The Role of Household Antimicrobials in the Proliferation of Antibiotic Resistance During Anaerobic Digestion

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THE ROLE OF HOUSEHOLD ANTIMICROBIALS IN THE PROLIFERATION OF ANTIBIOTIC RESISTANCE DURING ANAEROBIC DIGESTION

by

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ABSTRACT
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Antimicrobial chemicals in consumer personal care products have been found to increase antibiotic resistance in pure culture studies. Although many studies focus on antibiotic resistance development pertinent to medical scenarios, resistance developed in natural and engineered environments might be significant and has become an emerging concern for human health. This dissertation focuses on the antimicrobial chemicals triclosan and triclocarban. These compounds are distinctly different from antibiotics and are used in products like soaps that are labelled as "antibacterial". Municipal wastewater treatment plants receive triclocarban and triclosan loads higher than most contaminants of emerging concern because they are frequently used in consumer products and then discharged into the sewerage system. This research specifically focused on the impact of triclosan and triclocarban in lab-scale anaerobic digesters and investigated how they influenced digester function, the relative abundance of resistance genes, microbial community structure, and cross-resistance to antibiotics. Lab-scale anaerobic digesters were operated for 180 days and loaded with concentrations of triclocarban or triclosan ranging zero to inhibitory concentrations. Both triclosan and triclocarban selected for *mexB*, a gene that confers multidrug resistance in bacteria, at environmentally relevant concentrations. This is the first research to demonstrate that triclocarban can select for a multidrug resistance gene in anaerobic digesters. Relatively higher concentrations of these chemicals inhibited function in anaerobic digesters and further selected for some resistance genes and against others. The functional inhibition was not reversible when chemicals were removed. When these chemicals were removed from functioning digesters the *mexB* concentrations were no longer different from the control digesters suggesting that a decrease in consumer usage could have impacts on environmental antibiotic resistance. At higher concentrations of triclosan, and all concentrations of triclocarban, digester microbial community structures irreversibly shifted away from the control. In a separate set of experiments, addition of these antimicrobials altered how anaerobic digester microbial communities responded to the presence of three other antibiotics. Triclosan-amended communities had increased resistance to ciprofloxacin; triclocarban-communities were more sensitive to tetracycline and chloramphenicol. This research demonstrates that antimicrobials should be considered along with antibiotics when determining the role of chemical stress on the proliferation of antibiotic resistance.

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1 INTRODUCTION

1.1 Introduction to Contaminants of Emerging Concern

As detection methods become more sensitive, the ubiquity of contaminants in the environment at low concentrations has become more apparent (Bolong et al., 2009). Many pollutants at microgram per liter (μg/L) or nanogram per liter (ng/L) levels which were not previously detected have been categorized as "contaminants of emerging concern" (CECs) (Pal et al., 2010). Likewise, CECs are found in soils and sediments at or below mg/kg levels (Clarke and Smith, 2011). In 2015, one hundred chemicals were included on the draft Contaminant Candidate List 4 by the Environmental Protection Agency (USEPA, 2015); these chemicals may join the hundreds of other chemicals (regulated and unregulated) which the EPA suggests should be monitored in the environment and in sources of drinking water (USEPA, 2009a). These compounds include pharmaceuticals, steroids, sex hormones, illicit drugs, flame retardants, metals, polycyclic aromatic hydrocarbons, halogenated compounds and others (Díaz-Cruz et al., 2009).

CECs impact microbiota and macrobiota in the environment in a multitude of different ways and to various degrees. For example, estrogens have been shown to affect sex distribution of fish when in natural waters causing near collapse of a fish population (Kidd et al., 2007). Other chemicals bioaccumulate in bacteria or smaller organisms, then become toxic to organisms further up the food chain (Croteau et al., 2005; La Guardia et al., 2006). The dynamics and community structure of microorganisms can also be impacted by many different chemicals in natural systems (Tian et al., 2008; Yergeau et al., 2010). It is critical to quantify the impacts of CECs so that policy and engineering processes can be designed to target CECs that pose highest risks.

Although there are many routes for CECs into the environment, a major point source is from wastewater treatment plants (Verlicchi et al., 2012). Although many chemicals are degraded through the treatment process, some refractory compounds pass through the plant without transformation or are only partially degraded, and hence low levels of chemicals are detected in the treated water (Pal et al., 2010). Hydrophobic chemicals tend to accumulate in biosolids (USEPA, 2009b). After stabilization, perhaps through anaerobic digestion, biosolids are often applied to agricultural land as a source of nutrients (Hospido et al., 2010).

1.2 Antimicrobials of Concern: Triclosan and Triclocarban

Antimicrobials are a class of CECs that are commonly detected in the environment. In general, the term antimicrobial encompasses a wide range of chemicals which inhibit or kill microbiota (antibacterial, antiviral, antifungal, and antiprotozoal) (McDonnell and Russell, 1999). Antibiotics are a subset of antimicrobials which are used in the medical field to treat infection; (Kümmerer, 2004); antibiotics are also detected in the environment. The specific action of antibiotics, whereby they only inhibit bacteria and no other types of organisms, makes them useful in medicine to treat undesirable bacterial infections (Khachatourians, 1998).

Two broad spectrum antimicrobials widely found in consumer products are triclosan (TCS) and triclocarban (TCC). TCS is found in hand soap, deodorants, shower gels, lotions, toothpastes, and mouthwash at concentrations near 0.1-0.3 wt% (Jones et al., 2000; Villalaín et al., 2001). TCC is found most abundantly in bar soaps in concentrations near 1.5%, but also in detergents and cosmetics at 0.5 -5 wt% (Halden and

Paull, 2005). As of 2008, TCC or TCS is estimated to be in 45% of all soaps on the market (Ahn et al., 2008). These chemicals can be excreted with urine or rinsed down the drain after use and are sent to wastewater recovery facilities, where, due to their hydrophobic properties, they typically adsorb to solids in the treatment plant. Land application of biosolids presents a major route of these antimicrobials into the environment (Miller et al., 2008). In a survey of biosolids which includes antimicrobials and antibiotics, TCC and TCS were the most concentrated compounds found in biosolids (McClellan and Halden, 2010). These compounds are found to be 10-10,000 times more abundant than any given antibiotic in biosolids (McClellan and Halden, 2010).

TCC and TCS have similar structures (see Figure 1.1) and mechanism of action (Ahn et al., 2008). Each molecule is a binuclear structure with aromatic rings that bond one or two chlorine atoms. At the concentrations used in personal care products, the antimicrobial mechanism of TCC and TCS is thought to be disruption of the cell membrane; these chemicals intercalate into the membrane, allowing the intracellular fluid to leak into the environment and kill the cell (Villalaín et al., 2001). For TCS, distinctly different inhibitory actions have been identified at concentrations closer to 1 mg/L; TCS has been observed to inhibit intracellular proteins involved in fatty acid synthesis (McMurry et al., 1998). TCS (at concentrations much lower than application concentrations) can inhibit bacteria by a specifically targeting FabI; in a sense, this inhibitory action is similar to how an antibiotic inhibits bacteria.

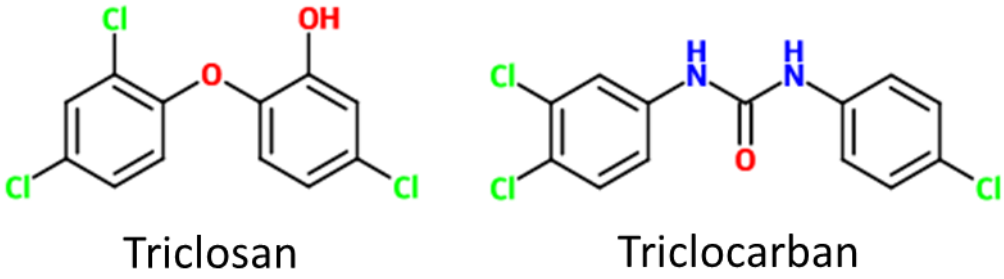

Figure 1.1 Chemical structure of triclosan (left) and triclocarban (right).

1.3 Antibiotic and Antimicrobial Resistance

Antibiotics and antimicrobials are a concern to public health because of their impacts on the spread of antibiotic resistance (CDC, 2013). Resistance, whereby bacteria counteract the deleterious effects of antibiotics, was detected shortly after the first medical use of antibiotics (Levy and Marshall, 2004). One or more resistance mechanisms may increase tolerance to a given antibiotic or many classes of antibiotics. Pathogenic bacteria which gain resistance mechanisms have become increasingly difficult to treat within medical patients (Levy and Marshall, 2004). Infections by antibiotic resistance bacteria lead to more than 23,000 deaths in the US each year (CDC, 2013). Further, it is estimated that approximately $50 billion in health care costs was spent in 2013 in attempts to counteract antibiotic resistant bacteria.

Resistance to antibiotics is gained on a genetic level (Alanis, 2005). Antibiotic resistance genes (ARGs) can be selected for in a bacterial population as well as transferred between bacteria. Plasmids and class 1 integrons play a major role in horizontal transfer of ARGs; the DNA fragment containing ARGs can even persist outside of a bacterial host (Berendonk et al., 2015). Even though ARGs are of biological

origin, they are also considered a CEC because they pose direct risks to public health (Martinez, 2009). The biological nature and negative impacts of ARGs are distinctly different than many other organic contaminants. In particular, ARGs multiply and transfer in many environments (Kümmerer, 2004).

Gene transfer is stimulated in bacteria that have been exposed to antibiotics or other chemical stressors (Russell, 2000). This phenomenon occurs in a variety of compartments, perhaps most recognizably in people and hospitals (Berendonk et al., 2015). Somewhat less recognized is the role of the natural environment on the resistome (i.e. the sum of antibiotic resistance genes). In the environment, not only can resistance be stimulated by stressors (both natural and anthropogenic), but transfer can occur on larger geographic scales (Pruden et al., 2006).The extent and rate of transfer in the environment remains an active area of research.

Antibiotics are a significant source of antibiotic resistance stimulation, although TCS has also been shown to have a role in stimulating antibiotic resistance (Yazdankhah et al., 2006). Perhaps because of the specific intracellular inhibition mechanisms at dilute concentrations, bacterial exposure to low concentrations of TCS has been shown to increase resistance to TCS (Saleh et al., 2011). Further, it has repeatedly been demonstrated that bacteria that have become resistant to TCS can also become resistant to antibiotics (Braoudaki and Hilton, 2004; Chuanchuen et al., 2001). Given the depth of the literature related to resistance and TCS, a detailed literature review on TCS resistance in the environment is included as **Chapter 2** in this dissertation.

