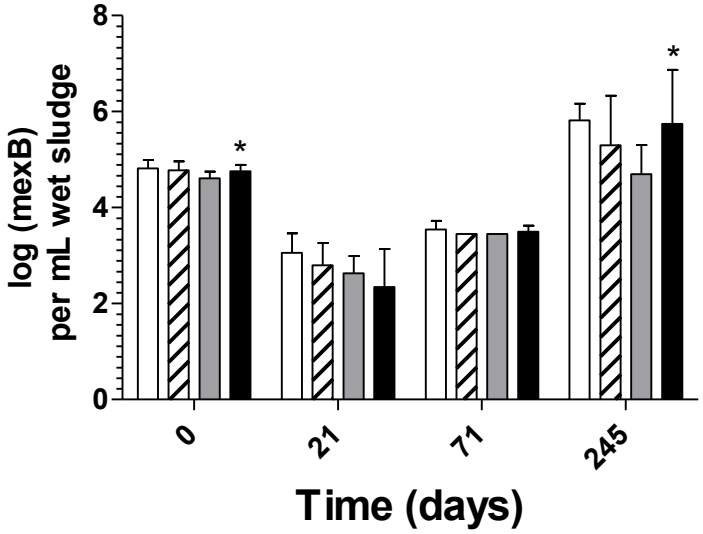
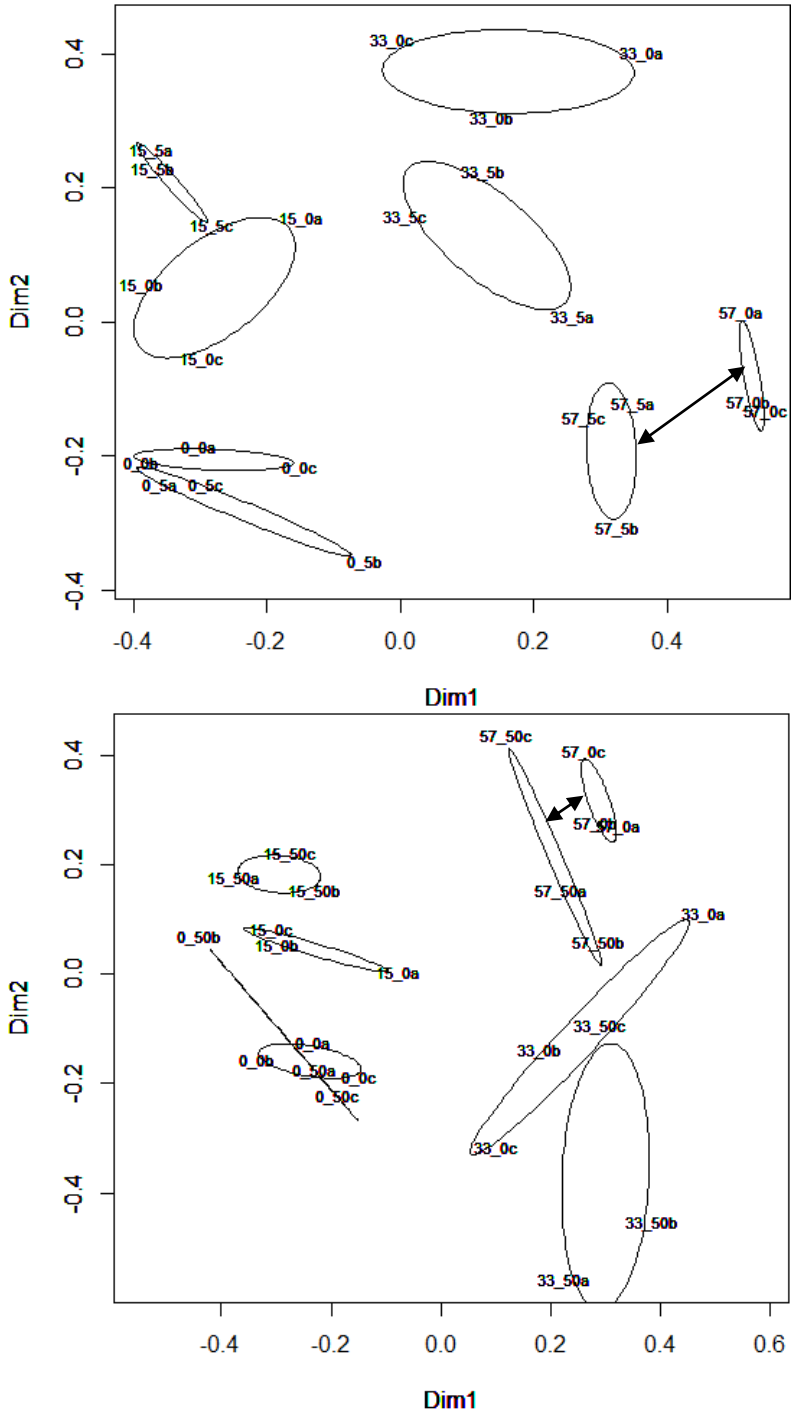


Figure A8. Impact of triclosan on abundance of *mexB* gene copies in unexposed communities in experiment from Part 1. Data taken from Tables A9-A10. Perturbed, triclosan-perturbed, control, and triclosan reactors are represented by white, slashed, grey, and black bars, respectively. Error bars depict standard deviation, n = 9 from triplicate PCR from triplicate reactors. At Day 245 n=6 for triclosan set. The (*) denotes statistical significance at $p < 0.05$.

Part II Supplemental Results



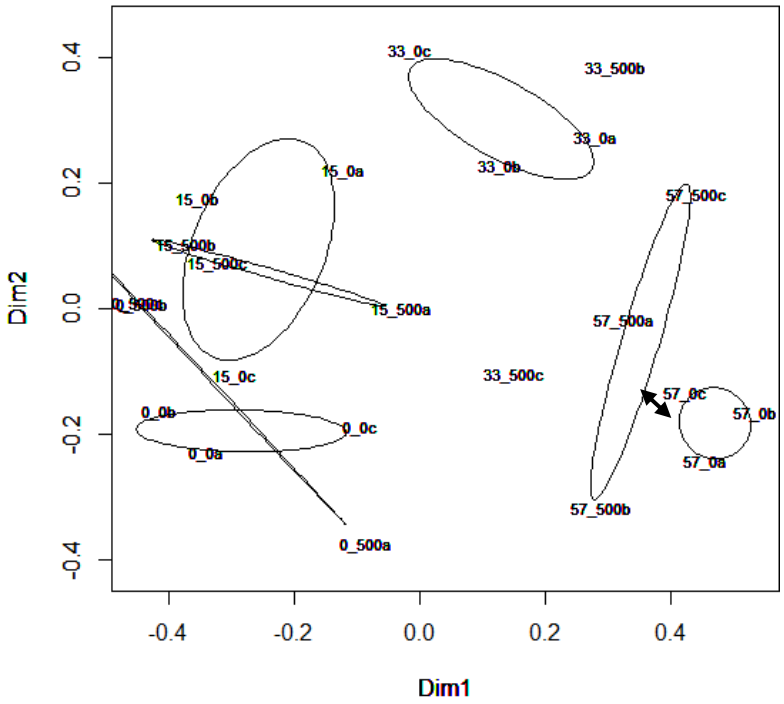


Figure A9. Impact of increased triclosan loading (5 mg/kg on top, 50 mg/kg in middle, 500 mg/kg on bottom) on Archaeal communities in previously exposed communities.

Table A12. 16S rRNA Gene Copies in Part 2, Day 0 and Day 33

Sample	16S (gene copies per g wet sludge)		Log transformed		Log transformed average		*p*-value control vs treatment	*p*-value control vs treatment
	Day 0	Day 33	Day 0	Day 33	Day 0	Day 33	Day 0	Day 33
1a	5.81E+11	1.75E+12	11.76	12.24	11.64	11.98		
1b	2.84E+11	7.61E+11	11.45	11.88				
1c	5.61E+11	1.46E+12	11.75	12.16				
2a	7.45E+11	1.76E+12	11.87	12.25				
2b	4.62E+10	6.56E+11	10.66					
2c	6.70E+11	1.29E+12	11.83	12.11				
3a	5.31E+11	1.17E+12	11.73	12.07				
3b	7.78E+11	4.39E+11	11.89	11.64				
3c	7.11E+11	2.84E+11	11.85	11.45				
4a	1.12E+12	1.18E+12	12.05	12.07	11.99	12.17	0.02	0.15
4b	7.08E+11	1.14E+12	11.85	12.06				
4c	7.79E+11	6.35E+11	11.89	11.80				
5a	8.02E+11	2.36E+12	11.90	12.37				
5b	7.63E+11	1.32E+12	11.88	12.12				
5c	9.96E+11	2.36E+12	12.00	12.37				
6a	1.49E+12	2.56E+12	12.17	12.41				
6b	1.09E+12	2.63E+12	12.04	12.42				
6c	1.34E+12	7.93E+11	12.13	11.90				
7a	1.07E+12	2.52E+12	12.03	12.40	11.99	12.24	0.02	0.03
7b	1.16E+12	2.13E+12	12.07	12.33				
7c	9.24E+11	1.60E+12	11.97	12.20				
8q	6.98E+11	1.77E+12	11.84	12.25				
8b	7.51E+11	2.01E+12	11.88	12.30				
8c	9.51E+11	7.78E+11	11.98	11.89				
9a	1.18E+12	2.19E+12	12.07	12.34				
9b	1.27E+12	1.94E+12	12.11	12.29				
9c	9.03E+11	1.54E+12	11.96	12.19				
10a	5.96E+11		11.77		11.88	12.18	0.11	0.31
10b	5.94E+11	1.04E+12	11.77	12.02				
10c	6.60E+11	1.52E+12	11.82	12.18				
11a	5.82E+11	8.72E+12	11.77	12.94				
11b	8.81E+11	4.31E+12	11.94	12.63				
11c	1.07E+12	1.42E+12	12.03	12.15				
12a	1.26E+12	2.11E+11	12.10	11.33				
12b	5.96E+11	1.20E+12	11.78	12.08				
12c	8.30E+11	1.35E+12	11.92	12.13				

1,2,3 = 0 mg/kg
4,5,6 = 5 mg/kg
7,8,9 = 50 mg/kg
10,11,12 = 500 mg/kg

Table A13. 16S rRNA Gene Copies in Part 2, Day 51 and Day 57

Sample	16S (gene copies per g wet sludge)		Log transformed		Log transformed average		*p*-value control vs treatment	*p*-value control vs treatment
	Day 51	Day 57	Day 51	Day 57	Day 51	Day 57	Day 51	Day 57
1a	7.14E+11	8.83E+12	11.85	12.95	12.04	12.50		
1b	1.41E+12	3.99E+12	12.15	12.60				
1c	9.41E+11	6.67E+12	11.97	12.82				
2a	1.30E+12	4.33E+12	12.11	12.64				
2b	8.97E+11	4.58E+12	11.95					
2c	9.36E+11	1.33E+12	11.97	12.12				
3a	1.35E+12	1.59E+12	12.13	12.20				
3b	1.18E+12	2.13E+12	12.07	12.33				
3c	1.39E+12	2.23E+12	12.14	12.35				
4a	4.16E+12	1.20E+12	12.62	12.08	12.17	12.46	0.24	0.75
4b	6.87E+11	2.09E+12	11.84	12.32				
4c	1.37E+12	2.21E+12	12.14	12.34				
5a	1.82E+12	5.12E+12	12.26	12.71				
5b	1.76E+12	3.70E+12	12.24	12.57				
5c	2.76E+12	4.19E+12	12.44	12.62				
6a	2.21E+12	3.85E+12	12.35	12.58				
6b	3.83E+11	2.55E+12	11.58	12.41				
6c	1.25E+12	3.29E+12	12.10	12.52				
7a	1.45E+12	2.41E+12	12.16	12.38	12.23	12.33	0.08	0.23
7b	2.90E+12	3.20E+12	12.46	12.50				
7c	4.20E+12	4.36E+12	12.62	12.64				
8q	3.58E+12	6.71E+11	12.55	11.83				
8b	1.18E+12	1.43E+12	12.07	12.16				
8c	2.10E+12	1.73E+12	12.32	12.24				
9a	6.05E+11	3.47E+12	11.78	12.54				
9b	1.03E+12	1.22E+12	12.01	12.08				
9c	1.08E+12	3.90E+12	12.04	12.59				
10a	7.10E+11	5.06E+12	11.85	12.70	12.01	12.55	0.77	0.73
10b	7.44E+11	6.49E+12	11.87	12.81				
10c	4.74E+11	3.75E+12	11.68	12.57				
11a	6.29E+11	3.79E+12	11.80	12.58				
11b	2.31E+12	4.79E+12	12.36	12.68				
11c	1.02E+12	3.24E+12	12.01	12.51				
12a	4.91E+12	6.24E+11	12.69	11.79				
12b	8.15E+11	5.70E+12	11.91	12.76				
12c	7.64E+11	3.67E+12	11.88	12.56				