Although cross-resistance has been associated with TCS, cross-resistance and related impacts have not been identified in the case of TCC. The structural similarities

and physical characteristic suggest that TCC may share a similar concern regarding the stimulation of resistance (Halden, 2014). This relationship has not been thoroughly investigated, though it is acknowledged by a variety of authors (Halden, 2014; Walsh et al., 2003). TCC is typically found in higher concentrations than TCS in biosolids and soils which further fortifies the importance of understanding the impact of TCC on antibiotic resistance in biosolids (McClellan and Halden, 2010).

1.4 Anaerobic Digesters as Prime Environments for Antimicrobials to Select Resistance Genes

Many CECs flow through wastewater treatment plants (Clarke and Smith, 2011). Inherent with the large and diverse bacterial populations, antibiotic resistance has been identified in biological treatment operations and products (i.e., reclaimed water and biosolids). Many chemical stressors, including antibiotics and antimicrobials, could play a role in the stimulation and transfer of antibiotic resistance, but specific roles of individual chemicals, especially antimicrobials, has not been well parsed. Due to their hydrophobic nature of TCC and TCS, these antimicrobials are relatively abundant CECs in biosolids. Biosolids are often anaerobically digested to stabilize pathogens and recover energy before dispersion into the environment via land application. Further, TCC and TCS are not readily degraded under anaerobic conditions (Pycke et al., 2014). The specific impact of TCC and TCS on ARGs in anaerobic digesters remains unknown.

1.5 Research Objectives

The overall objective of this dissertation was to quantify the impacts of TCC and TCS as chemical stressors on antibiotic resistance in anaerobic digesters. The general

approach was to use lab-scale anaerobic digesters that were given organic feed containing only TCC or TCS as a chemical stressor with no other chemical adulterants. Specifically, the first objective was to establish the effect of sustained concentrations of TCC and TCS on the abundance of ARGs, community structure, and functional performance. Additionally, the effect of adaption time was investigated with different antimicrobial loading rates. The TCC results are presented in **Chapter 3** and the TCS results are presented in **Chapter 4**.

As public policy or consumer usage of these products might change, it is important to understand how removing these chemicals might impact antibiotic resistance. The second objective was to determine the impact of removing antimicrobial stressors from anaerobic digesters on the abundance of ARGs, microbial community structure, and functional performance. The results of both TCC and TCS washout from digesters are presented in **Chapter 5**.

The final objective was to determine if exposure to TCS or TCC made anaerobic microbial communities more functionally resistant to antibiotics. In **Chapter 6**, biomass amended to tolerate high levels of TCS or TCC was tested for altered toxicity towards antibiotics. Finally, overall conclusions and directions for future research are highlighted in **Chapter 7**.

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2 LITERATURE REVIEW OF TRICLOSAN RESISTANCE

This chapter was published in *Frontiers in Microbiology*:

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2.1 Introduction

The World Health Organization warns that we may enter a post-antibiotic era in the 21st century due to the spread of antibiotic resistance (WHO, 2014). Antibiotic resistance is defined as the ability of bacteria to survive a concentration of antibiotics that typically inhibits growth of the majority of other bacteria (Russell, 2000). Antibiotics are extensively used in medicine to treat bacterial infections in humans and animals, and are widely used in agriculture to promote animal growth (Khachatourians, 1998; Kümmerer, 2004). Each year, in the United States (U.S.) alone, over 2 million people are infected by antibiotic resistant bacteria, leading to more than 25,000 deaths, and $50 billion spent managing antibiotic resistance (CDC, 2013). The associated cost continues to increase as bacteria acquire mechanisms to fight against the antibiotics that are typically employed (Levy and Marshall, 2004).

In addition to antibiotics, synthetic antimicrobial agents are also pervasive in households and hospitals, mainly for disinfection and sanitation purposes. The term ‘antimicrobial’ has been used to describe a broad range of compounds, including antibiotics that destroy or inhibit microorganisms (Kümmerer, 2004; McDonnell and Russell, 1999). For this paper, triclosan (TCS), which is not derived naturally, is referred to as an antimicrobial. Compounds produced or derived from microorganisms used *in-vivo* to treat bacterial infections in eukaryotes (e.g., erythromycin, tetracycline, ciprofloxacin, *etc.*) will be referred to as antibiotics (even though antibiotics are a subset of antimicrobials).

TCS is widely used for personal hygiene and disinfection purposes; in fact, 350 tons were produced for commercial use in the European Union in 2002. Based on 1998

records from the Environmental Protection Agency, approximately 500-5000 tons were produced in the U.S., and the industry has reported growth (Fang et al., 2010; Heidler and Halden, 2007; Singer et al., 2002; Venkatesan and Halden, 2014). With these approximations, it is estimated that 1 kg of TCS is produced for every 3 kg of antibiotics produced (FDA, 2011; DHHS, 2012). TCS is found in a wide range of consumer products including hand soap, toothpaste, deodorant, surgical scrubs, shower gel, hand lotion, hand cream, and mouthwash (Bhargava and Leonard 1996; Jones et al., 2000).

Because of its wide use, TCS is found in many natural and engineered environments, including surface water, wastewater, soil, drinking water, wastewater treatment plants (WWTPs), biosolids, landfills, and sediments (Bedoux et al., 2012; Benotti et al., 2009; Kumar et al., 2010; Mavri et al., 2012; Miller et al., 2008; Singer et al., 2002; Welsch and Gillock 2011; Xia et al., 2010). As TCS is commonly used in oral consumer products, it is widely found in human urine. In a survey of 181 pregnant women in an urban multiethnic population in Brooklyn, NY, TCS was found in 100% of urine samples (Pycke et al., 2014). In a geographically broader U.S. survey, 75% of people were found to have TCS in their urine (Calafat et al., 2008).

At application concentrations (0.1 – 0.3 w/v% or approximately 1,000 – 3,000 mg/L in hand soaps), TCS induces cell damage that causes cell contents to physically leak out of the membrane (Villalaín et al., 2001). At concentrations lower than 1 mg/L, TCS serves as an external pressure to select for TCS resistance as well as antibiotic resistance in many types of bacteria (Birosová and Mikulásová, 2009; Chapman 2003; Halden, 2014; Poole, 2002; Russell, 2000; Saleh et al., 2011; Schweizer, 2001; Yazdankhah et al., 2006). At low concentrations, TCS interacts with physiological

targets, and these interactions lead to numerous resistance mechanisms that are reviewed below (Bailey et al., 2008; Chuanchuen et al., 2001; Condell et al., 2012; Yu et al., 2010). In some cases, the mechanisms that convey resistance to TCS simultaneously confer resistance to more than one class of antibiotics (Alanis, 2005; Poole, 2002).

The wide use of TCS leads to concern about its potential to aid in the spread of antibiotic resistance (Kümmerer, 2004; Russell, 2000; Saleh et al., 2011). TCS exposure that leads to TCS resistance and antibiotic resistance has been widely reported, but the majority of these studies pertain to pure cultures of specific bacterial strains, and in most cases, pathogenic strains. This line of research is logical because antibiotic resistant pathogens are of greatest concern to public health. TCS might also impact the spread of resistance in environmental microbial communities as approximately $1.1x10^5$ to $4.2x10^5$ kg of TCS are distributed to the environment annually through WWTPs in the U.S. (Heidler and Halden, 2007). Studies on pure culture isolates provide insight into the *potential* impacts of TCS on antibiotic resistance in environmental bacterial communities. The important question then becomes: does TCS select for antibiotic resistance in these complex microbial communities?

Many engineered and natural processes are driven by microbes, and TCS is designed to impact microbes in homes and hospitals. Following discharge to the environment, the antimicrobial properties of TCS can impact complex microbial communities found in engineered and environmental systems. TCS has been linked to altered microbial community structure or function in wastewater operations, such as activated sludge and anaerobic digestion (Stasinakis et al., 2008; McNamara et al., 2014). Likewise, TCS can alter diversity and biofilm development in freshwater biofilms in

receiving streams (Lubarsky et al., 2012; Proia et al., 2011; Johnson et al., 2009). In soils, TCS impacts respiration rates and denitrification, and enriches for species capable of dehalogenation (Butler et al. 2011; Holzem et al., 2014; McNamara and Krzmarzick 2013). TCS induces responses in microbial communities, but the TCS concentrations that inhibit function are not often found in these complex microbial communities. At environmental concentrations, TCS is more likely to exert a stress that propagates resistance than to exert a stress that functionally inhibits complex microbial communities.

The purpose of this manuscript is to review the state of knowledge regarding the impact of TCS on antibiotic resistance in environmental systems and identify critical research questions that need to be addressed to better understand the impact of TCS-derived resistance in the environment on public health. This review describes TCS resistance and cross-resistance in pure cultures, and then considers the comparatively smaller amount of literature that addresses how TCS impacts antibiotic resistance in engineered environments containing complex microbial communities. Engineered environments are of prime interest because they contain TCS, bacteria and resistance genes that can be subsequently dispersed to terrestrial soils and surface waters, with the possibility of negative public health consequences (Burch et al., 2014; Cha and Cupples, 2009; Ghosh et al., 2009; LaPara et al., 2011; Ma et al., 2011; Munir et al., 2010; Pruden et al., 2012; Pruden et al., 2006; Yang et al., 2014; Baquero et al., 2008).

2.1 Genetic Targets of Triclosan

In 1998, TCS was first described by McMurry et al. (1998b) to have a specific target in *E. coli*. At 1 mg/L, approximately 1000-fold lower than the application concentration, TCS inhibits FabI, an enoyl-acyl carrier protein reductase (ENR). The

FabI protein catalyzes the elongation cycle in the synthesis of fatty acids, an essential process for cell viability (Bergler et al., 1996, Massengo-Tiassé and Cronan 2008; Massengo-Tiassé and Cronan, 2009). Prior to McMurry et al.'s (1998b) report, low concentrations of TCS were assumed to have minimal effects on cell viability.

Up-regulation of *fabI* is a response mechanism which may overcome the effects of intracellular TCS (Condell et al., 2012; Sheridan et al., 2013; Yu et al., 2012). Bacteria can up-regulate and down-regulate many more genes in response to TCS, although it can be difficult to determine which expression changes are casual. No universal response has been observed; however, many bacteria respond to some degree with the up-regulation of transport proteins and membrane bound proteins (Bailey et al., 2008; Chuanchuen and Schweizer 2012).

2.2 TCS Resistance in Pure Cultures

The most common resistance mechanisms based on pure culture studies are target site modification, membrane resistance, and efflux. The following sections briefly review resistance mechanisms to TCS and describe their impact on cross-resistance; a comprehensive review of TCS resistance mechanisms can be found by Schweizer (2001).

2.2.1 FabI Modification or Replacement

Target site modification is a resistance mechanism that involves a genetic alteration to the target site that reduces the effect of an inhibitory chemical (Hooper, 2005). Modification of TCS target site FabI is a common resistance mechanism observed in pure cultures. Mutation occurs whereby single or multiple amino acids are changed in the *fabI* gene, resulting in TCS-resistant FabI proteins (Brenwald and Fraise 2003; Yu et

al., 2010). Ciusa et al. (2012) suggested a resistance mechanism whereby an allele of a *fabI* gene is located on a mobile genetic element and transposed into *Staphylococcus aureus*. The presence of the *fabI* allele together with the intrinsic *fabI* gene increased the concentration of the FabI protein through heterologous duplication and increased bacterial tolerance to TCS. Alternatively, ENR isoenzymes, which perform similar functions to FabI, including FabL, FabK, and FabV, have been identified in TCS-resistant bacteria (Massengo-Tiassé and Cronan 2009). These isoenzymes are naturally found in some strains of bacteria. In fact, FabV has been found to functionally replace FabI, rendering *Pseudomonas aeruginosa* 2,000 times more resistant to TCS as seen by an increase in minimum inhibitory concentration (MIC) (Zhu et al., 2010). Similarly, FabK replaces function for FabI in *Streptococcus pneumonia*, leading to increased tolerance to TCS (Heath and Rock 2000), and FabL expression leads to increased resistance to TCS in *Bacillus subtilis* (Heath et al., 2000).

With respect to multidrug resistance, FabI alteration or replacement may specifically produce resistance to isoniazid, an important agent for the treatment of tuberculosis, which also targets FabI (Ciusa et al., 2012). However, FabI alterations are not generally known to cause resistance to other antibiotics. This type of resistance in environmental communities would not likely pose a threat to public health through increased multidrug resistance.

2.2.2 Membrane Alteration

Modifications through changes to the outer membrane is a less-studied TCS resistance mechanism in bacteria. Champlin et al. (2005) concluded that outer membrane properties were responsible for low-level resistance to hydrophobic antimicrobials and

antibiotics. The researchers compared *P. aeruginosa* strains that possessed highly refractory outer cell envelopes to strains that had highly permeable outer cell envelopes and discovered that the outer membrane properties conferred intrinsic resistance to TCS up to 256 mg/L. Tkachenko et al. (2007) suggested that TCS exposure could induce a genetic response which increases the concentration of branched chain fatty acids in the cell membrane in *S. aureus*; the membrane thereby sequesters the chemical agent and stops it from passing into the cell, preventing physiological disruption inside of the cell.

Outer membrane impermeability is a potential mechanism for cross-resistance to antibiotics. Particularly, non-specific rejection of hydrophobic chemicals could be a mechanism for resistance to TCS and other antibiotics that may be found in the environment.

2.2.3 Efflux Pumps

Efflux pumps are often associated with multidrug resistance, which is a public health concern. Active efflux, whereby a bacterium physically removes a constituent from its intracellular space by pumping the constituent across the membrane and back into the environment, is an effective mechanism against a wide range of antimicrobials and antibiotics, including TCS (Kern et al., 2000; Levy, 2002). The AcrAB efflux pump is responsible for efflux of TCS in *E. coli* and *S. enterica* (McMurry et al., 1998a; Webber et al., 2008). Non-specific multidrug efflux pumps (e.g., *mex* proteins) confer resistance to TCS as well as other antibiotics in *P. aeruginosa* and *R. rubrum*. (Chuanchuen et al., 2001; Pycke et al., 2010a; Pycke et al., 2010b). Most non-specific efflux pumps are capable of expulsing antibiotics. Thus, in cases where bacteria acquire non-specific efflux pumps through horizontal gene transfer after exposure to TCS, the

bacteria would likely acquire resistance to antibiotics as well. In some cases, specific efflux pumps confer resistance to TCS. TriABC-OpmH is a TCS-specific efflux pump in *P. aeruginosa* that is not known to expel other compounds such as antibiotics (Mima et al., 2007).

2.3 Triclosan and Cross-Resistance to Antibiotics

Resistance to TCS, incurred by exposure to TCS, can directly affect resistance to antibiotics. Cross-resistance has been tested for a wide range of antibiotics following exposure to TCS. Chloramphenicol and tetracycline are two antibiotics commonly included in antibiotic cross-resistance experiments. In studies done on *E. coli* and *P. aeruginosa*, resistance to chloramphenicol and tetracycline increased 10-fold following TCS exposure (Figure 2.1). Increased antibiotic resistance in *S. maltophilia* and *S. enterica* serovar Typhimurium following TCS exposure was also observed, but the increase was less severe. Cross resistance in *P. aruginosa* (Chuanchuen et al., 2001.), *S. maltophilia* (Sanchez at al., 2005), and *S. enterica* serovar Typhimurium (Karatzas et al., 2007) were attributed to efflux systems. Resistance mechanisms were not directly investigated in the studies on *E. coli* (Braoudaki and Hilton, 2004) and *S. enterica* serovar Typhimurium (Birosova and Mikulazova, 2009), however *acrAB* genes, which encode for efflux, are known to confer resistance to TCS, chloramphenicol, and tetracycline in both of these species (Karatzas et al., 2007). These findings highlight a main concern regarding the widespread dissemination of TCS, *i.e.*, that TCS exposure can spread multidrug resistance.

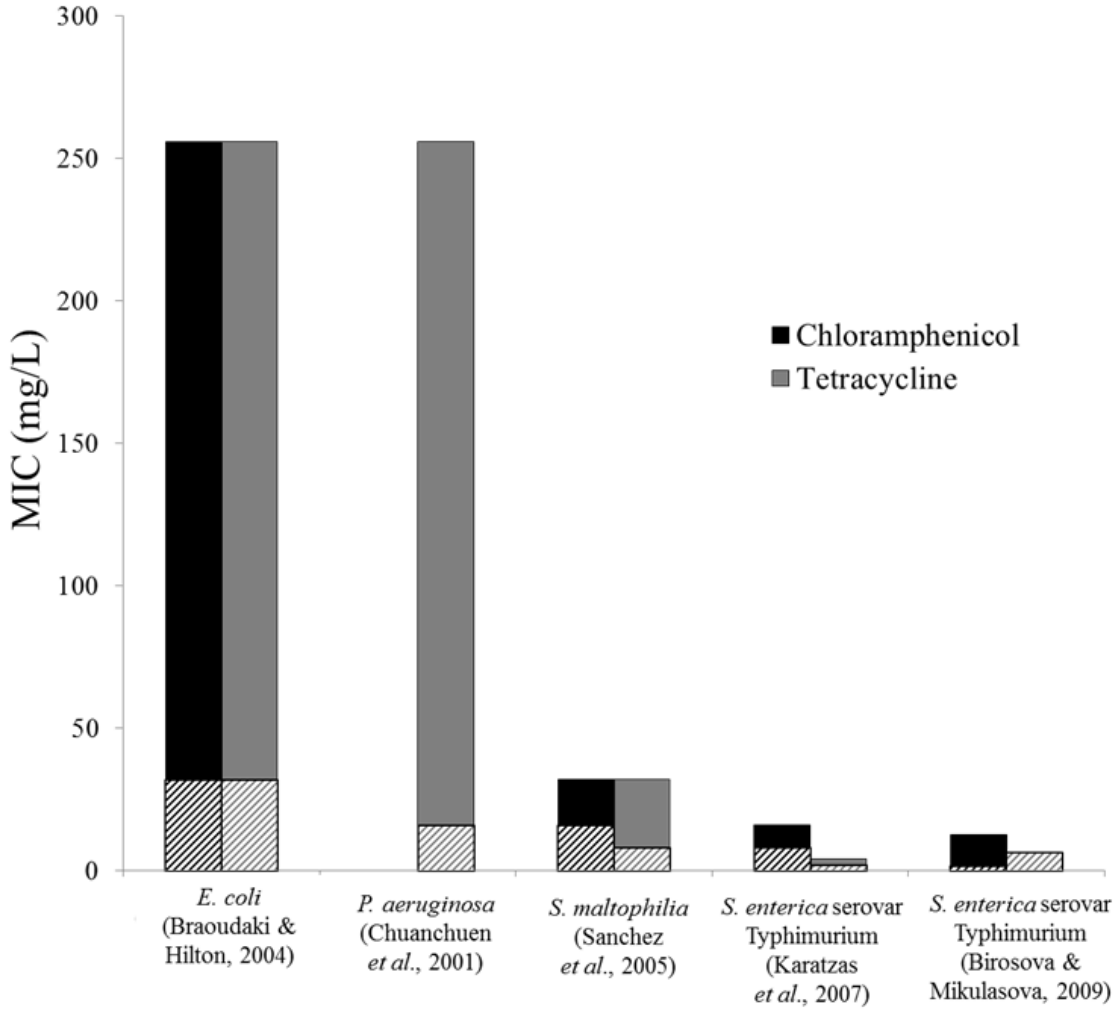

Figure 2.1 TCS exposure increases resistance to antibiotics. Minimum inhibitory concentrations (MIC) of chloramphenicol and tetracycline for control strains (striped bars) and TCS adapted strains (solid bars) are shown from various studies and bacteria. Significant differences were observed in most cases, however, no significant difference was found for tetracycline resistance for *S. enterica* in the study by Birosova and Mukulasova (2009). Chloramphenicol resistance was not tested in *Pseudomonas aeruginosa* (Chuanchuen et al., 2001).

TCS resistance and antibiotic resistance have been found together in clinical isolates. In a survey of 732 clinical isolates of *Acinetobacter baumannii* from hospitals, 3% of isolates were found to have reduced susceptibility to TCS (MIC > 1 mg/L) (Chen et al., *2009*). Those isolates which could tolerate higher than 4 mg/L also had increased tolerance to amikacin, tetracycline, levofloxacin and imipenem. Clinical isolates of *S. aureus*, which had MICs to TCS between 0.025 and 1 mg/L, were resistant to multiple antibiotics (Suller and Russell 2000). Some, but not all, of the strains showed increased resistance to gentamicin, erythromycin, penicillin, rifampicin, fusidic acid, tetracycline, methicillin, mupirocin and streptomycin. In some strains TCS resistance was stable when sub-culturing was performed in a TCS-free medium. In other strains TCS resistance was lost when the strain was propagated for 10 days in TCS-free media, indicating that the presence of TCS can select for resistance that is not regularly expressed. This finding implies that removing TCS from environmental systems through improved treatment processes or reduced consumer usage could lead to a decrease in TCS resistance. Research should be conducted to specifically test the impacts of removing TCS on TCS-derived resistance in complex microbial communities.

Conditions that perpetuate resistance to TCS frequently result in cross-resistance to antibiotics. TCS resistant *S. enterica* serovar Typhimurium strains were selected by daily sub-culturing of TCS-exposed cultures and increasing TCS concentrations in media from 0.05 mg/L to 15 mg/L over 15 days (Karatzas et al., 2007). The TCS MIC in the resulting strains increased from 0.06 mg/L to as high as 128 mg/L, and the strains were also more resistant to ampicillin, tetracycline, and kanamycin. The authors concluded that the overexpression of the *acrAB* efflux pump was likely involved in the increased

tolerance to TCS and antibiotics. In another study, TCS selected for ciprofloxacin resistant mutants in *S. enterica* serovar Typhimurium when exposed to 0.5 mg/L of TCS (Birosová and Mikulásová, 2009). These studies, along with the concentrations of TCS found in the environment, imply that TCS could select for bacteria in environmental communities that have efflux pumps.