1,2,3 = 0 mg/kg
4,5,6 = 5 mg/kg
7,8,9 = 50 mg/kg
10,11,12 = 500 mg/kg

Table A14. *intI1* Gene Copies in Part 2, Day 0 and Day 33

Sample	*intI1* (gene copies per g wet sludge)		Log transformed		Log transformed average		*p*-value control vs treatment	*p*-value control vs treatment
	Day 0	Day 33	Day 0	Day 33	Day 0	Day 33	Day 0	Day 33
1a	5.84E+08	3.96E+08	8.77	8.60	8.75	8.75		
1b	3.47E+08	7.01E+07	8.54	7.85				
1c	8.32E+08	2.74E+08	8.92	8.44				
2a	9.72E+08	1.96E+10	8.99	10.29				
2b	5.35E+07		7.73					
2c	8.54E+08	2.60E+10	8.93	10.42				
3a	6.84E+08	2.92E+08	8.83	8.47				
3b	1.15E+09	1.02E+08	9.06	8.01				
3c	9.57E+08	1.02E+08	8.98	8.01				
4a	1.21E+09	5.47E+08	9.08	8.74	9.09	8.74	0.03	0.93
4b	9.35E+08	5.37E+08	8.97	8.73				
4c	1.11E+09	3.84E+08	9.04	8.58				
5a	1.26E+09	1.83E+08	9.10	8.26				
5b	1.01E+09	7.37E+07	9.01	7.87				
5c	1.27E+09	1.44E+08	9.10	8.16				
6a	1.58E+09	1.93E+11	9.20	11.29				
6b	1.47E+09	4.68E+08	9.17	8.67				
6c	1.50E+09	1.36E+08	9.17	8.13				
7a	1.47E+09	1.25E+09	9.17	9.10	9.10	8.91	0.02	0.67
7b	1.59E+09	1.10E+09	9.20	9.04				
7c	1.23E+09	5.62E+08	9.09	8.75				
8q	1.44E+09	1.61E+09	9.16	9.21				
8b	1.04E+09	1.12E+09	9.02	9.05				
8c	1.30E+09	4.89E+08	9.11	8.69				
9a	1.23E+09	6.66E+08	9.09	8.82				
9b	1.35E+09	6.48E+08	9.13	8.81				
9c	8.83E+08	5.09E+08	8.95	8.71				
10a	7.82E+08	6.92E+08	8.89	8.84	8.94	8.64	0.20	0.76
10b	8.31E+08	5.52E+08	8.92	8.74				
10c	6.34E+08	2.81E+08	8.80	8.45				
11a	6.89E+08	2.52E+09	8.84	9.40				
11b	9.12E+08	2.34E+08	8.96	8.37				
11c	1.08E+09	3.51E+08	9.03	8.55				
12a	1.18E+09	8.09E+07	9.07	7.91				
12b	7.07E+08	5.27E+08	8.85	8.72				
12c	1.24E+09	6.49E+08	9.09	8.81				

1,2,3 = 0 mg/kg
4,5,6 = 5 mg/kg
7,8,9 = 50 mg/kg
10,11,12 = 500 mg/kg

Table A15. *intI1* Gene Copies in Part 2, Day 51 and Day 57

Sample	*intI1* (gene copies per g wet sludge)		Log transformed		Log transformed average		*p*-value control vs treatment	*p*-value control vs treatment
	Day 51	Day 57	Day 51	Day 57	Day 51	Day 57	Day 51	Day 57
1a	2.09E+07	4.01E+08	7.32	8.60	7.82	8.47		
1b	3.51E+07	2.31E+08	7.55	8.36				
1c	2.61E+07	1.25E+08	7.42	8.10				
2a	2.19E+08	1.71E+09	8.34	9.23				
2b	3.56E+08	1.97E+09	8.55					
2c	2.99E+08	4.71E+08	8.48	8.67				
3a	4.87E+07	1.82E+08	7.69	8.26				
3b	2.68E+07	1.96E+08	7.43	8.29				
3c	4.41E+07	1.88E+08	7.64	8.27				
4a	3.42E+08	1.83E+07	8.53	7.26	7.67	8.19	0.56	0.15
4b	6.29E+06	1.83E+08	6.80	8.26				
4c	3.78E+07	2.17E+08	7.58	8.34				
5a	5.19E+07	2.91E+08	7.72	8.46				
5b	7.48E+07	3.55E+08	7.87	8.55				
5c	1.70E+08	3.52E+08	8.23	8.55				
6a	8.59E+07	2.13E+08	7.93	8.33				
6b	9.52E+06	9.03E+07	6.98	7.96				
6c	2.68E+07	1.07E+08	7.43	8.03				
7a	3.01E+07	5.03E+08	7.48	8.70	8.06	8.51	0.32	0.90
7b	5.37E+07	1.47E+09	7.73	9.17				
7c	2.19E+08	1.12E+09	8.34	9.05				
8q	4.64E+08	1.06E+07	8.67	7.02				
8b	3.22E+07	3.80E+07	7.51	7.58				
8c	5.28E+08	1.39E+08	8.72	8.14				
9a	4.62E+07	1.62E+09	7.66	9.21				
9b	1.45E+08	3.63E+08	8.16	8.56				
9c	1.87E+08	1.56E+09	8.27	9.19				
10a	1.71E+07	6.90E+06	7.23	6.84	7.09	6.65	**0.00**	**0.00**
10b	1.43E+07	6.53E+06	7.15	6.81				
10c	7.65E+06	2.88E+06	6.88	6.46				
11a	4.23E+06	4.09E+06	6.63	6.61				
11b	1.66E+07	2.66E+06	7.22	6.42				
11c	1.17E+07	1.95E+06	7.07	6.29				
12a	6.01E+06	2.09E+07	6.78	7.32				
12b	2.44E+07	5.71E+06	7.39	6.76				
12c	2.96E+07	2.56E+06	7.47	6.41				

1,2,3 = 0 mg/kg
4,5,6 = 5 mg/kg
7,8,9 = 50 mg/kg
10,11,12 = 500 mg/kg

Table A16. *mexB* Gene Copies in Part 2, Day 0 and Day 15

Sample	*mexB* (gene copies per g wet sludge)		Log transformed		Log transformed average		*p*-value control vs treatment	*p*-value control vs treatment
	Day 0	Day 15	Day 0	Day 15	Day 0	Day 15	Day 0	Day 15
1a	1.24E+05	7.11E+04	5.09	4.85	5.01	4.69		
1b		2.56E+04		4.41				
1c		5.16E+04		4.71				
2a	8.98E+04	4.42E+04	4.95	4.65				
2b		5.74E+04						
2c		5.57E+04		4.75				
3a	9.42E+04	5.24E+04	4.97	4.72				
3b		5.53E+04		4.74				
3c		5.29E+04		4.72				
4a	2.16E+05	4.04E+04	5.34	4.61	5.30	4.73	0.18	NA
4b		4.05E+04		4.61				
4c		5.94E+04		4.77				
5a	9.47E+04	5.49E+04	4.98	4.74				
5b		5.80E+04		4.76				
5c		6.04E+04		4.78				
6a	3.99E+05	5.80E+04	5.60	4.76				
6b		7.52E+04		4.88				
6c		5.15E+04		4.71				
7a	2.40E+05	5.89E+04	5.38	4.77	5.26	4.96	0.10	NA
7b		5.84E+04		4.77				
7c		5.93E+04		4.77				
8q	1.10E+05	6.55E+04	5.04	4.82				
8b		5.31E+04		4.73				
8c		6.45E+04		4.81				
9a	2.28E+05	2.37E+05	5.36	5.38				
9b		1.35E+05		5.13				
9c		2.70E+05		5.43				
10a	2.02E+05	9.69E+05	5.31	5.99	5.26	5.10	0.02	NA
10b		1.17E+06		6.07				
10c		5.37E+05		5.73				
11a	1.45E+05	5.23E+04	5.16	4.72				
11b		5.99E+04		4.78				
11c		4.26E+04		4.63				
12a	2.15E+05	5.63E+04	5.33	4.75				
12b		4.36E+04		4.64				
12c		4.35E+04		4.64				

1,2,3 = 0 mg/kg
4,5,6 = 5 mg/kg
7,8,9 = 50 mg/kg
10,11,12 = 500 mg/kg

NA = not applicable because only reactors #9 and #10 yielded quantities above detection limit at 15 days.

Table A17. *mexB* Gene Copies in Part 2, Day 57.

Sample	*mexB* (gene copies per g wet sludge)	Log transformed	Log transformed average	*p*-value control vs treatment	*p*-value control vs treatment
	Day 57	Day 57	Day 57	Day 57	
1a	2.13E+05	5.33	5.14		
1b	1.37E+05	5.14			
1c	8.87E+04	4.95			
2a	1.74E+05	5.24			
2b	1.76E+05	5.24			
2c	1.05E+05	5.02			
3a	1.11E+05	5.05			
3b	1.43E+05	5.15			
3c	1.27E+05	5.10			
4a	1.82E+05	5.26	5.18	0.42	
4b	1.22E+05	5.09			
4c	1.05E+05	5.02			
5a	1.69E+05	5.23			
5b	1.15E+05	5.06			
5c	1.72E+05	5.24			
6a	1.87E+05	5.27			
6b	1.48E+05	5.17			
6c	1.87E+05	5.27			
7a	2.14E+05	5.33	5.14	0.96	
7b	1.21E+05	5.08			
7c	1.79E+05	5.25			
8q	1.46E+05	5.16			
8b	1.68E+05	5.23			
8c	1.10E+05	5.04			
9a	1.14E+05	5.06			
9b	8.91E+04	4.95			
9c	1.40E+05	5.15			
10a	1.40E+05	5.15	5.05	0.39	
10b	1.99E+05	5.30			
10c	1.14E+05	5.06			
11a	1.07E+05	5.03			
11b	2.02E+05	5.31			
11c	1.28E+05	5.11			
12a	2.91E+04	4.46			
12b	1.12E+05	5.05			
12c	1.08E+05	5.03			

1,2,3 = 0 mg/kg
4,5,6 = 5 mg/kg
7,8,9 = 50 mg/kg
10,11,12 = 500 mg/kg

Table A18. pH in Previously Exposed Serum Bottle Reactors

Day	Before/After Feed*	0-A	0-B	0-C	5-A	5-B	5-C	50-A	50-B	50-C	500-A	500-B	500-C
0	Before	7.89	8	7.93	7.98	7.98	7.93	7.9	7.95	8	7.87	8	7.99
1	After	7.44	7.52	7.5	7.5	7.51	7.48	7.48	7.47	7.5	7.47	7.49	7.49
5	Before	6.38	6.53	6.55	6.59	6.46	6.48	6.46	6.49	6.49	5.64	6.47	6.42
5	After	6.92	6.98	6.96	7.04	6.85	6.88	6.93	6.91	6.93	6.22	6.89	6.86
7	Before	6.43	6.42	6.49	6.39	6.33	6.24	6.29	6.33	6.29	5.71	6.24	6.22
7	After	7.37	7.21	7.4	7.23	7.41	7.3	7.26	7.34	7.29	7.24	7.5	7.4
10	Before	6.21	6.2	6.26	5.94	6.38	6.24	6.31	6.33	6.18	7.12	6.45	6.29
	After	7.28	7.43	7.28	7.16	7.32	7.23	7.46	7.2	7.4	7.16	7.14	7.36
12	Before	6.44	6.61	6.35		6.52	6.35	6.49	6.4	6.42	6.31	7.42	6.26
	After	7.31	7.15	7.28	7.51	7.47	7.3	7.31	7.31	7.35	7.23	7.64	
15	Before	6.47	6.6	6.5	6.25	6.72	6.45	6.7	6.59	6.69	6.79	6.51	6.16
18	Before	6.24	6.54	6.3	6.25	6.48	6.36	6.56	6.36	6.31	6.52	5.8	5.93
21	Before	6.44	6.49	6.36	6.14	6.61	6.51	6.82	6.24	6.48	6.33	6.22	6.15
	After	6.64	6.63	6.51									
24	Before	6.54	6.54	6.52	6.4	6.81		6.99	6.56	6.64	6.36	6.4	6.3
	After	7.26	7.12	7.24	7.15	7.01	6.99	7.08	7.06	7.15	7.26	7.36	7.67
27	Before	6.4	6.32	6.31	6.4	6.5	6.73	6.86	6.38	6.44	6.38	6.06	6.03
	After	7.39	7.18	7.22	7.12	6.94	6.93	6.98	7.08	7.17	7.03	7.16	7.14
30	Before	6.53	6.33	6.31	6.68	6.6	6.84	6.8	6.43	6.499	6.14	6.12	6.1
	After	7.29	7.08	7.14	7.12	7.11	6.91	7.01	7.08	7.36	6.91	7.16	7.2
33	Before	6.6	6.08	6.47	6.93	6.51	7.42	6.78	6.73	6.73	6.14	6.16	6.22
	After	7.19	7.24	7	7.07	7.09	7.33	6.92	6.89	6.93	6.99	7.33	7.05
36	Before	6.81	6.33	6.75	7.19	6.74	6.83	7.06	7.11	5.48	6.31	6.29	6.34
	After	7.02	6.95	6.95	7.31	6.95	7.03	7.18	7.24	7.23	7.07	7.12	7.04
39	Before	6.41	5.84	6.22	6.85	5.94	6.38	6.84	6.53	6.16	6.06	5.96	6.13
42	Before	6.57	6.34	6.25	6.86	6.09	6.41	6.9	6.39	6.04	6.23	6.12	6.19
45	Before	6.39	6.31	6.09	6.93	6.47	6.61	7.05	6.48	6.23	6.37	6.34	6.23
	After	7.06	6.91	7.24	7.16	6.97	7.13	7.25	7.03	6.88			
48	Before	6.67	6.38	6.42	6.91	6.55	6.54	7.16	6.54	6.29	6.48	6.82	6.61
51	Before	6.12	5.97		6.3	5.98	5.96	6.37	5.97	5.96	5.95	5.87	6.16
	After	7.07	7.05	7.19	7.04	7.09	7.18	7.02	6.93	7.06	7.07	6.97	7.01
54	Before	5.86	5.91	5.84	5.95	5.96	5.86	5.69	5.94	5.97	6.43	6.21	6.26
	After	6.89	6.84	7.15	6.97	6.88	7.14	6.93	7.04	7.01	6.95	7.2	7.15
57	Before	5.86	5.63	5.65	5.76	5.79	5.78	5.76	6.24	5.75	6.58	6.5	6.23
	After	7.12	7.1	7.09	7.11	7.17	7.11	6.99	7.05	6.92	7.01	7.23	7.1

*Before = value prior to feeding, After = value after feed with sodium carbonate addition

Part III Supplemental Results

Table A19. 16S rRNA Gene Copies in Part 3, Day 0 and Day 17.