Efflux is a common method of resistance, but the specific efflux system used and the resulting cross-resistance profile can vary between species. In *P. aeruginosa*, MexAB-OprM, MexCD-OprJ and MexEF-OprN, contribute to TCS resistance (Chuanchuen et al., 2001). Exposure to TCS selected for up-regulation of these efflux systems due to mutations in the regulatory gene, *nfxB,* which increased the tolerance to tetracycline, ciprofloxacin, trimethoprim, erythromycin and gentamicin. In some cases the TCS resistant strains could tolerate up to 500-fold higher antibiotic concentrations than the non-TCS resistant strains. Strains which lacked these efflux systems showed increased sensitivity to antibiotics. In the opportunistic pathogen *S. maltophilia*, TCS binds to the repressor SmeT, allowing expression of an efflux pump, SmeDEF (Hernández et al., 2011). Expression of this efflux pump following exposure to TCS resulted in increased resistance to the antibiotics ciprofloxacin, norfloxacin, nalidixic and ofloxacinin. Sanchez et al. (2005) also found that TCS-resistant mutants of *S. maltophilia* (tolerant up to 64 µg/L of TCS) overexpress SmeDEF. These mutants had an increased tolerance to tetracycline, chloramphenicol, and ciprofloxacin. Even though SmeDEF is intrinsically contained in the genome of *S. maltophilia,* TCS exposure selected for up-regulation of this efflux pump which increased antibiotic resistance.

In addition to variances between genera, cross-resistance varies within genera. TCS-adapted *E. coli* O157:H7 exhibited increased resistance to chloramphenicol, tetracycline, amoxicillin, amoxicillin/clavulanic acid, trimethoprim, benzalkonium chloride and chlorohexidine, while TCS-adapted *E. coli* O55 exhibited resistance to only trimethoprim (Braoudaki and Hilton 2004).

Although most evidence supports the notion that TCS increases resistance to antibiotics, this is not necessarily true for all classes of antibiotics. In one case, TCS-resistant mutants of *S. enterica* were more (or no less) susceptible to antibiotics (Rensch et al., 2013). *S. enterica* that were selected to have overexpression of *fabI* or a *fabI* mutation had increased susceptibility to the aminoglycoside antibiotics kanamycin and gentamicin.

The cross-resistance profiles vary among the bacteria surveyed in this review, and other types of bacteria yet to be studied are likely to have unique cross-resistance profiles. While resistance profiles vary, the overarching theme is the same: resistance to TCS can yield cross-resistance to multiple antibiotics. Given that TCS is not an antibiotic, resistance to TCS alone is not a public health threat. TCS-derived proliferation of multidrug resistant bacteria, however, could be a severe threat to public health. These pure-culture studies indicate that TCS is likely to select for multidrug resistant bacteria above a critical concentration. In environmental communities, such as anaerobic digesters or sediments, TCS is found at 2 to 1000 fold higher concentrations than any given antibiotic (McClellan and Halden, 2010). Is TCS selecting for resistant bacteria in the environment? The role of TCS on the selection of antibiotic resistance genes and

multidrug resistance genes in the environment needs to be quantified to determine what steps, if any, are necessary for protecting public health.

2.4 Triclosan Derived Resistance in Complex Environmental Communities

Environmental systems, including WWTPs and sediments, represent the most likely sites for TCS resistance to develop because of the high abundance of TCS and high density of bacteria. Wastewater treatment systems should be given special focus because they contain and discharge TCS and resistance genes to the environment. To understand the role of TCS and the remaining research gaps, the fate of TCS in the environment is summarized to highlight locations of prime interest, and the state of knowledge regarding TCS and resistance in complex microbial communities is assessed.

2.4.1 Fate of Triclosan

TCS is discharged into the environment with treated liquid and solid effluents from WWTPs. In the U.S. alone, WWTPs are estimated to receive approximately 100 tons of TCS each year, but the prevalence of TCS in treated effluent is not restricted to U.S. facilities. A survey of WWTPs in Germany found TCS in treated effluents at concentrations ranging from $1x10^{-5}$ to $6x10^{-4}$ mg/L (Bester 2005). The concentrations of TCS and its aerobic degradation products in receiving waters were less than $3x10^{-6}$ mg/L. A study of 8 WWTPs in Switzerland revealed that, on average, six percent of the influent TCS was found to discharge with the effluent water at concentrations of $4.2x10^{-5}$ to $2.13x10^{-4}$ mg/L (Singer et al., 2002); these receiving streams had concentrations at $1.1x10^{-5}$ to $9.8x10^{-5}$ mg/L. A more recent study found TCS in WWTP effluents at $9.7x10^{-5}$ mg/L, and in nearby sediments at 0.018 mg/kg (Blair et al., 2013). Several other studies

have found TCS in surface water in concentrations ranging from $<2x10^{-7}$ mg/L up to 2.2 $x10^{-2}$ mg/L (Bedoux et al., 2012).

TCS that is discharged with liquid effluent often partitions to sediments. Miller et al. (2008) found that TCS accumulated in sediments near WWTP outfalls for approximately 50 years, and similar results were found by other researchers (Anger et al., 2013; Buth et al., 2010). Sediment concentrations have been found at 53 mg/kg (Chalew et al., 2009). TCS is prevalent in liquid effluents and abundant in sediments, but this discharge route does not account for the majority of TCS that enters the environment. One study estimated that 0.24 kg/day of TCS are released with liquid effluent, but 5.37 kg/day are released with the treated residual solids from a midsized WWTP (Lozano et al., 2013).

Indeed, nearly half (or even higher) of the influent TCS load to WWTPs is captured by solids following sorption (Heidler and Halden 2007; Lozano et al., 2013). The concentration of TCS in biosolids is often much higher than in aqueous systems because of the hydrophobic nature of TCS. A nationwide U.S. survey of TCS in biosolids found the median concentration in treated biosolids to be 3.9 mg/kg and the maximum level was 133 mg/kg (USEPA 2009). The high levels found in biosolids can lead to high levels in soils when biosolids are land applied. TCS was found in biosolids-amended soils which had been receiving biosolids for 33 years (Xia et al., 2010). The concentrations in the soil ranged from approximately 1 mg/kg in the first 15 cm of soil to less than 0.1 mg/kg at a depth of 60 – 120 cm. The half-life of TCS in soil under aerobic conditions was found to be 104 days, and TCS is even more persistent under anaerobic conditions (McAvoy et al., 2002; Ying et al., 2007). These fate data, along with the hydrophobic

nature of TCS, indicate that TCS is most likely to impact microbial communities that contain high concentrations of organic matter, including anaerobic digesters, sediments, and soils, and these communities should receive special focus when investigating TCS-derived resistance in the environment.

The range of TCS concentrations found in the environment is depicted in Figure 2.2 along with the MIC of TCS-acclimated and TCS-unacclimated pathogenic strains of bacteria. The concentrations in the biosolids and sediments are higher than the MICs of TCS-sensitive strains, indicating that TCS-sensitive strains would not thrive in these environments and TCS-resistant strains may be present. The MICs of TCS-acclimated strains, however, are higher than the current environmental TCS concentrations and could tolerate an increase in TCS concentrations. A future increase in TCS concentrations may select for resistance rather than functionally inhibit complex microbial communities. This figure indicates that biosolids and sediment environments with high TCS concentrations likely have TCS-resistant bacteria. However, this figure does not indicate the level of TCS required to select or enrich for resistance in the environments with lower TCS concentrations. What happens when TCS is below the MIC? Certainly environments with very high levels of TCS will have TCS-resistant strains, but do environments with TCS concentrations below the MIC of acclimated strains select for resistance? What concentration of TCS is required to select for resistance in various environmental communities? These questions represent critical research gaps. By answering these questions with further research we can determine if and where TCS is selecting for resistance. Research plans are outlined in the final section to address these questions.

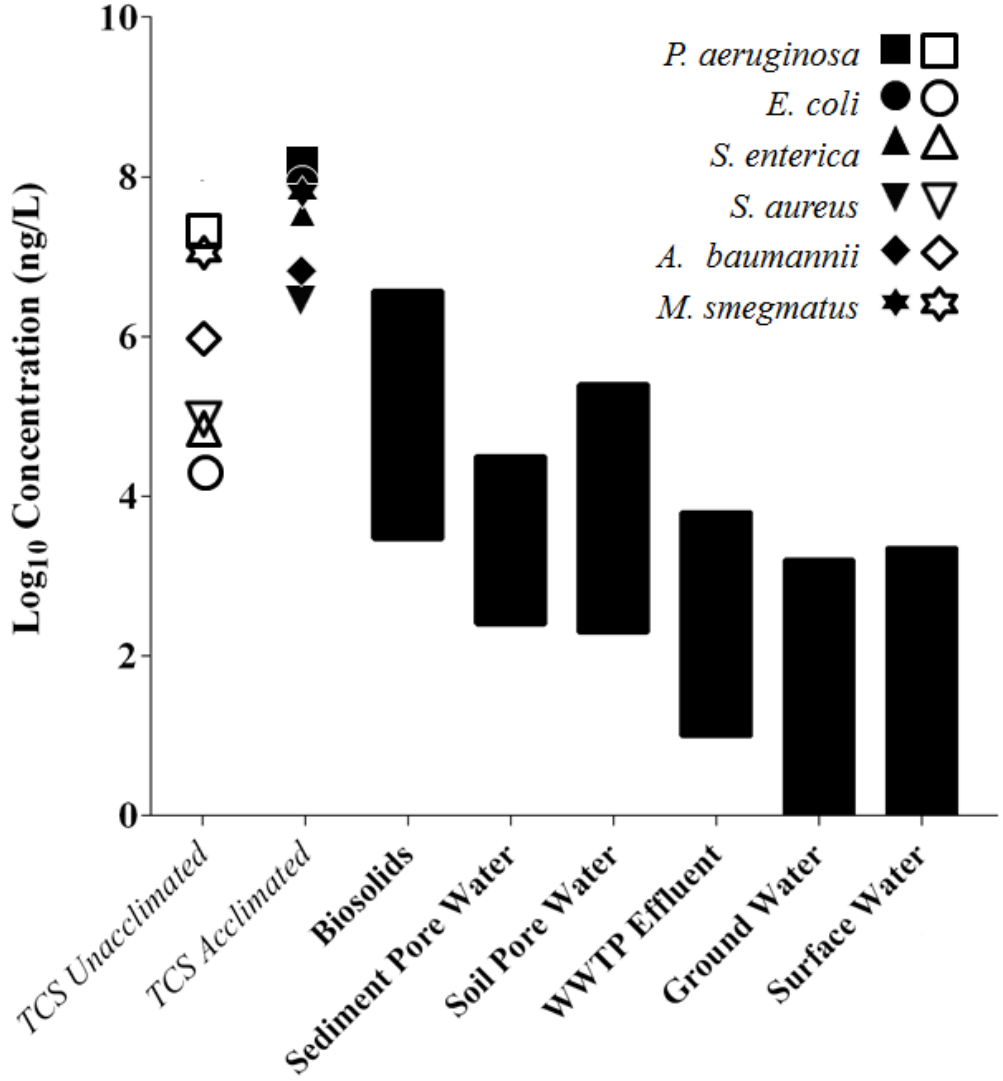

Figure 2.2 The MIC of TCS-acclimated and TCS-unacclimated strains relative to environmental TCS concentrations. Open symbols represent the MIC for TCS sensitive strains, while closed symbols represent the MIC for TCS adapted strains. Black bars are ranges of TCS concentrations found in each environmental setting. Biosolids concentrations were converted from mg/kg to mg/L by assuming 3% total solids in digesters that produce biosolids (Bedoux et al., 2012; Bailey et al., 2008; Chalew and

Halden 2009; Chen et al., 2009; Chuanchuen et al., 2001; Fan et al., 2002; Karatzas et al., 2007; McClellan and Halden, 2010; McMurray et al., 1998a; McMurry et al., 1999; Mima et al. 2007; Saleh et al., 2011; Slater-Radosti et al., 2001; Tkachenko et al., 2007; Webber et al., 2008; Yazdankhah et al., 2006; Yu et al., 2010).

2.4.2 Triclosan Resistance in Complex Microbial Communities

Bacteria with resistance to TCS are found in the environment, and experiments have been performed to determine whether TCS could be the cause for resistance. Drury et al., (2013) constructed artificial streams to control for other selective pressures such as antibiotics. The artificial streams were inoculated with approximately 8 mg/L of TCS. Over 34 days, the relative abundance of benthic bacteria which were able to be cultivated in 16 mg/L of TCS in agar climbed from 0% to 14%. In a similar study, TCS was added to artificial stream mesocosms at $1x10^{-4}$, $5\ x10^{-4}$, $1\ x10^{-3}$, $5\ x10^{-3}$ and $1\ x10^{-2}$ mg/L, and resistance to TCS significantly increased in bacterial populations exposed to TCS concentrations over $5x10^{-4}$ mg/L (Nietch and Quinlan 2013). This study was conducted at environmentally relevant concentrations, and suggested that TCS exposure leads to TCS-resistance. Middleton and Salierno (2013) discovered that TCS resistance was detected in 78.8% of fecal coliform samples from streams receiving wastewater, and 89.6% of these samples were resistant to 4 classes of antibiotics. *Escherichia*, *Enterobacter*, *Serratia* and *Citrobacter* were also found in the stream with resistance to TCS and multiple antibiotics. This study investigated real-world surface water samples which are implicitly associated with many uncontrolled variables. Accordingly, it infers, but does not prove, that TCS may be an external stressor that results in increased abundance of resistance genes.

Studies on the impacts of TCS on anaerobic digesters, where TCS is of highest abundance, are limited. Lab-scale studies revealed that TCS can affect multidrug resistance genes in anaerobic bioreactors. McNamara et al. (2014) found that TCS at 500 mg/kg selected for *mexB* in lab-scale anaerobic digesters inoculated with cow manure. In anaerobic digesters that were seeded with municipal biosolids, 500 mg/kg did not select for *mexB*, but methane production was inhibited. It is not yet known if anaerobic communities need to carry resistance genes in order to maintain function at these high TCS levels. The findings indicated that the microbial community structure, in addition to the concentration of TCS, influences the selection of resistance genes. Also, this research demonstrated that TCS can select for resistance, but does selection happen at environmental concentrations of TCS? Similarly, in activated sludge mesocosms, TCS selected for *tetQ* at 0.3 mg/L of TCS (Son et al., 2010). These two wastewater studies found a correlation between the presence of a resistance gene and TCS, but each study only investigated a single gene. A much more thorough research effort is required to determine the breadth of genes, with a special emphasis on multidrug resistance genes that are selected for when environmental concentrations of TCS are applied to the complex microbial communities found in WWTPs.

It is also possible that TCS-resistant bacteria are formed in premise plumbing which can feed into municipal WWTPs. In a sink drain biofilm, TCS was shown to affect the bacterial population structure when a 0.2% (~2000 mg/L) solution of soap containing TCS was pumped over the biofilm (Mcbain et al., 2003). Overall bacterial diversity was reduced and several TCS-resistant bacteria related to *Achromobacter xylosoxidans* increased in abundance, while other species including *aeromonads, bacilli*,

chryseobacteria, *kebsielellae*, *stenotrophomonads* and *Microbacterium phyllosphaerae* were reduced. TCS in a drain following consumer usage may result in resistant bacteria which are then sent to WWTPs. Research is needed to determine if these bacteria survive in the sewer system and whether these resistant bacteria influence the resistance profile in WWTPs.

These studies show that TCS in the environment could select for resistance genes. It seems likely that TCS resistance coincides with TCS-derived cross-resistance to antibiotics in the environment, but further studies are required to validate this point.

2.5 Research Gaps and Conclusions

It is noted that pathogenic bacteria, such as *S. epidermidis*, are less susceptible to TCS today than they were in the past (Skovgaard et al., 2013). Although resistance to TCS alone is not a threat to human health, antibiotic resistance is a major public health concern. Triclosan is widespread throughout the environment, but the direct role of triclosan on antibiotic resistance in environmental systems is not yet defined. Four specific research questions, which are outlined below, need to be answered to identify the role of triclosan on antibiotic resistance in environmental systems and ultimately determine the impact on human health.

2.5.1 Identify the role of Triclosan on Antibiotic Resistance in Environmental Systems

What is the threshold concentration of TCS that triggers resistance? TCS is found at a wide range of concentrations in a wide range of environments (see Figure 2.2), and previous work found that TCS *can* select for a resistance gene in a complex microbial

community (McNamara et al., 2014; Son et al., 2010). Moving forward, it is important to determine the concentrations of TCS that trigger an increase in antibiotic resistance genes. Answering this question will also help address the question framed by the lack of data in Figure 2.2, *i.e.* what is the effect of TCS concentrations below the MIC? Do low levels of triclosan select for resistance? Chronic exposure experiments using lab mesocosms should be performed at a range of steady-state TCS concentrations. In most real world cases, TCS levels will slowly increase, and lab experiments should be designed to reflect this slow loading rate. TCS levels should be slowly increased over time and held constant during steady-state operation of the mesocosm to determine the concentration of TCS that sustains changes in antibiotic resistance profiles. Metagenomics can be used with an Antibiotic Resistance Genes Database (Liu and Pop, 2009) to determine how the concentration of TCS impacts the relative abundance of antibiotic resistance genes. Additionally, qPCR can be employed to quantify changes in resistance gene abundance. After completion of these experiments we will have a better understanding about the concentrations of TCS that trigger increases in antibiotic resistance genes.

What is the role of the microbial community composition on TCS-derived antibiotic resistance? Previous work revealed that the same concentration of TCS can lead to different impacts on the abundance of a resistant gene depending on the microbial community (McNamara et al., 2014). Experiments outlined in the question above should be performed on several different microbial communities. For example, communities found in river sediments, soils, and anaerobic digesters should be investigated, and experiments should also be performed on several different communities from each type

of environment. Wastewater communities can vary widely in their structure and so could the impact of TCS on resistance in these communities. Mesocosms should be inoculated with biosolids from several different cities to quantify how the same TCS concentrations impact the antibiotic resistance profiles of different communities. Is there a universal TCS concentration that is of concern in anaerobic digester communities, in sediments, or in soils? Illumina sequencing on 16S rRNA genes should be performed as well to determine if a link exists between certain microbes in a community and the TCS-impacted resistance profile.

What is the impact of TCS on resistance profiles in environments that are also perturbed by antibiotics? Some resistance mechanisms, mainly efflux pumps, which are triggered by TCS are also triggered by antibiotics. In environments perturbed by TCS, antibiotics are also present (McClellan and Halden, 2010). Does the presence of TCS impact the acquisition of antibiotic resistance genes through horizontal gene transfer when antibiotics are already present? In other words, if TCS were not in these environments would the resistance profile look the same? To help answer this question, mesocosms could be inoculated with complex microbial communities from environments that are not heavily impacted by antibiotics or triclosan. One set of mesocosms could be amended with antibiotics and another set would be amended with antibiotics and TCS. It is important to add TCS and antibiotics at ratios typically found in the environment. Granted, this question is difficult to answer because complex microbial communities from pristine environments will have inherent differences from the communities that are typically exposed to TCS and antibiotics. Another possibility would be to use a microbial community that has been widely exposed to antibiotics but not exposed to triclosan; this

type of community might be readily found in countries that have not adopted wide-spread use of TCS. Molecular techniques described above could be employed to determine the added impact of TCS on antibiotic resistance gene profiles.

Will the abundance of resistance genes decrease if TCS concentrations decrease? It is important to know the concentrations of TCS that select for resistance and the communities that are most vulnerable to resistance caused by TCS, but it is equally important to know if resistance caused by TCS is reversible. Mitigated use of TCS has been proposed in the U.S. in part because of the potential concerns over antibiotic resistance (Landau and Young, 2014). If there were a sudden decline in consumer usage, would TCS-resistance and associated multidrug resistance decrease? Experiments should be performed where TCS is slowly increased to encourage TCS-resistance and the mesocosms should be operated at steady-state with a constant supply of TCS. After the resistance profile is determined, TCS should be removed from the system while the mesocosms are maintained under TCS-free conditions. The resistance profile can then be quantified after TCS is washed out of the mesocosms to determine if TCS-derived resistance will decrease as TCS levels decrease. This set of experiments would help to determine the potential impacts of reducing TCS from environmental systems.

2.5.2 Identify the Impact of Triclosan-derived Resistance in the Environment on Public Health

Complex microbial environments can be highly conducive for the transfer of resistance genes (Baqeuro et al., 2009). Locations with high densities of bacteria, such as WWTPs, produce conditions which are suitable for proliferation and exchange of resistance genes, and TCS may serve as a selective pressure to increase the abundance of

resistance genes in these communities. In a study focusing on plasmid genes found in activated sludge, a wide array of resistance genes, including genes that confer resistance to TCS in pure cultures (*mexB*, and other efflux pump homologues including *acrB* and *smeE*) were found on plasmids (Zhang et al., 2011). Research is needed to address the fate of environmentally-derived resistance genes to understand how they impact human health.