Sample	16S (gene copies per g wet sludge)		Log transformed		Log transformed average		*p*-value control vs treatment	*p*-value control vs treatment
	Day 0	Day 17	Day 0	Day 17	Day 0	Day 17	Day 0	Day 17
1a	2.02E+12	1.22E+12	12.31	12.09	12.27	12.21		
1b	1.78E+12	1.67E+12	12.25	12.22				
1c	1.89E+12	2.33E+12	12.28	12.37				
2a	7.98E+11	9.85E+11	11.90	11.99				
2b	1.66E+12	9.82E+11	12.22					
2c	2.10E+12	1.87E+12	12.32	12.27				
3a	2.78E+12	2.22E+12	12.44	12.35				
3b	2.44E+12	2.06E+12	12.39	12.31				
3c	2.10E+12	1.26E+12	12.32	12.10				
4a	1.91E+12	2.98E+12	12.28	12.47	12.28	12.32	0.91	0.18
4b	1.80E+12	1.15E+12	12.26	12.06				
4c	1.55E+12	1.36E+12	12.19	12.13				
5a	1.30E+12	2.09E+12	12.11	12.32				
5b	1.88E+12	2.45E+12	12.27	12.39				
5c	2.20E+12	1.37E+12	12.34	12.14				
6a	2.49E+12	2.85E+12	12.40	12.46				
6b	2.11E+12	2.22E+12	12.32	12.35				
6c	2.04E+12	3.52E+12	12.31	12.55				
7a	2.34E+12	3.12E+12	12.37	12.49	12.19	12.11	0.27	0.31
7b	1.82E+12	1.18E+12	12.26	12.07				
7c	1.23E+12	7.52E+11	12.09	11.88				
8q	2.77E+12	1.76E+12	12.44	12.25				
8b	1.47E+12	1.58E+12	12.17	12.20				
8c	1.56E+12	1.56E+12	12.19	12.19				
9a	7.99E+11	5.03E+11	11.90	11.70				
9b	1.22E+12	1.92E+12	12.09	12.28				
9c	1.44E+12	8.64E+11	12.16	11.94				
10a	1.33E+12	1.77E+12	12.12		11.96	12.27	0.00	0.54
10b	7.16E+11	1.38E+12	11.85	12.14				
10c	9.23E+11	1.68E+12	11.97	12.23				
11a	5.40E+11	2.24E+12	11.73	12.35				
11b	1.17E+12	1.36E+12	12.07	12.13				
11c	1.10E+12	2.12E+12	12.04	12.33				
12a	9.17E+11	1.11E+12	11.96	12.04				
12b	6.73E+11	1.48E+12	11.83	12.17				
12c	1.16E+12	6.16E+12	12.07	12.79				

1,2,3 = 0 mg/kg
4,5,6 = 5 mg/kg
7,8,9 = 50 mg/kg
10,11,12 = 500 mg/kg

Table A20. *intI1* Gene Copies in Part 3, Day 0 and Day 17

Sample	*intI1* (gene copies per g wet sludge)		Log transformed		Log transformed average		*p*-value control vs treatment	*p*-value control vs treatment
	Day 0	Day 17	Day 0	Day 17	Day 0	Day 17	Day 0	Day 17
1a	4.86E+07	4.91E+07	7.69	7.69	7.74	7.98		
1b	7.90E+07	5.62E+07	7.90	7.75				
1c	2.76E+07	7.14E+07	7.44	7.85				
2a	3.85E+07	3.26E+07	7.59	7.51				
2b	4.74E+07	3.31E+07	7.68					
2c	5.55E+07	5.98E+07	7.74	7.78				
3a	8.60E+07	4.75E+08	7.93	8.68				
3b	6.70E+07	2.80E+08	7.83	8.45				
3c	7.70E+07	1.42E+08	7.89	8.15				
4a	5.75E+07	5.46E+07	7.76	7.74	7.81	7.78	0.33	0.21
4b	4.54E+07	4.01E+07	7.66	7.60				
4c	7.91E+07	4.22E+07	7.90	7.62				
5a	4.71E+07	5.52E+07	7.67	7.74				
5b	5.22E+07	4.92E+07	7.72	7.69				
5c	6.86E+07	2.59E+07	7.84	7.41				
6a	1.13E+08	1.08E+08	8.05	8.03				
6b	9.20E+07	1.32E+08	7.96	8.12				
6c	5.74E+07	1.05E+08	7.76	8.02				
7a	1.08E+08	3.50E+08	8.03	8.54	7.82	8.27	0.29	0.10
7b	6.14E+07	2.55E+08	7.79	8.41				
7c	8.08E+07	1.32E+08	7.91	8.12				
8q	1.01E+08	3.13E+08	8.00	8.50				
8b	6.91E+07	1.92E+08	7.84	8.28				
8c	7.64E+07	2.18E+08	7.88	8.34				
9a	4.10E+07	8.42E+07	7.61	7.93				
9b	4.10E+07	3.07E+08	7.61	8.49				
9c	5.33E+07	6.84E+07	7.73	7.83				
10a	4.93E+07	1.04E+08	7.69	8.02	7.64	7.69	0.36	0.11
10b	2.54E+07	7.88E+07	7.40	7.90				
10c	3.43E+07	1.10E+08	7.54	8.04				
11a	2.25E+07	4.74E+07	7.35	7.68				
11b	3.77E+07	3.47E+07	7.58	7.54				
11c	1.13E+08	3.85E+07	8.05	7.59				
12a	1.20E+08	1.40E+07	8.08	7.14				
12b	2.33E+07	2.61E+07	7.37	7.42				
12c	5.26E+07	8.32E+07	7.72	7.92				

1,2,3 = 0 mg/kg
4,5,6 = 5 mg/kg
7,8,9 = 50 mg/kg
10,11,12 = 500 mg/kg

Table A21. *mexB* Gene Copies in Part 3, Day 0 and Day 17

Sample	*mexB* (gene copies per g wet sludge)		Log transformed		Log transformed average		*p*-value control vs treatment	*p*-value control vs treatment
	Day 0	Day 17	Day 0	Day 17	Day 0	Day 17	Day 0	Day 17
1a	6.86E+04	3.63E+04	4.84	4.56	4.77	4.79		
1b	6.04E+04	7.12E+04	4.78	4.85				
1c	5.40E+04	7.62E+04	4.73	4.88				
2a	4.64E+04	6.55E+04	4.67	4.82				
2b	4.97E+04	7.00E+04	4.70					
2c	5.79E+04	7.37E+04	4.76	4.87				
3a	7.00E+04	5.52E+04	4.85	4.74				
3b	5.84E+04	5.70E+04	4.77	4.76				
3c	7.60E+04	7.22E+04	4.88	4.86				
4a	6.04E+04	5.53E+04	4.78	4.74	4.81	4.74	NA	NA
4b	5.66E+04	5.62E+04	4.75	4.75				
4c	6.31E+04	5.74E+04	4.80	4.76				
5a	5.55E+04	5.13E+04	4.74	4.71				
5b	6.55E+04	4.74E+04	4.82	4.68				
5c	6.72E+04	5.76E+04	4.83	4.76				
6a	7.61E+04	6.13E+04	4.88	4.79				
6b	6.71E+04	3.90E+04	4.83	4.59				
6c	7.51E+04	7.57E+04	4.88	4.88				
7a	6.68E+04	6.13E+04	4.82	4.79	4.83	4.71	NA	NA
7b	6.95E+04	7.46E+04	4.84	4.87				
7c	6.23E+04	3.19E+04	4.79	4.50				
8q	7.29E+04	5.89E+04	4.86	4.77				
8b	6.68E+04	7.63E+04	4.82	4.88				
8c	7.59E+04	6.27E+04	4.88	4.80				
9a	6.33E+04	3.25E+04	4.80	4.51				
9b	6.27E+04	4.22E+04	4.80	4.62				
9c	6.92E+04	4.25E+04	4.84	4.63				
10a	7.97E+04	1.10E+07	4.90		4.82	6.20	NA	**0.00**
10b	7.63E+04	8.41E+06	4.88	6.92				
10c	6.68E+04	1.35E+07	4.82	7.13				
11a	5.89E+04	1.83E+06	4.77	6.26				
11b	5.93E+04	1.51E+06	4.77	6.18				
11c	6.39E+04	2.86E+06	4.81	6.46				
12a	6.55E+04	1.81E+05	4.82	5.26				
12b	5.98E+04	2.08E+05	4.78	5.32				
12c	7.26E+04	1.09E+06	4.86	6.04				

1,2,3 = 0 mg/kg
4,5,6 = 5 mg/kg
7,8,9 = 50 mg/kg
10,11,12 = 500 mg/kg

NA = not applicable because gene copies were not above detection limit. Only samples from reactors #10, #11, #12 on Day 17 were above detection limit.

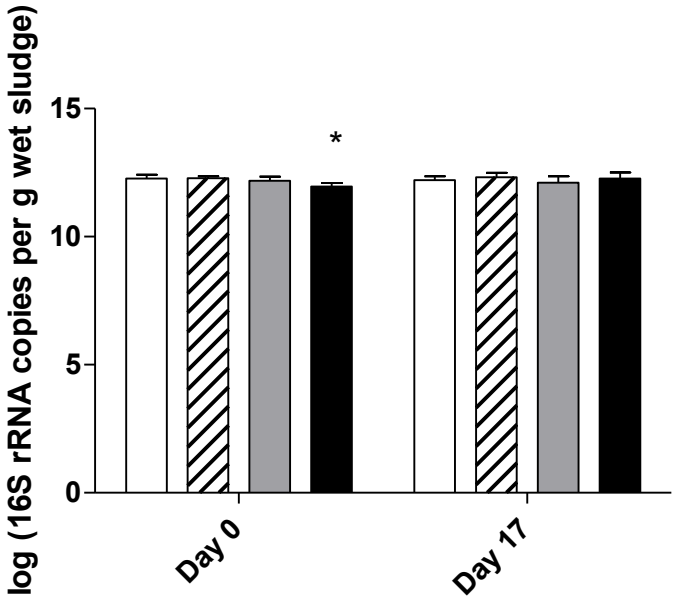


Fig A10. Impact of triclosan on abundance of 16S rRNA in previously unexposed communities. 0, 5, 50, and 500 mg/kg are represented by white, slashed, grey, and black bars, respectively. Error bars represent standard deviation of the mean, n = 9 for triplicate extractions from triplicate reactors. The (*) denotes statistical significance at $p < 0.05$ between a particular triclosan reactor set and the 0 mg/kg reactor set at a given time point.

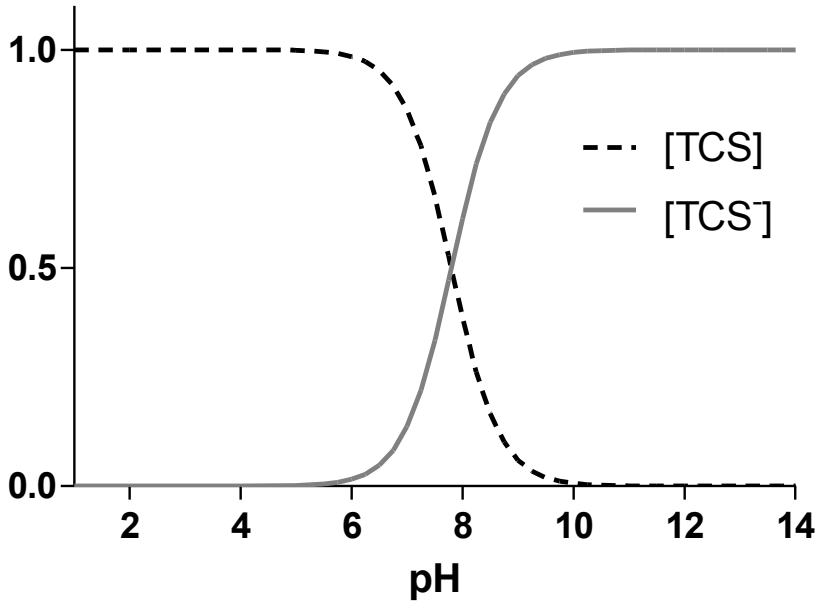


Figure A11. Speciation of triclosan as a function of pH; pKa = 7.8. Typical methanogenic pH values were between pH = 6.5 and pH =8, corresponding to approximately 95%-40% of total triclosan being in the undissociated form, *i.e.* concentration of undissociated form did not change by an order of magnitude.