The fate and transport of these resistance genes in the environment following discharge from WWTPs is not well defined. Transport of genes can occur through direct uptake of DNA (transformation), by viral infection (transduction), or by transfer of plasmids and other mobile genetic elements (conjugation); the resulting pathways for genetic transport are challenging to model (Baqeuro et al., 2009). Genetic tracking of resistance in the environment would require vast resources; using established models of viruses or bacteria may be an appropriate place to begin modeling resistance gene transport.

The rate of transfer of antibiotic resistance genes in the environment to humans is also under investigation (Viau et al., 2011; Ashbolt et al., 2013). Better understanding the threat of environmentally-derived antibiotic resistance genes on human health is required to determine the role of TCS on public health. Employing quantitative microbial risk assessment for antibiotic resistance genes in environmental systems may be a useful avenue for pursuing this topic.

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3 THE INFLUENCE OF TRICLOCARBAN ON ANAEROBIC DIGESTION: FUNCTION, ANTIBIOTIC RESISTANCE AND MICROBIAL COMMUNITY STRUCTURE

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3.1 Introduction

Triclocarban (TCC) is a polychlorinated, binuclear, aromatic antimicrobial agent commonly used in bar soaps, detergents, cosmetics and other personal care products to prevent products from cultivating bacteria and spoiling (USEPA, 2002; Halden and Paull, 2005). Following consumer usage, TCC typically flows to wastewater treatment plants (WWTPs), and approximately 275,000 kg of TCC are sent to WWTPs each year (Heidler et al., 2006). TCC is not readily biodegraded in WWTPs, and approximately 75% of the TCC that enters a WWTP partitions to the biosolids (Pycke et al., 2014). In a US nationwide survey on micropollutants in biosolids, TCC was found in 100% of municipal biosolids at a median concentration of 22 mg/kg and an average concentration of 39 mg/kg (USEPA, 2009). Of the personal care products, pharmaceuticals and other analytes screened in this survey, TCC was detected most frequently and at the highest concentrations. The high abundance of TCC is concerning because it has been found to be persistent, toxic, and potentially bioaccumulative in biological systems (Halden and Paull, 2005).

Because TCC is designed to act against bacteria, the pervasiveness of TCC in biological engineered and environmental systems could impact microbial antibiotic resistance profiles (Oggioni at al., 2013; Halden, 2014). To date, very little research is available that describes the impacts of TCC on antibiotic resistance. Triclosan (TCS), which is also a polychlorinated, binuclear, aromatic antimicrobial agent, is a chemical analog of TCC and has been studied much more thoroughly for its impact on antibiotic resistance (Halden and Paull, 2005; Chapter 2). Specific molecular targets of TCS in *E. coli* were discovered in 1998, and since then multiple TCS resistance mechanisms have

been found in many bacterial genera (McMurry et al., 1998; Brenwald and Fraise, 2003; Chen et al., 2009; Pycke et al., 2010). The most prevalent forms of resistance to TCS are efflux through surface proteins, cell wall modification, and mutation of the target protein FabI, a key enzyme in the fatty acid elongation cycle (see Chapter 2, section 2.2.1). Pathogenic and environmental bacteria have been shown to exhibit these resistance properties towards TCS as previously reviewed (see Chapter 2, section 2.2 and 2.3).

Of greatest concern is that resistance acquired by exposure to TCS or TCC could lead to cross-resistance to other antibiotics (Son et al., 2010; Levy, 2002). Indeed, the expression of an efflux pump which confers TCS resistance can lead to resistance to other antibiotics with similar physicochemical properties (Son et al., 2010; Chuanchuen et al., 2001; Braoudaki and Hilton, 2004). Although little research has been performed to determine links between TCC and antibiotic resistance, many authors acknowledge TCC may present the same concerns as TCS with respect to cross-resistance to antibiotics (Halden, 2014; Walsh et al., 2003). Son et al. found that TCC, as well as TCS, selected for *tet*(Q) in aerobic activated sludge microcosms (2010). Resistance to TCC is most likely to occur in environments where TCC is pervasive at sub-inhibitory concentrations. Anaerobic digesters could be prime environments where TCC exerts selective pressure on antibiotic resistance genes (ARGs) because TCC sorbs to biosolids in a WWTP, and these biosolids are often stabilized using anaerobic digestion (Holzem et al., 2014). Moreover, retention times in anaerobic digesters are not long enough for significant biological transformation of TCC, yet the retention times are much longer than other unit operations providing bacteria a longer exposure time to adapt to TCC (Pycke et al., 2014; Heidler et al., 2006; Venkatesan and Halden, 2014).

The objective of this research was to determine if TCC impacts the abundance of ARGs as well as the microbial community structure and function in anaerobic digesters. Lab-scale anaerobic digesters were operated for 110 days with concentrations ranging from the background TCC levels (found in the seed biosolids) to inhibitory TCC concentrations that were twice the maximum concentration reported in the nationwide biosolids survey (USEPA, 2009). An additional goal of this research was to determine if the rate at which TCC concentrations increased in digesters would impact the ARG profiles and community structure. Various digester TCC concentrations were either immediately administered or attained after gradual increase over approximately four solids retention times (SRTs). It was hypothesized that microbial communities that were provided more time to adapt to higher TCC levels would maintain function, have an increased abundance of ARGs, and exhibit community structure changes compared to communities that were immediately amended with increased levels of TCC.

3.2 Materials and Methods

3.2.1 Experimental Setup

Lab-scale anaerobic digesters were operated for 110 days to determine the impacts of TCC loading rates and concentrations on ARGs and community structure. The digesters were inoculated with anaerobic digester biosolids taken from municipal digesters at South Shore Water Reclamation Facility (Oak Creek, WI, USA). Background TCC levels were measured at 27±3 mg/kg (average± average deviation of triplicate samples). Method details for TCC extraction and analysis by LC-MS are provided in the

Appendix A; recovery of ^{13}C-labeled TCC was 53% ± 10% (average ± standard deviation).

An SRT of 10 days was maintained. The digesters consisted of 160 mL serum bottles with a 50 mL working volume and were capped with butyl stoppers. Each digester was fed daily with synthetic primary sludge at a loading rate of approximately 3.6 g COD/(L_r-day). The synthetic primary sludge consisted of dog food (Nutro- Natural Choice, Franklin, TN, USA) at 3% solids in a nutrient medium (see Appendix B). TCC was added to the synthetic primary sludge. TCC was dissolved in acetone then applied to a 1 cm layer of dog food and allowed to dry for 48 hours. The dried dog food that contained the TCC was then mixed with the nutrient solution for daily digester feeding. Digesters were incubated at 35^{o}C and mixed on a shaker table at 100 rpm.

Eight sets of triplicate digesters were operated at different quasi steady-state TCC concentrations and ramp-rates (see Figure 3.1) to test if stress induced by TCC would result in an increase in ARG abundance. Three different stress levels (in addition to the background level) were tested: a low level that did not inhibit digester function, a medium level that moderately inhibited digester function, and a high level that severely inhibited digester function. The medium and high concentrations were determined based on preliminary anaerobic toxicity tests and equated to the IC_{10} (450 mg/kg) and IC_{50} (850 mg/kg), respectively (see Appendix C for description of anaerobic toxicity tests and results). All digester sets were maintained with the background TCC concentration detected in the seed biosolids (30 mg/kg) for the first 45 days with the exception of the control digesters that received no TCC. After 45 days, five different quasi steady-state TCC concentrations were used and labelled as control (0 mg/kg), background (30 mg/kg),

low (130 mg/kg), medium (450 mg/kg), and high (850 mg/kg). The low concentration was equivalent to the 95th percentile environmental concentration of TCC found in a nationwide survey of biosolids (*i.e.*, 5% of samples surveyed were at concentrations of 130 mg/kg or higher in USEPA, 2009). The medium concentration in this study was nearly equivalent to the environmental maximum concentration detected in the EPA biosolids survey of 440 mg/kg, and the high concentration was approximately twice the environmental maximum concentration (USEPA, 2009).

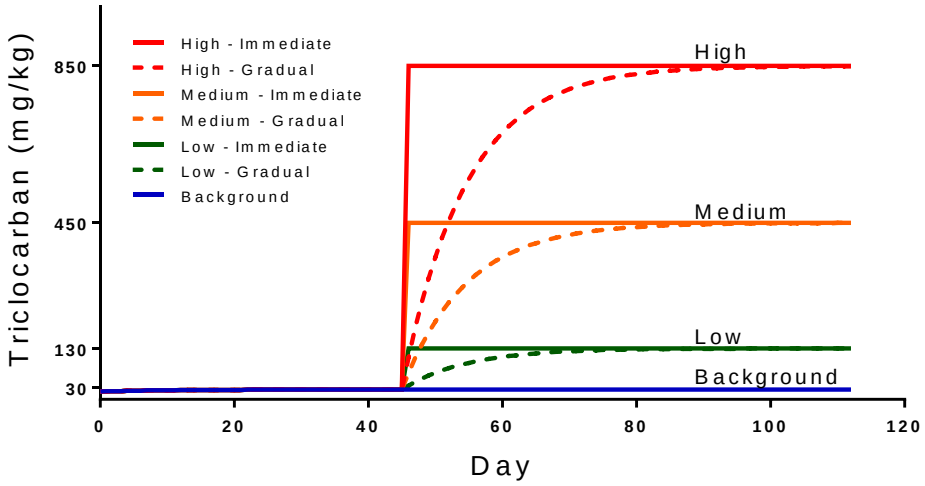

Figure 3.1 Nominal TCC concentrations (normalized to total solids) in triplicate sets of lab-scale anaerobic digesters. Not shown is a set of triplicate control digesters that received no TCC. All digesters, other than the control, were allowed to acclimatize to TCC feed at background concentrations that matched the TCC concentration of the original biosolids seed for 45 days before further addition of TCC.

On Day 46 the contents of three sets of digesters were immediately amended with TCC to their nominal TCC quasi steady-state concentration, and TCC was continuously added to maintain this concentration for the duration of the experiment. These digester

sets are referred to as low-immediate, medium-immediate, and high-immediate. Three other sets of digesters were fed TCC more gradually such that the nominal TCC concentration was not reached until after approximately three SRT values. These digester sets are referred to as low-gradual, medium-gradual, and high-gradual. Digester TCC concentrations were measured at Day 0, 33, 47, and 110. All measured values were within 20% of expected concentrations. See Appendix A for measured TCC values.

3.2.2 Molecular Methods

3.2.2.1 Microbial Sampling and DNA Extraction.

Approximately 1.8 mL of biomass slurry from each digester was taken prior to TCC addition on Day 45 and on Days 105, 107, and 110 after quasi steady-state conditions were established. DNA was extracted using MP FastDNA SPIN kits (Solon, Ohio) and modified to include 3 freeze-thaw cycles for improved lysis as described previously (McNamara et al., 2014). This extraction method may have an inherent bias towards the extraction of Bacterial DNA over Archaeal DNA (Urakawa et al., 2010).

3.2.2.2 Detection and Quantification of ARGs

ARGs were quantified for differences between TCC and control digesters at quasi steady-state. Quantitative PCR (qPCR) was carried out on several genes. The gene *mexB* was selected because it is part of the MexAB-Opr multidrug efflux pump that has been associated with triclosan resistance (McNamara et al., 2014; Ramsden et al, 2010); *tet*(L) was selected because it is also an efflux pump (Diehl and LaPara, 2010)*; erm*(F) was selected as a negative control because it is not an efflux pump, rather it confers

macrolide resistance through methylation and was therefore not anticipated to be selected for by TCC (Rasmussen et al., 1986); the integrase of class 1 integrons (*intI1*) was selected as an indicator of horizontal gene transfer (Ramsden et al, 2010; Mazel, 2006). These ARGs and *intI1* were normalized to the Bacterial 16S rRNA gene (Pruden et al., 2012). Primers, annealing temperatures, efficiencies, limits of quantification, and qPCR conditions are found in Appendix D.

3.2.2.3 Illumina Sequencing and Bioinformatic analysis

Sequencing of partial 16S rRNA gene amplicons and analysis was done to evaluate the microbial community structure of digesters and analysis was performed according to previously described protocols (Slapeta et al, 2015; Kolderman et al., 2015). Universal primers targeting the V4 variable region of 16S rRNA, 515F and 806R, were used for PCR amplification with HotStarTaq Plus Master Mix Kit (Qiagen, USA). PCR conditions consisted of 94°C for 3 minutes, followed by 28 cycles of 94°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, and a final elongation at 72°C for 5 minutes. PCR product was purified utilizing Ampure XP beads. The purified PCR product was used to prepare DNA libraries by following the Illumina TruSeq DNA library preparation protocol. Sequencing was performed at MRDNA (Shallowater, TX, USA) with Illumina MiSeq v3 300 base pair sequencing platform (Illumina, San Diego, CA, USA). Raw un-joined sequence data were quality filtered (Q25). Barcodes and primers were removed from reads. Further sequences were removed including: those with ambiguous base reads, those which are less than 200 base pairs, and those with homopolymer sequences of 7 base pairs or longer. The denoised sequences were then clustered in operational taxonomic units which have 97% similarity. Each taxonomical

unit was then compiled into taxonomic ‘counts’ and classified using BLASTn against a highly curated database derived from GreenGenes, RDPII and NCBI. Sequencing was carried out on 48 samples (one sample was taken from each of the triplicate digesters for eight different TCC conditions on day 45 and day 110).

3.2.3 Analytical Methods

Gas production from each digester was measured daily with a 150 mL wetted glass syringe. Approximately every 10 days biogas methane content was measured by gas chromatography and thermal conductivity detection (7890A, Angilent Technologies, Santa Clara, CA, USA) using a method described previously (Schauer-Gimenez et al., 2010). Volatile fatty acids (VFAs) were measured using a GC-FID as described previously (GC System 7890A, Angilent Technologies, Irving, TX, USA) (Schauer-Gimenez et al., 2010). The pH was measured using a pH meter and probe (Orion 4 Star, Thermo, Waltham, MA, USA).

3.2.4 Analysis and Statistics

Average, standard deviation, and average deviation values were calculated using Excel (Microsoft, 2013), whereas one-way ANOVA and t-test calculations were performed using GraphPad Prism (V 6.04) for methane production and relative gene abundance. Custom R scripts were used to perform dual hierarchal clustering (utilizing R commands hclust of covariance, heatmap, and gplots library) and nonmetric multidimensional scaling (nMDS) of anaerobic community sequence data gathered from Illumina (McNamara and Krzmarzick, 2010).

3.4 Results and Discussion

3.4.1 Influence of TCC on Anaerobic Digestion Performance

During initial steady state before TCC addition (days 31-44), the average COD conversion to methane was 90% ± 17% (average ± standard deviation) for all digesters, while average biogas methane concentration was 68% ± 2.5%. All digesters maintained a pH near neutral; pH data can be seen in Appendix E. Following functional digester operation at background TCC levels, addition of TCC resulted in decreased methane production at 850 mg/kg, but TCC concentrations of 130 mg/kg and below did not impact methane production (Figure 3.2). The control, background, low-level, and medium-gradual feed digester sets produced 67±8.5 mL of methane per day (corresponding to a COD conversion rate of 90± 16%) during quasi steady-state operation through Day 110, and methane production was not statistically different between these digesters (ANOVA, $p = 0.06$). The medium-immediate, high-gradual, and high-immediate digester sets produced only 3.0±1.0 mL of methane per day (corresponding to a COD conversion rate of 4± 1%) between days 80 and 110. The high-immediate digesters received 850 mg/kg of TCC on Day 45 and decreased methane production was observed on Day 46. The observed decrease in methane production might indicate that TCC directly inhibits methanogens, but could also stem from the inhibition of Bacteria that convert larger VFAs to acetate causing the digesters to sour. This high-TCC digester set had an immediate drop in biogas production and an associated rise in VFA concentration and drop in pH (see in Appendix F for total VFA data). In the high-gradual digesters, a greater than 10% difference in average methane production (relative to the control) occurred by Day 55 when TCC was approximately 560 mg/kg. By Day 60, when TCC

was only at 680 mg/kg, average methane production had decreased by over 90%. Although the decrease in methane production was more gradual than observed in the high-immediate digesters, these digesters also had a rise in VFA concentrations and a drop in pH and both digesters sets seemed to cease function due to a secondary buildup of VFAs. These conditions were too extreme for the microbial communities to successfully adapt and maintain methane production.

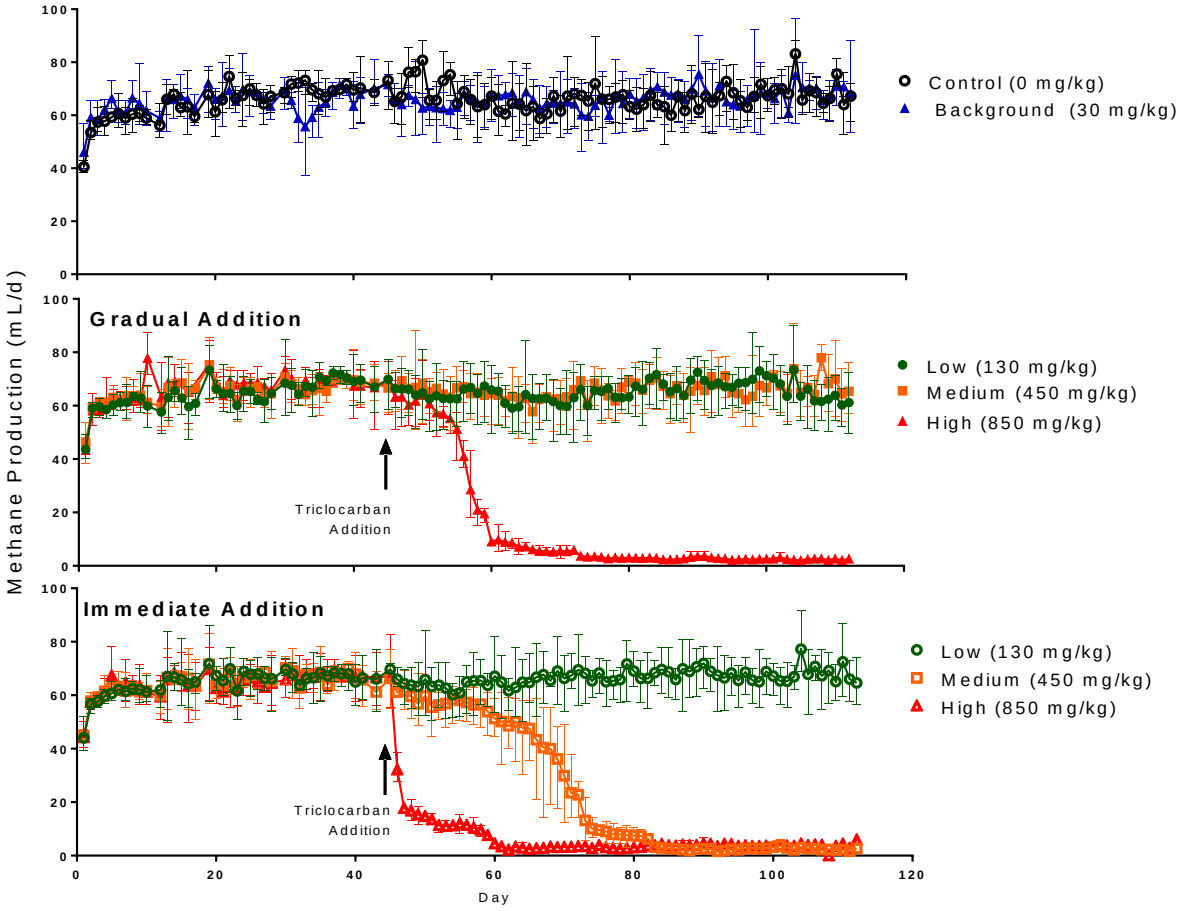

Figure 3.2 Methane production over 110 days of operation. Data points represent average values from triplicate digesters and error bars represent range of data. Total biogas production can be seen in Appendix I.

For the medium TCC concentration of 450 mg/kg, the TCC loading rate determined whether or not methane production was maintained (Figure 3.2). In the medium-immediate digesters methane production nearly ceased while the medium-gradual digesters maintained methane production throughout the experiment. The microbial communities in the gradual digesters were able to adapt to the slow buildup of TCC from 30 mg/kg to 450 mg/kg over three SRT values. The medium-immediate digesters were shocked with a 15x increase in TCC and did not have adequate time to adapt to this concentration of TCC; this digester set also had increased VFA concentrations (see Appendix F). An increase in VFAs is common following shock additions of toxicants (Ahring et al., 1995; Hickey and Switzenbaum, 1991).

Based on the functional data in Figure 3.2, full-scale anaerobic digesters should be able to maintain methane production if TCC concentrations increase slowly over time. The slow ramp-up used in this experimental study, as opposed to the immediate addition, is more similar to how concentrations would likely increase in full-scale anaerobic digesters if consumer usage and population density increases. These results imply that bacteria will have time to adapt to increasing TCC concentrations up to a certain threshold. This ability to acclimate to TCC is fortunate from a digester health standpoint because the medium concentration of 450 mg/kg used in this study that required acclimation time is similar to the environmental maximum concentration of 440 mg/kg already detected in biosolids. The slower ramp-up in TCC concentration to 850 mg/kg in the high digesters, however, did not allow the microbial communities to adapt and maintain function. In fact, the high-gradual digesters became inhibited well below the

850 mg/kg level as methane production was substantially reduced when TCC was only at 680 mg/kg. This inhibitory concentration is less than 2x the environmental maximum detect so it is feasible full-scale digesters could see these levels if consumer usage continues. It is noted the 50th percentile concentration of TCC measured in biosolids is only 21.7 mg/kg, and the 90th percentile is 88 mg/kg, which means the TCC concentrations in the majority of anaerobic digesters are still well below inhibitory concentrations (USEPA, 2009).