Appendix B - Supporting Information for Chapter 4

Community Fingerprints

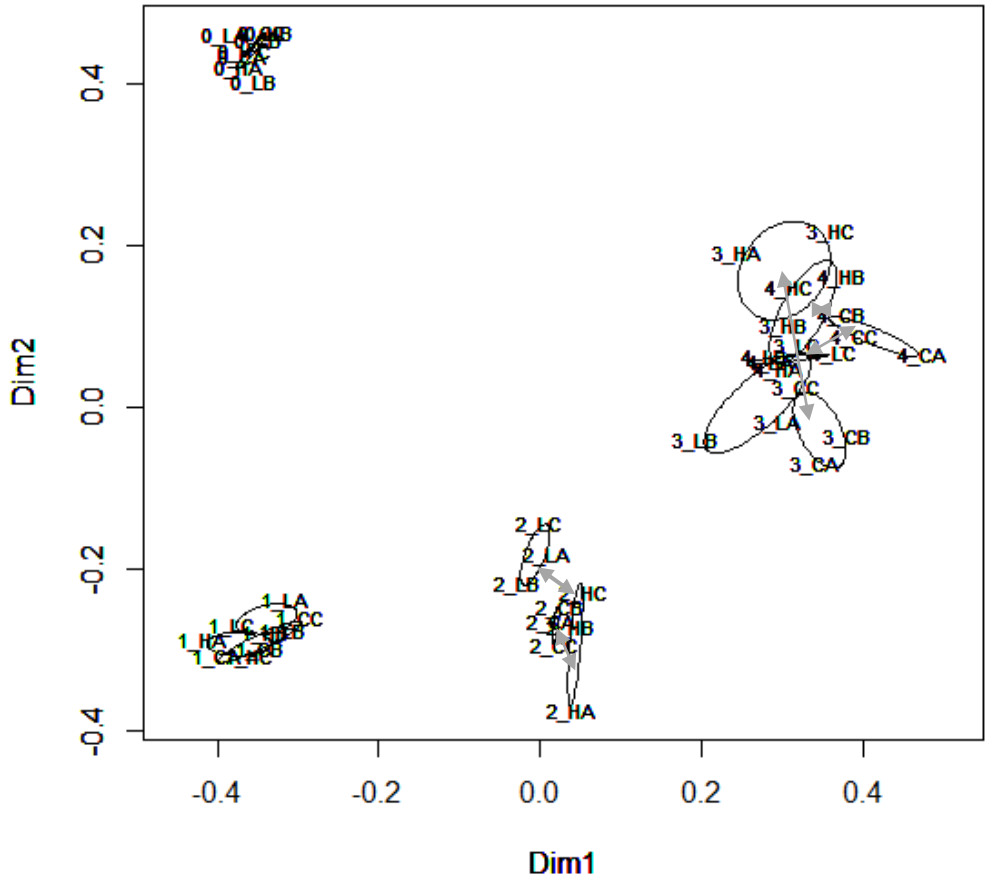


Figure B1. nMDS on *Bacteria* in unperturbed reactors. Control vs Low PFOS (60) vs High PFOS (800). (0 = 0 days, 1 = 15 days, 2 = 50 days, 3 = 115 days, 4 = 140 days). Grey arrows indicate difference between triplicate communities.

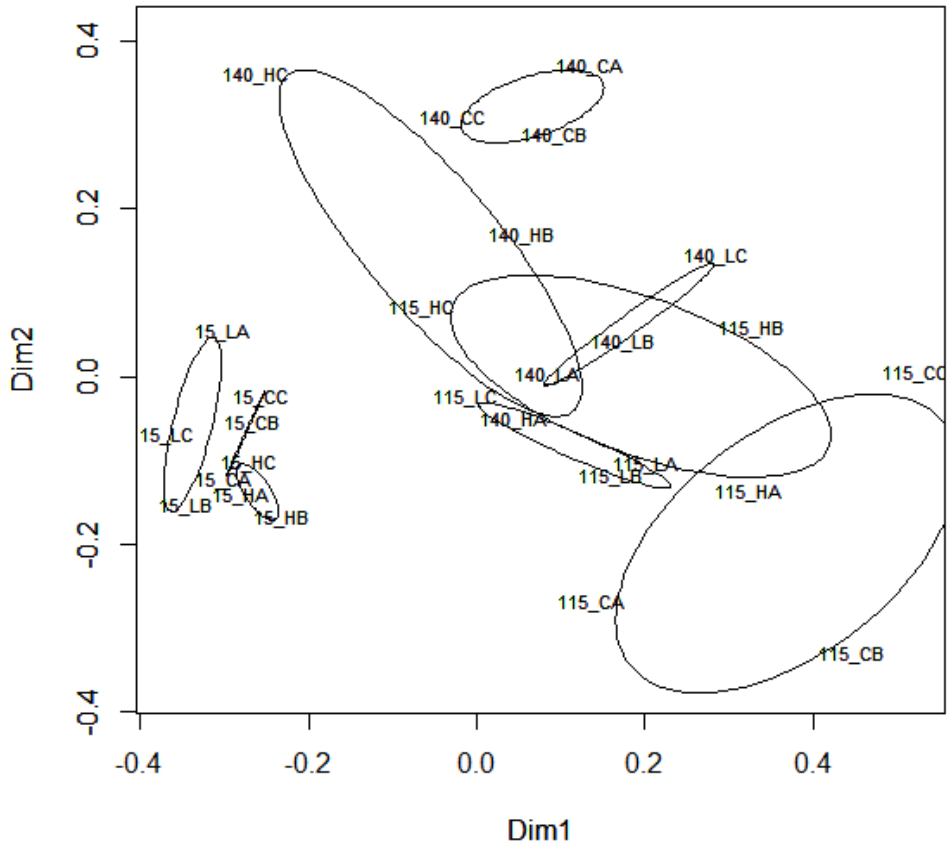


Figure B2. nMDS on *Archaea* in unperturbed reactors. Control vs Low PFOS-60 (L) vs High PFOS-H (800). Days 15, 115, 140 shown.

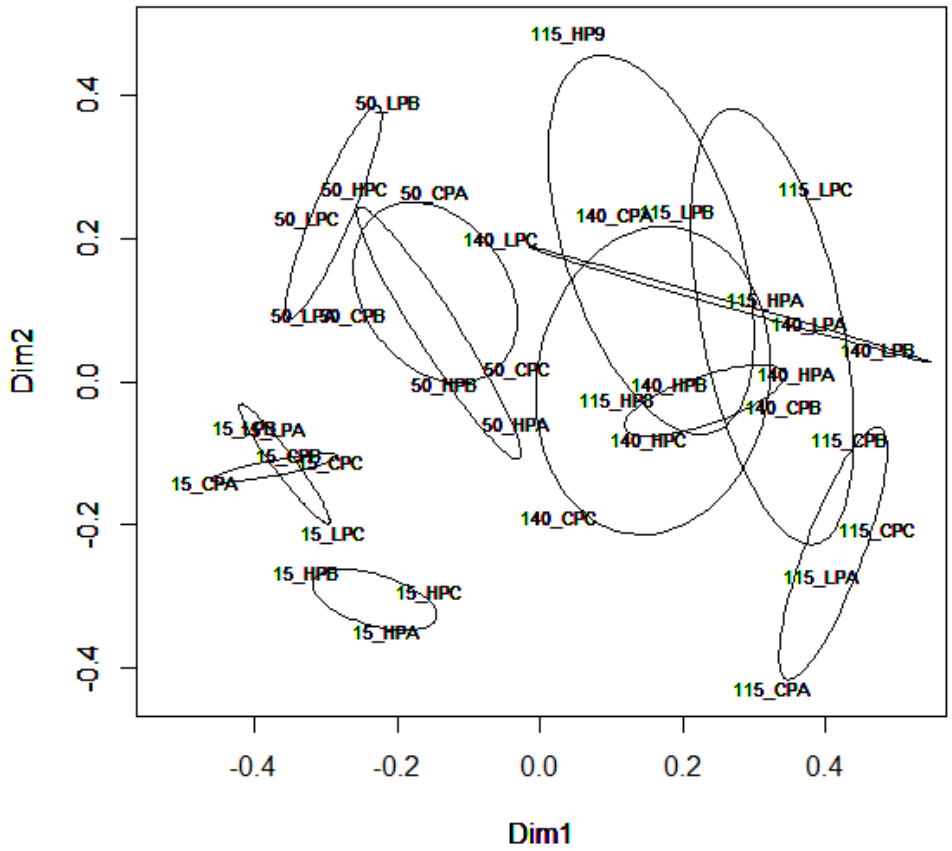


Figure B3. nMDS on *Archaea* in perturbed reactors. Control vs Low PFOS-60 (L) vs High PFOS-800 (H). Days 15, 50, 115, 140 shown.

Functional Data

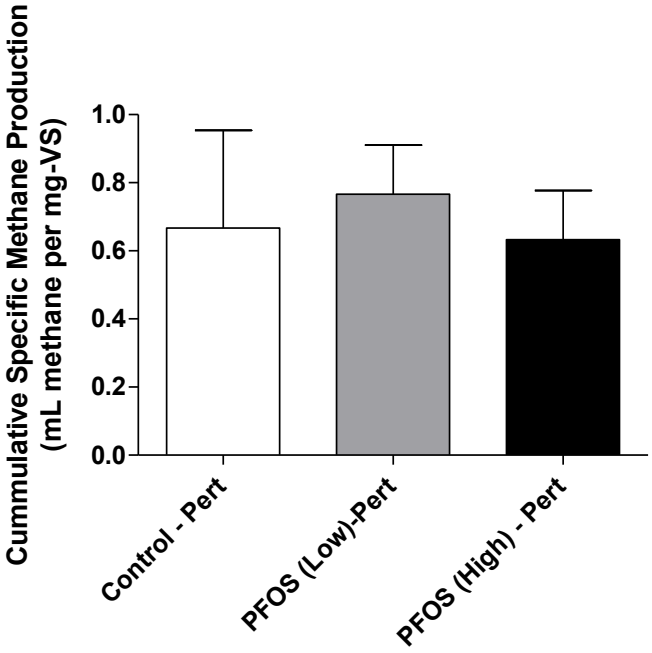


Figure B4. Impact of PFOS on methane production in perturbed reactors after 14 days during long-term experiment, error bars are 95% confidence intervals, n = 3.

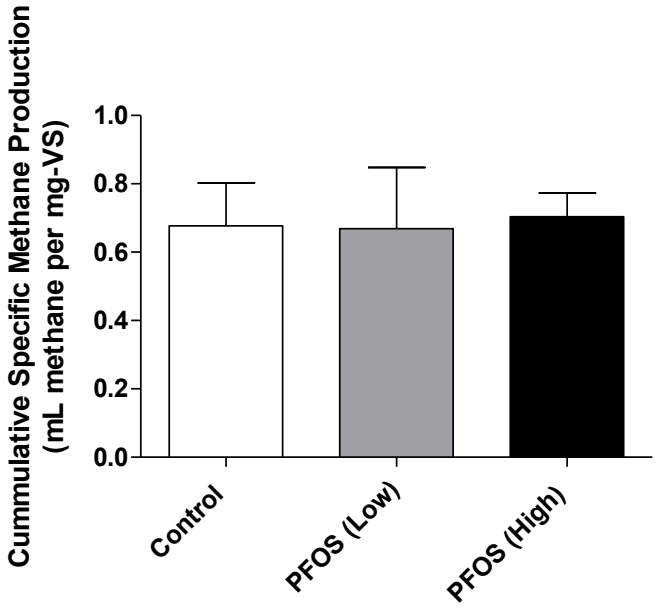


Figure B5. Impact of PFOS on methane production in unperturbed reactors after 14 days during long-term experiment, error bars are 95% confidence intervals, n=3.

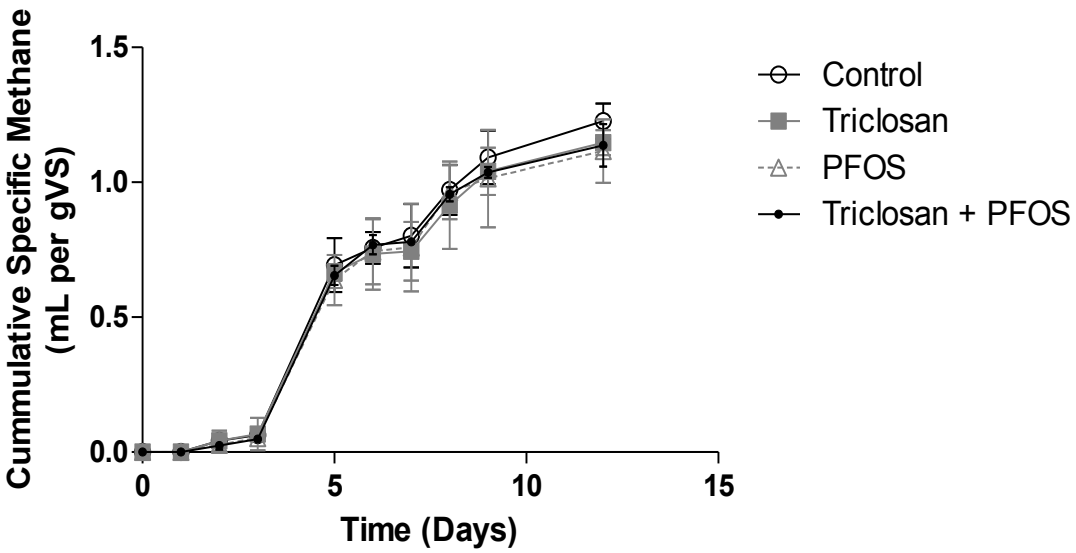


Figure B6. Cumulative specific methane production during short-term 14 day exposure experiment. Error bars are 95% confidence intervals, n=3.

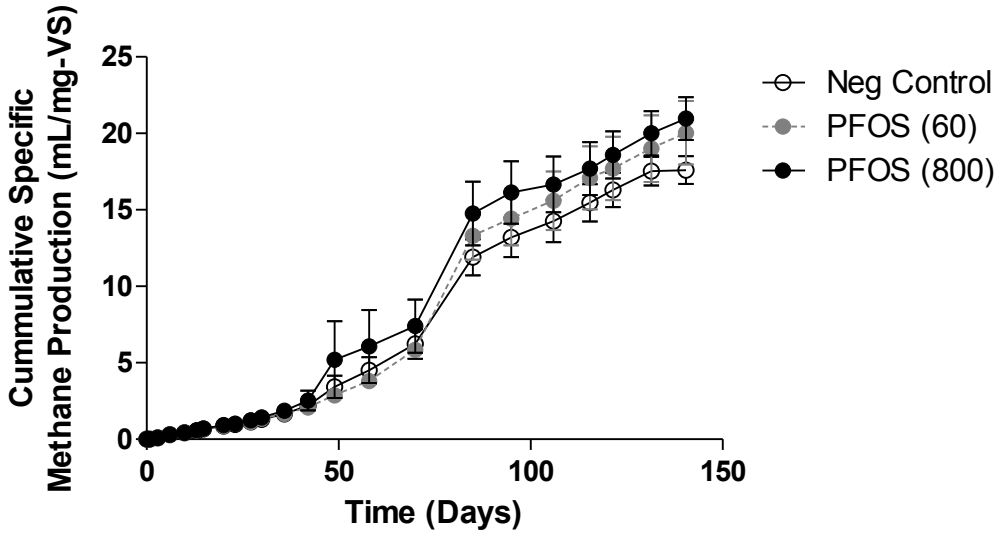


Figure B7. Impact of PFOS on cumulative methane production in long-term 140 day exposure experiment (unperturbed reactors). Error bars are standard error of the mean, n=3. ANOVA p = 0.3439. Data prior to Day 84 were normalized to total volatile solids; data after Day 84 were normalized to total volatile suspended solids.

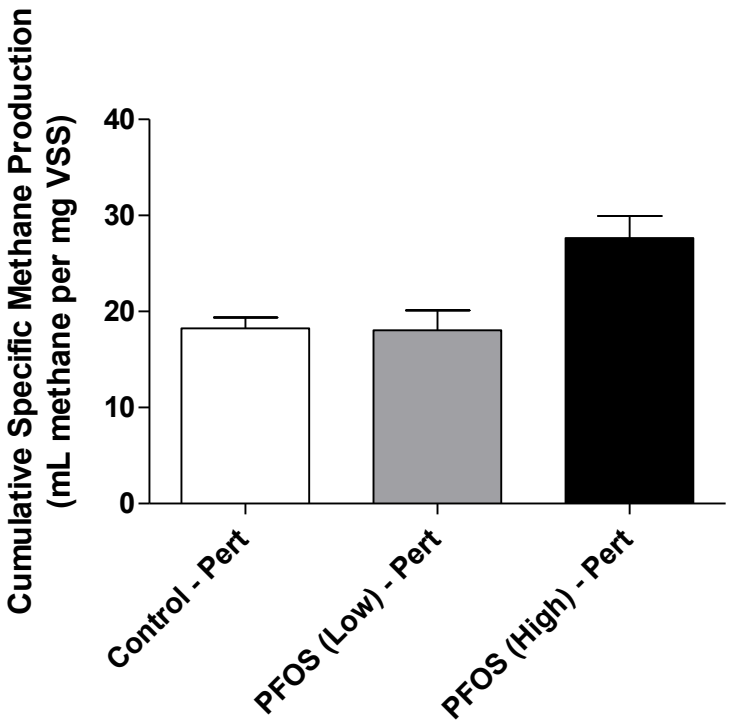


Figure B8. Impact of PFOS on methane production in perturbed reactors after 140 days (error bars are standard error of the mean, n = 3)

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pH and Total Solids

Table B1. pH in Long-Term 140 Day Reactors

Day	C-A	C-B	C-C	CP-A	CP-B	CP-C	L-A	L-B	L-C
30	8.09	8.11	8.03	8.13	8.20	8.14	8.04	8.03	8.04
36	7.93	7.90	7.85	7.91	7.98	7.95	7.94	7.93	7.90
49	7.86	7.83	7.82	7.94	7.86	7.91	7.81	7.75	7.71
84	7.84	7.82	7.71	7.82	7.79	7.80	7.77	7.70	7.82
115	7.75	7.83	7.80	7.77	7.85	7.63	7.76	7.75	7.95
140	7.49	7.67	7.89	8.06	7.64	7.44	7.68	7.65	7.74

Day	LP-A	LP-B	LP-C	H-A	H-B	H-C	HP-A	HP-B	HP-C
30	8.09	8.09	7.99	7.99	8.05	8.02	8.11	8.12	8.12
36	7.85	7.96	7.91	7.90	7.90	7.93	7.89	7.86	7.89
49	7.84	7.87	7.82	7.76	7.83	7.89	7.88	7.90	7.93
84	7.70	7.80	7.74	7.69	7.69	7.71	7.68	7.71	7.76
115	7.76	7.91	7.74	7.67	7.64	7.73	7.72	8.00	7.45
140	7.83	7.62	7.85	7.90	7.74	7.70	7.63	7.89	7.08

C = control, P = perturbed, L = PFOS(Low), H = PFOS(High)

Table B2. Total Solids Measurements in Long-Term 140 Day Experiment

	Total Solids			Volatile Solids		%VS	
Reactor	**Day 0**	**Day 10**	**Day 49**	**Day 10**	**Day 49**	**Day 10**	**Day 49**
CP-A	1.46%	1.60%	0.98%	0.40%	0.09%	24.8%	8.8%
CP-B	1.48%	1.54%	1.00%	0.34%	0.11%	21.9%	11.2%
CP-C	1.18%	1.56%	0.99%	0.35%	0.10%	22.7%	10.1%
C-A	1.58%	1.61%	0.96%	0.37%	0.09%	22.7%	9.7%
C-B	1.51%	1.60%	1.07%	0.36%	0.19%	22.7%	17.3%
C-C	1.55%	1.60%	1.01%	0.36%	0.10%	22.6%	10.0%
LP-A	1.54%	1.52%	1.04%	0.32%	0.12%	21.0%	11.1%
LP-B	1.53%	1.56%	1.02%	0.34%	0.10%	21.6%	9.6%
LP-C	1.51%	1.51%	1.03%	0.30%	0.14%	20.1%	13.6%
L-A	1.50%	1.54%	1.00%	0.35%	0.13%	22.6%	12.5%
L-B	1.48%	1.56%	1.06%	0.33%	0.16%	21.2%	15.4%
L-C	1.52%	1.60%	1.08%	0.39%	0.15%	24.6%	13.8%
HP-A	1.56%	1.67%	0.95%	0.38%	0.08%	23.0%	8.3%
HP-B	1.54%	1.65%	0.99%	0.44%	0.10%	26.4%	10.4%
HP-C	1.53%	1.59%	0.97%	0.37%	0.09%	23.2%	9.0%
H-A	1.48%	1.56%	1.00%	0.37%	0.19%	24.0%	18.5%
H-B	1.50%	1.55%	0.95%	0.36%	0.04%	23.1%	4.2%
H-C	1.52%	1.55%	0.98%	0.38%	0.14%	24.2%	14.2%

CP = control-perturbed; C = control; LP = Low PFOS-Perturbed; L = Low; HP = High PFOS – Perturbed; H = High PFOS.

Table B3. Volatile Suspended Solids in Long-term 140 Day Exposure Experiment

Reactor	Day 84	Day 115	Day 140
CP-A	240	600	467
CP-B	340	500	800
CP-C	176	733	500
C-A	340	767	500
C-B	280	567	567
C-C	350	600	300
LP-A	120	533	1,133
LP-B	280	333	567
LP-C	280	533	567
L-A	460	467	467
L-B	400	700	433
L-C	200	300	567
HP-A	240	500	367
HP-B	180	367	533
HP-C	180	367	300
H-A	180	600	333
H-B	280	800	433
H-C	220	500	233

Table B4. Estimated Mass of Volatile Matter in Reactors

	Total Volatile Solids (mg)		Total VSS (mg)			
Reactor	**Day 10**	**Day 49**	**Day 84**	**Day 115**	**Day 140**	**Avg 84-140**
CP-A	199.3	42.9	12.0	30.0	23.3	21.8
CP-B	168.9	56.3	17.0	25.0	40.0	27.3
CP-C	177.2	49.8	8.8	36.7	25.0	23.5
C-A	182.9	46.7	17.0	38.3	25.0	26.8
C-B	182.0	93.2	14.0	28.3	28.3	23.6
C-C	180.9	50.6	17.5	30.0	15.0	20.8
LP-A	159.4	57.6	6.0	26.7	56.7	29.8
LP-B	168.3	49.0	14.0	16.7	28.3	19.7
LP-C	152.2	70.5	14.0	26.7	28.3	23.0
L-A	174.1	62.6	23.0	23.3	23.3	23.2
L-B	165.8	81.2	20.0	35.0	21.7	25.6
L-C	197.2	74.2	10.0	15.0	28.3	17.8
HP-A	192.3	39.6	12.0	25.0	18.3	18.4
HP-B	217.8	51.9	9.0	18.3	26.7	18.0
HP-C	184.1	43.8	9.0	18.3	15.0	14.1
H-A	187.5	92.6	9.0	30.0	16.7	18.6
H-B	179.7	19.8	14.0	40.0	21.7	25.2
H-C	187.6	69.8	11.0	25.0	11.7	15.9

ATP Data

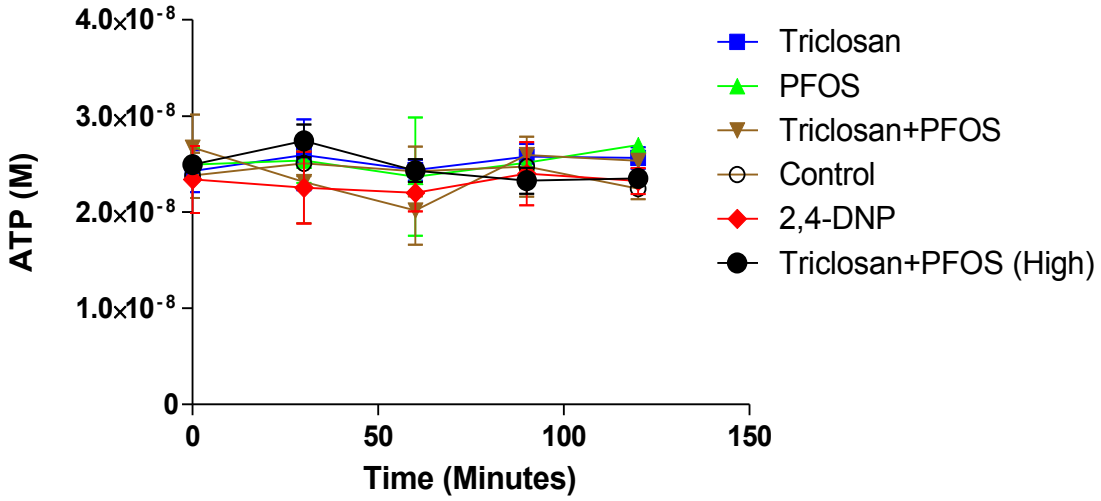

Figure B9. Impact of micropollutants on ATP levels over two hours.

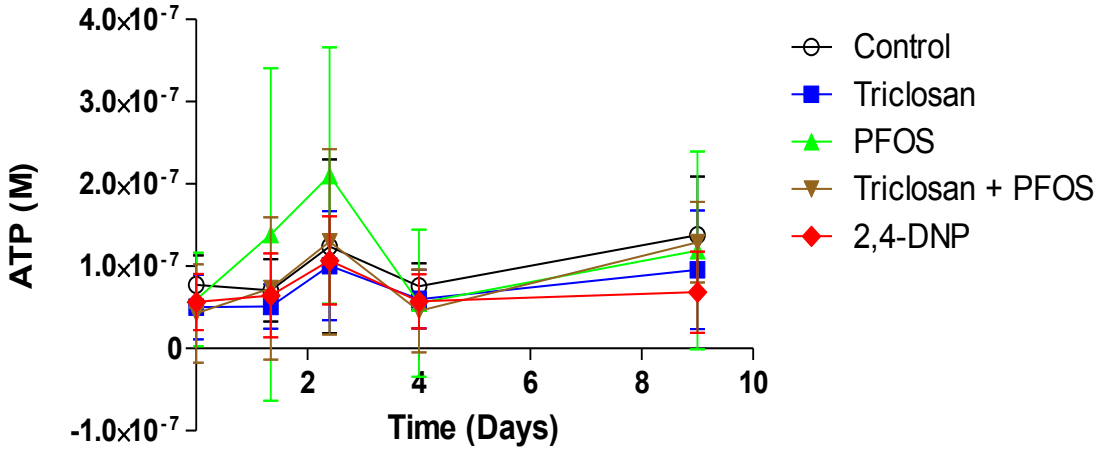

Figure B10: Impact of micropollutants on ATP levels in over nine days.

Appendix C - Impact of Micropollutants on Pure Culture Methanogens

Pure culture methanogens were grown to test the direct impacts of micropollutants on methanogenesis. *M. thermophila* (DSM 1825) and *M. acetivorans* (DSM 2834) were purchased from Deutsche Sammlung von Mikroorganismen (DSMZ), and grown according to supplier's instructions (www.dsmz.de). A typical growth curve is shown in Figure C1. The total methane was normalized to volatile suspended solids. The amount of methane produced under various micropollutant concentrations are shown in Table C1. Both cultures were able to grow when exposed to triclosan at levels greater than 10,000 mg/kg (10 μM), and thus these cultures would not be susceptible to triclosan at environmentally relevant levels (≤133 mg/kg). These results suggest that triclosan does not inhibit function of methanogens at environmental levels. At much higher levels, however, triclosan likely lyses methanogens as suggested by Figure C2.

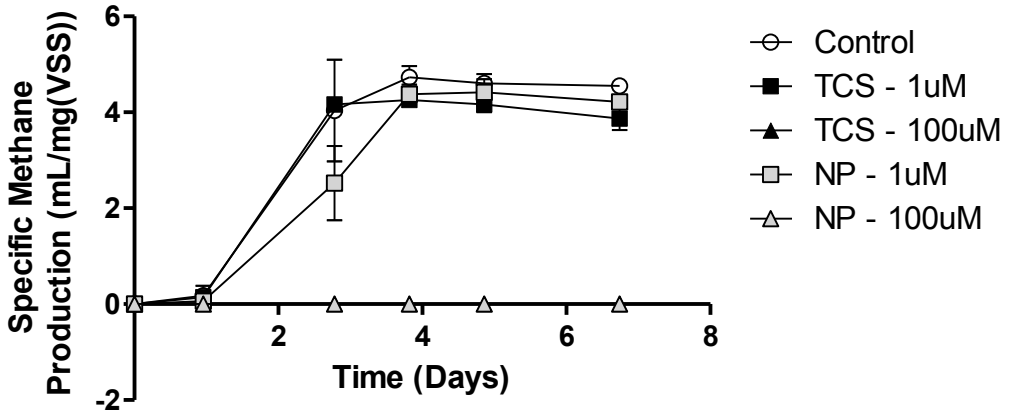

Figure C1. Impact of triclosan and nonylphenol on *M. thermophila.*

Table C1. Impact of Micropollutants on Growth of Pure Culture Methanogens

	M. acetivorans			*M. thermophila*			
	X	[C] (mg/kg TS)	*mL CH_4 per mg VS*		[C] (mg/kg TS)	X	*uM*
Triclosan	0.E+00	0	3.78	*4.84*	0	0	0
	9.E-01	5	4.63				0.01
	1.E+02	493	4.68	4.25	781	156	1
	0.E+00		No Growth	No Growth	598,643	119,729	10
	1.E+04	53,148	17.8	7.16	19,704	3,941	
	0.E+00	87,756	No Growth	No Growth	1,621,296	324,259	100
Nonylphenol	0	0	3.78	*4.84*	0	0	0
	99	497	1.89	4.43	596	119	1
	995		No Growth	No Growth	1,602,290	1,192	10
	9,947	118,097	No Growth	No Growth	1,385,185	11,920	100
PFOS	24	8,710	4.70	*9.06*	19,369	54	10
Gen		2	*4.78*	*9.09*			0.004
2,4-DNP				*7.89*	11,224		*10*
PFOS/TCS				7.89			*5 & 5*

X = 5 mg/kg

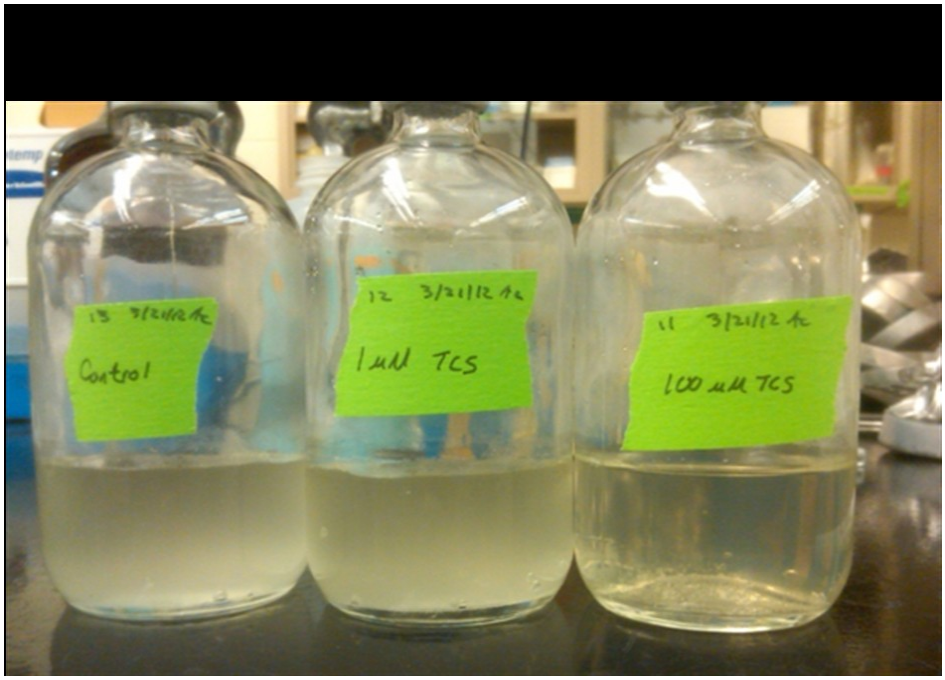

Figure C2. Impact of triclosan on visible turbidity in *M. acetivorans*.

Appendix D - Triclosan Enriches for *Dehalococcoides*-Like *Chloroflexi* in Anaerobic Soils Despite Minimal Impacts on Microbial Community Structure

The following work was a collaborative effort between my colleague, Dr. Mark Krzmarzick, and myself, Patrick McNamara.

INTRODUCTION

Triclosan is a biologically active micropollutant[1] that poses a range of environmental health concerns. Recent work on its impacts to multicellular organisms found that triclosan decreases aggression in fathead minnows[2] and muscle functioning in mice.[3] Triclosan, which is used as a household antimicrobial agent, is commonly detected in human urine[4] and enters the environment via discharge of effluent liquids and solids from wastewater treatment plants.[5,6,7,8,9] An additional environmental concern associated with triclosan is that it is a precursor for dioxin in the environment following photolysis in surface waters.[10,11] A substantial portion of triclosan that enters a treatment plant, however, sorbs to solids and undergoes solids handling treatment.[6] Triclosan has been shown to be recalcitrant under anaerobic conditions[12,13,14] and passes through advanced solids handling treatment processes unremoved.[15] It is through these wastewater solids that approximately 50,000 kg of triclosan is discharged into the environment via land application of biosolids.[8]

So what does triclosan, a biologically active micropollutant, do to *Bacteria* after land-application? This question has been partially answered with respect to aerobic communities, but little is known about its impacts in anaerobic soils where it will likely accumulate.[16,17] Triclosan can persist in soil for hundreds of days,[18,19] where it may bioaccumulate in worms[20] or be taken up into roots.[21] In aerobic soils, it has been shown to shift community structure.[22] Under anaerobic conditions, the effect of triclosan on microbial communities has not been investigated. Furthermore, triclosan, being a

chlorinated organic molecule, may be degraded through reductive dechlorination in anaerobic environments by halorespiring organisms, which are ubiquitous in natural soil environments and sediments.[23,24] The ability for halorespiring bacteria to use triclosan as a terminal electron acceptor has not been studied. Additionally, halorespirers may simply become enriched in the microbial community as these bacteria have shown tolerance for some chlorinated pollutants[25,26] and some antibiotics,[27,28] though no research to date has documented tolerance of halorespirers specifically to triclosan. In this manuscript, we hypothesized that triclosan would enrich for *Dehalococcoides*-like *Chloroflexi* bacteria and structurally alter the *Bacterial* community in batch reactors containing agricultural soil—a common sink for triclosan following land application of biosolids. We used two community fingerprinting analyses to study community structure and qPCR to enumerate *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes and *Bacteria* 16S rRNA genes over 618 days. Understanding the effect of triclosan on anaerobic microbial communities is important considering the widespread distribution of triclosan in the environment.

EXPERIMENTAL SECTION

Batch Reactors. Anaerobic batch reactors were inoculated with soil to test the impacts of triclosan on anaerobic microbial community structure. Agricultural soil from a corn-soybean rotation field was collected one foot below surface from a farm near Fairfax, MN in June 2010. Collected soil was filled and packed into a glass container to limit atmospheric headspace. Soil was stored in glass jars in an anaerobic glovebag until reactor startup (approximately 1 week later). The soil chosen has no previously known

exposure to triclosan from municipal waste or from agricultural practices. No background concentration of triclosan was detected (see Table S1 for LC-MS-MS analysis results of triclosan in soil).

Serum glass bottles (160 mL) capped with Teflon-lined septa were used for batch reactors. Reactors amended with farm soil and triclosan were operated in triplicate, and one control set was not-amended with triclosan but operated under identical conditions. The triclosan reactors were spiked with 40 μg of triclosan with a methanol stock solution; the methanol was allowed to evaporate to dryness. Each reactor received 40 g farm soil, 40 mL mineral media[29] reduced with titanium citrate, 1.0 mL of potassium acetate (10 mM), and 1.0 mL yeast extract solution (0.1 g/L). Reactors were set-up and kept in an anaerobic glovebag with a 1-3% H_2 headspace (Coy Labs) during the duration of the experiment.

Samples were taken at days 0, 42, 108, 190, 275, 406, 472, and 618 from each reactor. During sampling, bottles were manually shaken for 5 minutes to mix contents, opened in the glovebag, and 1.5 mL of mixed slurry was transferred with sawed-off Pasteur pipettes to 1.5 mL microcentrifuge tubes. An additional 1.0 mL aliquot of potassium acetate (10 mM) was added after sampling at days 275 and 406. Batch reactors became pressurized between samplings, indicative of methanogenesis. Microbial samples were stored at -20^{o}C until DNA extraction. Aliquots for triclosan analysis were also taken during sampling and stored at -20^{o}C.

LC-MS-MS Analysis. The collected soil and selected reactor samples were analyzed for triclosan concentration. Samples were freeze-dried, extracted with an Accelerated Solvent Extractor (Dionex), and analyzed with Waters NanoAcquity ultra performance liquid chromatograph (UPLC) connected to a Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer (MS-Q^3). Background levels in the soil were below the limits of quantification (see Table S1). Measuring triclosan concentrations for reactor samples were attempted but were not quantitatively reliable, likely due to strong initial sorption to the glass bottles followed by desorption. Nonetheless, triclosan was detected in the soil matrix for the duration of the experiment.

Bacterial Community Profiling. DNA was extracted with a PowerSoil DNA extraction kit (MoBio Laboratories). From this DNA extraction, the microbial community was profiled with automated ribosomal intergenic space analysis (ARISA) and denaturing gradient gel electrophoresis (DGGE). Quantitative-PCR (qPCR) was also used to quantify *Dehalococcoides*-like *Chloroflexi* and *Bacteria* 16S rRNA genes.

Community fingerprints for the reactors were assessed by ARISA as described previously[30] for the samples from days 0, 108, 275, 472, and 618. ITSF and ITSReub primers from Cardinale *et al.,* 2004[31] were used to amplify the intergenic spacer region of *Bacteria*. Peak areas were analyzed with the open software PeakScanner Software v 1.0 (Applied Biosystems). The amplified regions of the 16S and 23S genes in conjunction with primers were deleted to yield intergenic spacer length.[31] Fragments greater than 1000 bp were also eliminated. Only peaks that accounted for ≥ 0.5% of the total area for

the sample were used for further analysis. Fragments were binned using the R code "interactive binner" provided by Ramette, 2009.[32] The window size was 2 bp and the shifting window was 0.1 bp. For each sample the PCR reaction was carried out in triplicate. Only fragments that occurred in at least two of three replicates were used. The average percent area for the amplified replicates was used for final analysis.

Denaturing gradient gel electrophoresis (DGGE) was performed as previously described.[33,34] 16S rRNA genes were amplified in 30 μL reactions with 10X PCR reaction buffer (Bioline), 2.5 mM $MgCl_2$, 30 μg bovine serum albumin, 0.13 mM dNTPs, 1 unit of *Taq* polymerase (Bioline), 0.5 μM each of primers 338F (5'-ACT CCT ACG GGA GGC AGC AG-3') attached to a GC clamp and 518R (5'ATT ACG GCC GCT GCT GG -3'),[35] and 1 μL of DNA extract. PCR thermocycling conditions are described previously.[33] PCR amplifications were analyzed on a polyacrylimide gel (8% wt/vol) ranging from 30-55% denaturant (100% denaturant contains 7 M urea and 40% formamide) with a D-Code apparatus (BioRad). Electrophoresis was performed for 4 hours at 200 V and at 60^{o}C. The gel was then stained with SYBR Green I (Molecular Probes) and imaged with Quantity One software (BioRad).

The abundance of *Dehalococcoides*-like *Chloroflexi* 16S rRNA gene copies[23] and *Bacteria* 16S rRNA genes[36] were determined by qPCR using standards and methods previously described.[23] For both *Bacteria* and *Dehalococcoides*-like *Chloroflexi*, 16S rRNA genes were quantified in duplicate and the means were used for further analysis.

Results reported from qPCR represent means and standard errors of the values from triplicate reactors for triclosan-amended and unamended controls.

Statistical Analysis. The DGGE gel image was analyzed using QuantityOne Software (BioRad). Lanes were manually anchored and the software then subtracted background signal, detected bands, and matched bands using input parameters sensitive enough to detect all visible bands. Band matching and sizing was manually inspected. The peak intensities of each band, relative to the total intensity for the lane, were determined and used for nonmetric multidimensional scaling analysis.

Nonmetric multidimenionsal scaling (nMDS) was used to analyze ARISA and DGGE data as described by Nelson *et al.,* 2010.[37] The *vegan* package in R was used for nMDS analysis.

Student t-tests were used to compared qPCR values and Shannon's diversity indexes between triclosan amended and unamended controls. For *Bacteria* 16S rRNA gene copies and diversity indexes, two-tailed t-tests (equal variances) were used to determine significance ($P<0.05$). For *Dehalococcoides*-like *Chloroflexi* 16S rRNA gene copies, one-tailed t-tests (equal variances) were used to determine if triclosan-amended reactors had statistically greater numbers than the controls. Two-tailed Student t-tests were also used to compare relative peak areas for the DGGE bands and ARISA fragments between triclosan and control reactors. Spearman's rank correlation was used to determine the covariance between the change in *Bacteria* 16S rRNA genes and the

change in *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes in both the control and triclosan-amended reactors.

RESULTS

DGGE Analysis. DGGE was performed to determine i) if overall community structure changed and ii) if abundant members were systematically enriched as a result of triclosan. DGGE community fingerprints from the initial community (Day 0) and from the triclosan-amended and control reactors at days 472 and 618 are shown in Figure 1. From visual inspection of Figure 1, the communities appear similar with the exception of two bands that appear prominently in one triplicate reactor amended with triclosan (Triclosan A). These bands, however, are present at lower relative intensities in the other triclosan-amended and control reactors, and the relative intensities of these bands are not significantly higher in the triclosan-amended reactors than in the control reactors (For band A, $p = 0.16$; for band B, $p = 0.06$). Bands similarly positioned also appear prominently at earlier time points in some control reactors as well (see Figure S1) and thus these bands are not considered connected to triclosan amendment. No bands appeared in the triplicate triclosan-amended reactors that did not appear in the triplicate control reactors and vice-versa, and no bands had significantly greater relative intensities in the triclosan reactors compared to the control reactors or vice versa at Day 472 or Day 618. The relatively high complexity of the communities in all reactors is evident from the multiple banding patterns and multiple regions which appear as smears. It is thus not surprising that DGGE results revealed no clear differences in individual enriched

members of these complex communities, but DGGE results certainly revealed that communities were complex.

To further examine these, the relative band intensities for the triclosan-amended and control reactors at days 472 and 618 were used for nMDS analysis. This analysis confirms that the overall structure of the triclosan-amended and control reactors were not statistically different, as indicated by their occupation of overlapping space (Figure 2). The Shannon's Diversity index and evenness coefficients, based on DGGE analysis, were not significantly different ($p = 0.23$ and $p = 0.23$, respectively) between the triclosan-amended and control reactors, further supporting the notion that triclosan did not cause large enough shifts in community structure to be statistically-significantly detected with DGGE analysis.

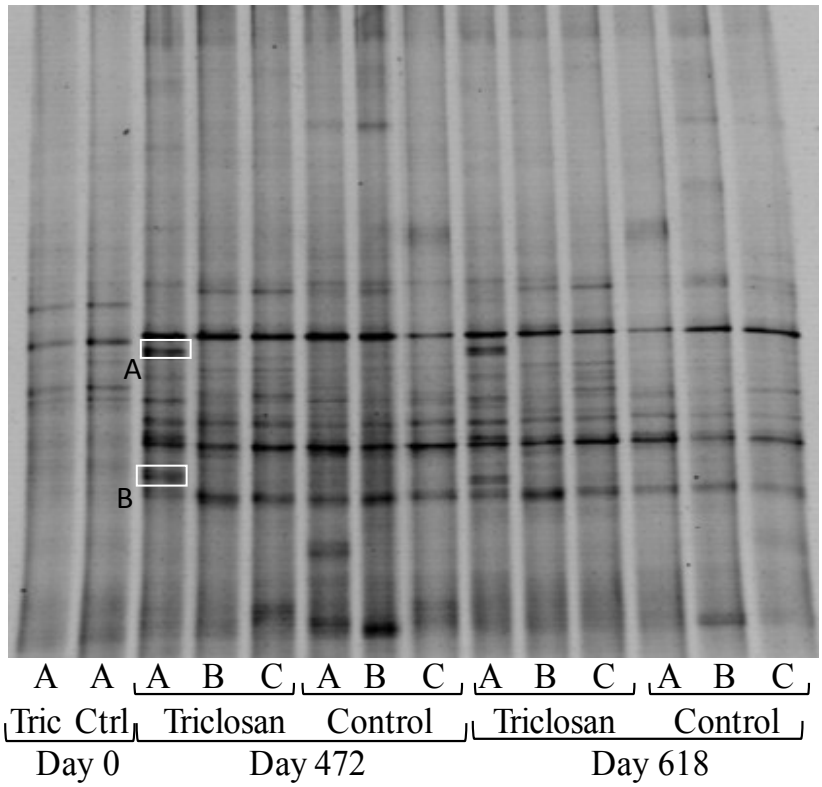

Figure 1. PCR-DGGE fingerprints of initial reactor samples (Day 0) from triclosan-amended reactor A (Tric) and control reactor A (Ctrl) and fingerprints of triplicate triclosan-amended reactors and control reactors at Day 472 and Day 618. No overall change in community structure as a result of triclosan amendment is evident from the DGGE gel image.

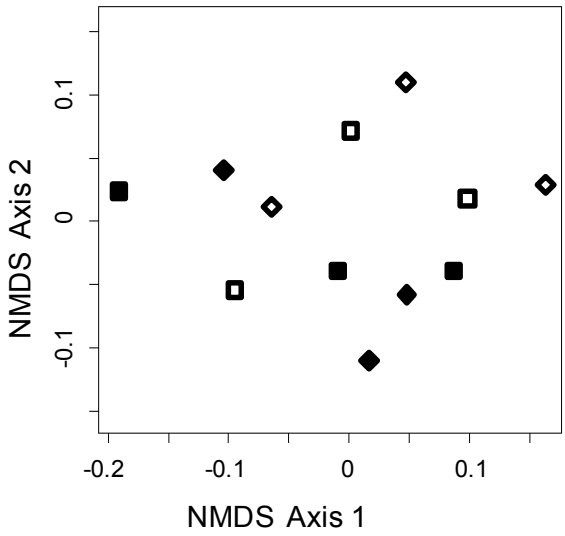

Figure 2. Results of nonmetric multi-dimensional scaling (nMDS) analysis for the *Bacteria* based on DGGE gel analysis. Triclosan amended reactors are depicted as solid symbols and control reactors are depicted as clear symbols. Squares correspond to Day 472 samples and diamonds correspond to Day 618 samples. 16S rRNA communities are similar between the triclosan-amended and control samples based on DGGE analysis.

ARISA. Automated ribosomal intergenic spacer analysis (ARISA) was performed to validate the DGGE results and more quantitatively determine if community structures shifted as a result of triclosan over the duration of the experiments. NMDS was used to analyze ARISA data (Figure 3). The triclosan-amended and control communities grouped very closely at Day 0. The community profiles at later sampling time points shifted away from the Day 0 communities, and the communities at Day 618 were the most dissimilar from the communities at Day 0. The ellipses, which encompass triplicate reactors at a given time point, intersect at Day 108, Day 275, and Day 472, indicating that communities are quite similar in triclosan-amended and control reactors. By Day 618, however, the ellipses do not overlap indicating a small shift in community structure as a

result of triclosan. This shift, though, was not statistically significant at the 95% confidence interval. Shannon's Diversity and evenness coefficients, as calculated from ARISA data, did not yield significant differences between the two sets of reactors at any given time point either. When comparing the impact of time on evenness using a paired t-test, the triclosan-amended reactors had a significant increase in evenness between Day 0 and Day 618 ($p = 0.01$), while the control reactors had an increase less than statistical significance ($p = 0.07$). Since triclosan has antimicrobial capabilities it is feasible that some dominant members of the original community were inhibited and the community became more even. The DGGE and ARISA data both reveal that triclosan, spiked at environmentally relevant concentrations, did not substantially alter the macro-microbial community structure, despite its antimicrobial capabilities. Additionally, the change between the initial and subsequent sampling points were similarly different for triclosan and unamended reactors over time indicating the community change was most strongly affected by setup and treatment of the reactors, not triclosan amendment.

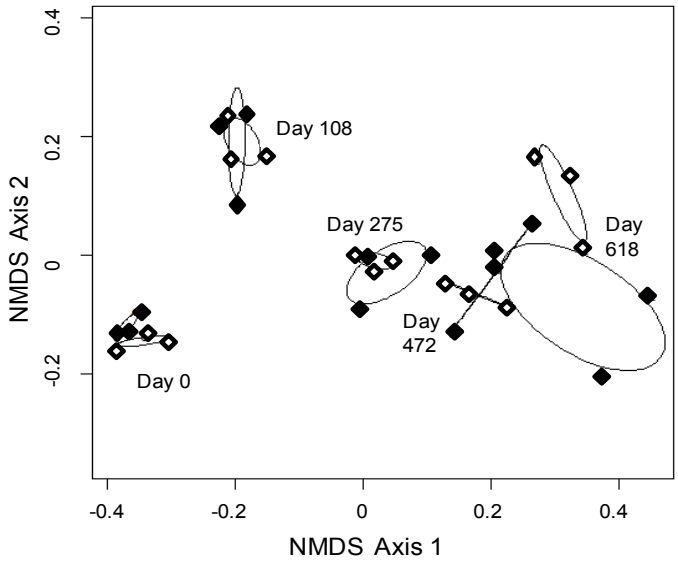

Figure 3. Community composition as analyzed by nonmetric multidimensional scaling for ARISA data. Communities from triclosan-amended reactors are depicted by solid symbols and communities from control reactors are depicted by open symbols.

Quantification of *Bacteria* and *Dehalococcoides*-like *Chloroflexi*. Quantitative-PCR was performed to determine if triclosan presence correlated to the abundance of *Dehalococcoides*-like *Chloroflexi,* which are known to tolerate and breathe chlorinated organic compounds.[25,27,28] Although the ARISA and DGGE analysis can be used to determine shifts in the community of dominant members,[31,38] changes in less represented members are not detected. Indeed, at the end of the experiment the abundance of *Dehalococcoides*-like *Chloroflexi* was significantly higher ($p = 0.03$) in the triclosan-amended reactors than in the control reactors, despite no difference in the abundance of total *Bacterial* 16S rRNA genes between reactor sets at that time (Figure 4). The changes in *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes in the control reactors also strongly

correlated with the changes in the *Bacterial* 16S rRNA genes (Spearman's $\rho = 1.00$, $p < 0.001$), with the largest increases occurring directly after addition of acetate to the reactors. In the triclosan-amended reactors, however, the changes of *Dehalococcoides*-like *Chloroflexi* were independent of changes in *Bacterial* 16S rRNA genes (Spearman's $\rho = 0.25$; $p = 0.59$).

The impact of triclosan on *Bacteria* 16S rRNA genes was variable. At 108 days, the number of *Bacteria* 16S rRNA genes was significantly lower in the triclosan-amended reactors than in the control reactors, which is not surprising given that triclosan is an antimicrobial agent. This effect was not sustained though, and by 190 days the triclosan-amended reactors had significantly greater numbers of 16S rRNA genes. Any difference in *Bacteria* 16S rRNA genes no longer existed between the triclosan and control reactors by the end of the experiment.

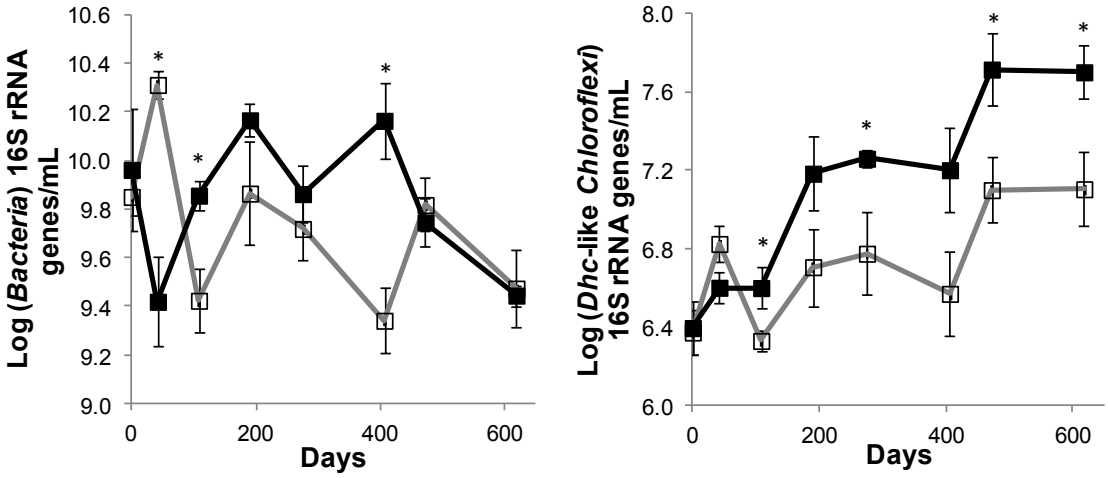

Figure 4. Number of *Bacteria* 16S rRNA genes per mL of reactor slurry (left) and number of *Dehalococcoides*-like (*Dhc*-like) *Chloroflexi* 16S rRNA genes per mL of reactor (right) over the duration of the experiment. Samples from triclosan-amended reactors are depicted by black symbols, black line and samples from control reactors are depicted by white symbols, grey line. Error bars indicate standard error of the mean for the triplicate reactors. Asterisks correspond to statistically significant differences between the triclosan- amended reactors and control reactors (Student t-test, $p < 0.05$).

DISCUSSION

Overall, triclosan had negligible impacts on the macro-level community structure in our study as determined by ARISA and DGGE. To our knowledge, our study is the first to analyze community structure changes resulting from triclosan amendment in strictly anaerobic batch reactors. Somewhat contradictory results concerning the effect of triclosan on bacterial community structure has been reported in aerobic systems. In one study, triclosan shifted the community based on TRFLP analysis,[22] but in another study, high dosages of triclosan were not shown to dramatically alter the microbial community structure over 70 days as determined from phospholipid fatty-acid analysis.[39] In both

studies the communities changed temporally, corroborating the temporal shifts observed in our study. Other biologically active micropollutants, such as pharmaceuticals and the antimicrobial sulfamethoxazole, have been shown to have subtle effects on microbial community structure,[40,41] and these impacts can be exacerbated when mixed with other micropollutants.[42] These results indicate that the impacts of micropollutants on community structure should be taken into account when evaluating the environmental impacts of a micropollutant, especially since microbial changes can lead to altered functional performance.[40,41,43]

Dehalococcoides-like *Chloroflexi* 16S rRNA genes were significantly higher in triclosan-amended reactors than in control reactors. The growth in the control reactors was not unexpected as *Dehalococcoides*-like *Chloroflexi* have been shown to likely dechlorinate naturally produced organochlorines.[23] The soils used in this study were rich in organic matter and thus likely contained some natural organochlorines. Nonetheless, the abundance of *Dehalococcoides*-like *Chloroflexi* grew 86 percent greater with triclosan amendment (1.3 ± 0.2 log units in the triclosan-amended reactors compared to 0.7 ± 0.2 log units in the unamended controls) which was statistically significant ($p = 0.05$). This result indicates that the presence of triclosan was favorable towards the growth of *Dehalococcoides*-like *Chloroflexi*. Two main explanations exist for the relative growth of *Dehalococcoides*-like *Chloroflexi* with triclosan amendment: 1) *Dehalococcoides*-like *Chloroflexi* were able to gain a competitive advantage for nutrients, carbon, or electron donor, and/or 2) *Dehalococcoides*-like *Chloroflexi* were

able to use triclosan as a terminal electron acceptor and thus grew as a result. Resistance to triclosan in *Bacteria* is variable.[1,44,45] Outer membrane permeability limitations have been linked to triclosan resistance[46] and the S-layer type cell wall found in *Dehalococcoides* spp.[27] may theoretically contribute to triclosan resistance by limiting permeability. It is evident in our results that, though *Bacteria* 16S rRNA genes decreased in the first 42 days in the triclosan reactors, the *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes actually increased, thus suggesting possible resistance. It is possible that *Bacteria* that compete with *Dehalococcoides*-like *Chloroflexi* for nutrients, carbon, or electron donor were lost from triclosan exposure while *Dehalococcoides*-like *Chloroflexi* were unaffected, thus allowing *Dehalococcoides*-like *Chloroflexi* a relative competitive advantage for growth and activity. In the enrichment of *Dehalococcoides*, antibiotics are often used as a selective agent to aid in isolation.[27,28] In one study, the suppression of methanogenesis activity with bromoethanesulfonic acid resulted in higher dechlorination activity, and the use of the antibiotic vancomycin resulted in an increase in abundance of *Dehalococcoides* spp. with a concomitant decrease in abundance of other Gram-positive bacteria.[47] The increase of *Dehalococcoides*-like *Chloroflexi* from triclosan amendment in our reactors may have been from a similar selective enrichment for *Dehalococcoides*-like *Chloroflexi* that were presumably using natural organochlorines nascent in the organic matter of the soil.

The second possibility for increased *Dehalococcoides*-like *Chloroflexi* is that these halorespirers may have actively used triclosan for growth; however, the ability of

Dehalococcoides-like *Chloroflexi* to use triclosan as a terminal electron acceptor has not been established. The dehalogenation of triclocarban—a similar antimicrobial compound that occurs concomitantly with triclosan[12,48] has been observed.[12] In one study, triclosan and triclocarban concentration trends tracked similarly in Jamaican Bay sediments where dehalogenation products of triclocarban were only minimally observed.[12] In the same study, Chesapeake Bay sediments, however, showed strong evidence of triclocarban dehalogenation, and in these sediments triclosan was not detected above limits of quantification despite historical contamination.[12] The fact that triclosan was not detected in the sediments showing triclocarban dechlorination but persisted in the sediments showing only low levels of triclocarban dechlorination is curious due to the expected commingling of these contaminants and the otherwise pervasive nature of triclosan in anaerobic sediments.[13,14] This result implores the possibility that triclosan was removed via reductive dehalogenation. Nevertheless, evidence of dehalogenated triclosan metabolites were not investigated in that study, and therefore dehalogenation was not conclusively determined. In other work, a trichloroethene reductively dechlorinating culture (DehaloR^2) was developed from the Chesapeake Bay sediment contaminated with triclosan and triclocarban, but not with chlorinated ethenes,[26] further inferring the possibility that triclosan may be dechlorinated and used as a terminal electron acceptor. In our work a decrease in triclosan concentration over time could not be established (see Methods). Other triclosan removal pathways, such as methylation of the aromatic rings by other microorganisms[49,50] prior or after a reductive dechlorination process is also

possible, but these metabolites were not investigated. Careful work with labeled triclosan should be performed to conclusively determine if *Dehalococcoides* spp. or other halorespirers can use triclosan as a terminal electron acceptor.

Although the effect on the macro-community structure appeared to be minimal, a significant increase in the abundance of *Dehalococcoides*-like *Chloroflexi* 16S rRNA genes indicates that these halorespiring bacteria are nonetheless enhanced and thus triclosan does affect the microbial community. The global application of triclosan through biosolids may thus pose inadvertent affects on the natural biogeochemical cycling of organochlorines in addition to its already menacing qualities to environmental health, or, alternatively, our research indicates that triclosan could purposefully aid the dehalogenation of toxic anthropogenic compounds at contaminated sites.

ASSOCIATED CONTENT

Supporting Information

Table S1 details LC-MS-MS quantification of triclosan in the soil used for this study, which was below limit of quantification. Figure S1 contains gel images from the DGGE analysis of each reactor over the duration of the experiment.

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Supporting Information

Table S1: Background Levels of Triclosan in Soil, Measured in Triplicate, were Below Limit of Quantification

Sample	g/L in Extract	mg/kg	13C-Recovery
Soil (Replicate 1)	3.55E-06	0.00313	56%
Soil (Replicate 2)	2.55E-06	0.00156	39%
Soil (Replicate 3)	3.10E-06	0.00213	51%
Sand Blank	4.17E-06	0.00041	19%
LOQ	2.18E-05	NA	NA

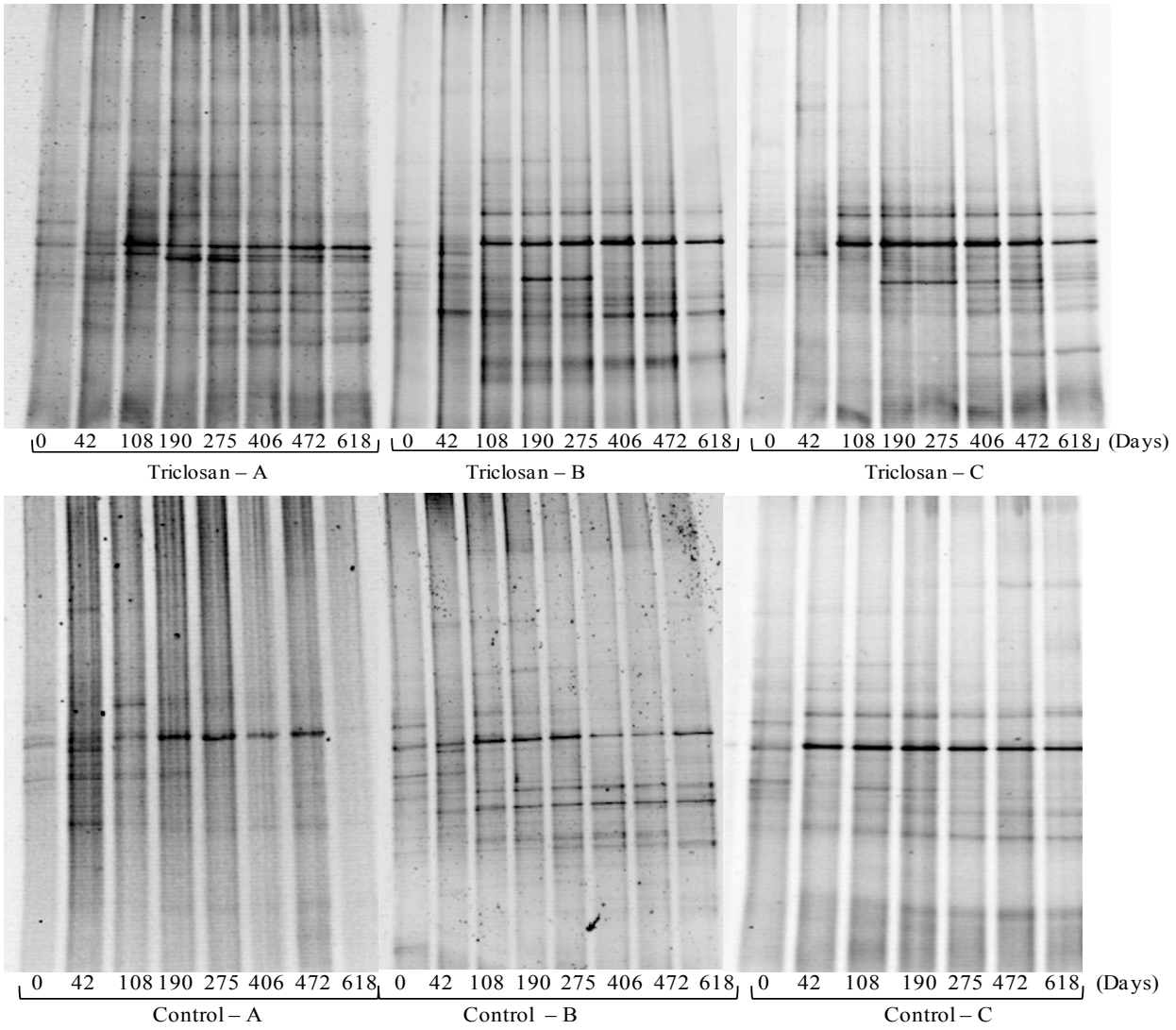

Figure S1. DGGE profiles of the *Bacteria* 16S rRNA genes for each reactor during the duration of the experiments. No unique bands common to all triclosan-amended reactors but not detected in control reactors were found from this analysis; each reactor shows some temporal shifts.