3.4.2 Influence of TCC on Abundance of ARGs and *intI1*

The continued functioning of the lab-scale anaerobic digesters upon the addition of TCC might be explained by the proliferation of TCC resistance mechanisms through horizontal gene transfer within the microbial community or the selection of individual bacteria with established resistance to TCC. Resistance mechanisms have been identified for other biocides, such as TCS and quaternary ammonium compounds, and many of these mechanisms also produce resistance to antibiotics (McMurry et al., 1998; Russell, 2000; Chapman, 2003). Efflux pumps are a resistance mechanism to many small molecules including antibiotics and the pumps are capable of eliciting cross-resistance (Levy, 2002). For these reasons, the antibiotic resistance genes encoding efflux pumps, *mexB* and *tet*(L) found in Bacteria, were investigated in this study.

The relative abundance of *mexB* was statistically higher in all TCC digesters during quasi steady-state relative to the control, as seen in Figure 3.3 (ANOVA, $p < 0.05$); gene concentrations normalized to digester volume can be found in Appendix G. The abundance of *mexB* in high-gradual digesters was significantly higher than in the background digester set as well ($p < 0.05$). TCC may have acted directly or indirectly to

select for the presence of the *mexB* gene, but increases in TCC concentrations did not consistently correlate with an increase in the relative abundance of *mexB*. As these data are a measurement of *mexB* gene copies, it is possible that expression of *mexB* increased as TCC concentration increased; alternatively, *mexB* may be capable of providing resistance up to a threshold concentration of TCC, beyond which, other resistance mechanisms become dominant. Also noteworthy is that the presence of the *mexB* gene was not sufficient for the anaerobic digesters to maintain function. This gene was likely maintained in Bacteria that were not critical for digester function, and moreover, Bacteria that had critical roles in maintaining function did not carry sufficient resistance mechanisms.

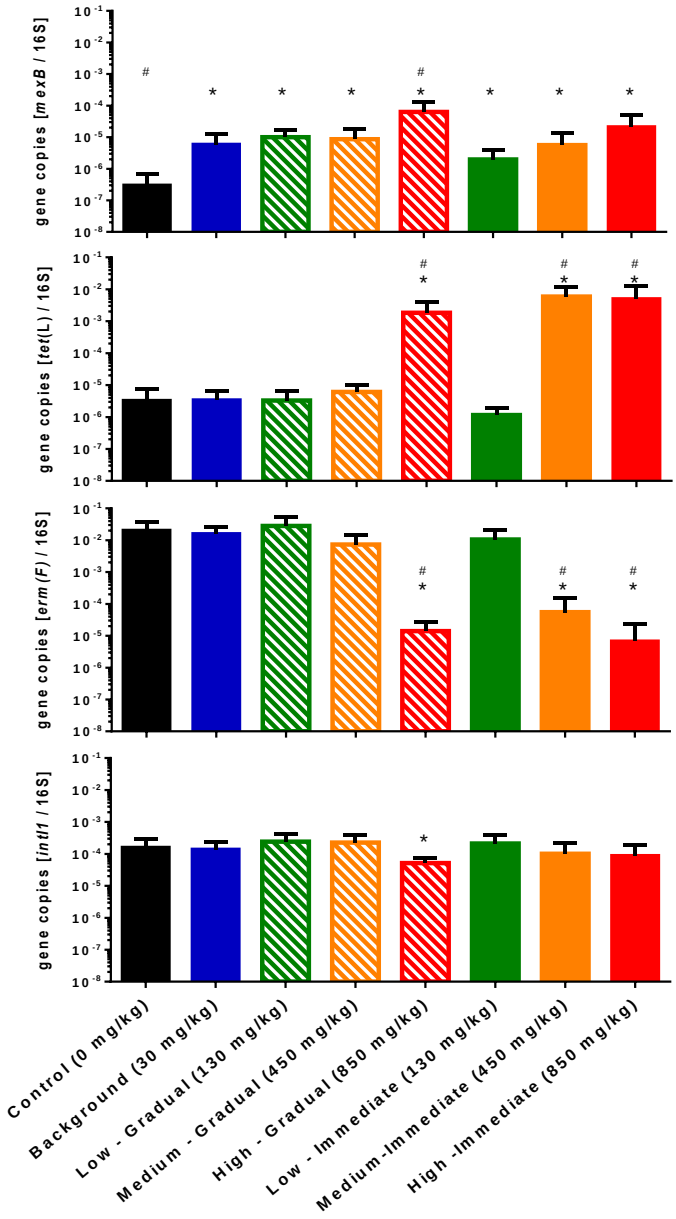

Figure 3.3 Abundance of ARGs and *intI1* at quasi steady-state. Each gene was normalized to 16S rRNA gene copies. Averages are shown with standard deviations of log values (n=9, triplicate digesters were sampled on three different days during quasi steady-state: Day 105, 107, and 110). A (*) denotes a statistical difference between the sample noted and the control ($p < 0.05$); A (#) denotes a statistical difference between the

sample noted and the background digester set which maintained a TCC concentration equivalent to what was found in the seed biosolids throughout the experiment ($p < 0.05$).

This research demonstrates that TCC can select for a multidrug resistance gene in anaerobic environments, and this selection occurs at concentrations that were observed in full-scale anaerobic digesters. The *mexB* gene encodes for the MexB subunit of the MexAB multidrug efflux pump (Li et al., 1995; De Angelis et al., 2010). The MexAB system is able to pump antibiotics, organic dyes, detergents and organic solvents from within a cell, and can decrease bacterial susceptibility to several classes of antibiotics and TCS (De Angelis et al., 2010; Li et al., 1994). The genera *Pseudomonas* and *Cupriavidus* are known to carry the *mexB* gene, along with other bacteria (Poole et al., 1996; Pycke et al, 2010). Results from Zhang et al., suggest that *mexB* is found on plasmids as well and may be mobile in the environment (2011).

The presence of TCC in anaerobic digesters could be selecting for multidrug-resistant Bacteria. The proliferation of the *mexB* gene has been observed in anaerobic digesters previously in response to biocides. In short-term 17-day experiments the *mexB* gene was selected for in anaerobic digesters as a response to TCS at 500 mg/kg, but not at 50 mg/kg (McNamara et al., 2014). In the longer-term experiments on TCC presented in this study, however, *mexB* selection occurred at background levels of 30 mg/kg. While the abundance of ARGs can be decreased during stabilization techniques such as lime stabilization or air drying beds, they still persist when biosolids are land-applied to the environment (Zhang et al., 2011; Munir and Xagoraraki, 2011). No research is available to describe the impacts of biosolids stabilization specifically on the *mexB* gene.

The abundance of the *tet*(L) gene was substantially increased (Figure 3.3) under

TCC loading conditions that also resulted in decreased pH (Appendix E) and methane production (Figure 3.2). The relative abundance of *tet*(L) gene copies was at least three orders of magnitude higher in the inhibited digesters (high-gradual, high-immediate, medium-immediate) than in the control digesters ($p < 0.05$). The relative abundance of *tet*(L) was not statistically different in any of the uninhibited digesters (ANOVA, $p = 0.47$). The pH in the inhibited digesters dropped from approximately pH 7 to approximately pH 5 following high TCC additions (See Appendix E). Therefore, the low pH was likely the selective pressure that selected for genera carrying the *tet*(L) gene, and TCC did not specifically select for *tet*(L). The importance of conditions associated with a drop in pH, as opposed to solely the TCC levels, on the selection of *tet*(L) is supported by the results from the two medium digester sets that were receiving the same amount of TCC at quasi steady-state. The medium-immediate digesters, in which methane production nearly ceased because of the immediate addition of TCC, had an increase in relative abundance of *tet*(L) and lower pH. The medium-gradual digesters, which slowly received TCC, had no increase in *tet*(L) and neutral pH was maintained. Perhaps acid tolerant clades harbor *tet*(L) more frequently.

The *tet*(L) gene has not been previously implicated as a response to TCC or other biocides. In this study, since no difference existed between control digesters and TCC-amended digesters which maintained methane production, it can be concluded that TCC does not impact the abundance of *tet*(L) in digesters that maintain function. Previous research found mesophilic anaerobic digestion can actually decrease the abundance of *tet*(L) gene copies, corroborating this result that digester operation which maintains efficient COD conversion minimizes the discharge of *tet*(L) resistance genes (Diehl and

LaPara, 2010).

The *erm*(F) gene was quantified as a control because it encodes for macrolide resistance by altering the molecular target (23S protein) of erythromycin and was not expected to perpetuate from TCC exposure (Rasmussen et al., 1986). Indeed, the *erm*(F) gene was not enriched in the functional TCC digesters relative to the control, but was selected against in digesters which were significantly inhibited. The decrease in *erm*(F) in the inhibited digesters was likely due to the shift in microbial community structure and function, similar to how *tet*(L) was increased in the inhibited digesters. Previous research found functioning mesophilic anaerobic digesters did not influence the relative abundance of *erm*(F) (Ma et al., 2011).

One mechanism by which resistance gene abundance can be increased is through horizontal gene transfer mediated through class 1 integrons (Burch et al., 2013). The relative abundance of class 1 integrons was not different between any digester groups except for the high-immediate digester which was significantly different, albeit lower, than the control ($p < 0.05$). Based on the results found in Figure 3.3, TCC did not select for *intI1*. The similar relative abundance of *intI1* indicates equal potential in the digesters for Bacteria to transfer genetic material through integrons.

3.4.3 The Impact of TCC on Microbial Community Structure of Anaerobic Digesters

The TCC concentrations and loading conditions that inhibited methane production also substantially altered the microbial community structure at the class and genus levels (Figure 3.4). Illumina sequencing generated an average of approximately 20,000 reads from each digester sampled. Significant differences in microbial community composition

were observed in the inhibited digesters at the class level (See Figure 3.4 [bottom]), while the digesters that maintained function were more similar. In the inhibited digesters the Archaeal class Methanobacteria was enriched, likely because the inhibited digesters had a pH of approximately 5.5 and some Methanobacteria are known to tolerate moderate acidity (Ma et al., 2011). The Bacterial classes Actinobacteria and Clostridia were enriched in the inhibited digesters as well; both of these classes contain pathogenic bacterial strains which may have antibiotic resistance, and the Actinobacteria class contains many acid tolerant bacteria (Ghosh et al., 2009; Patel et al., 1990).

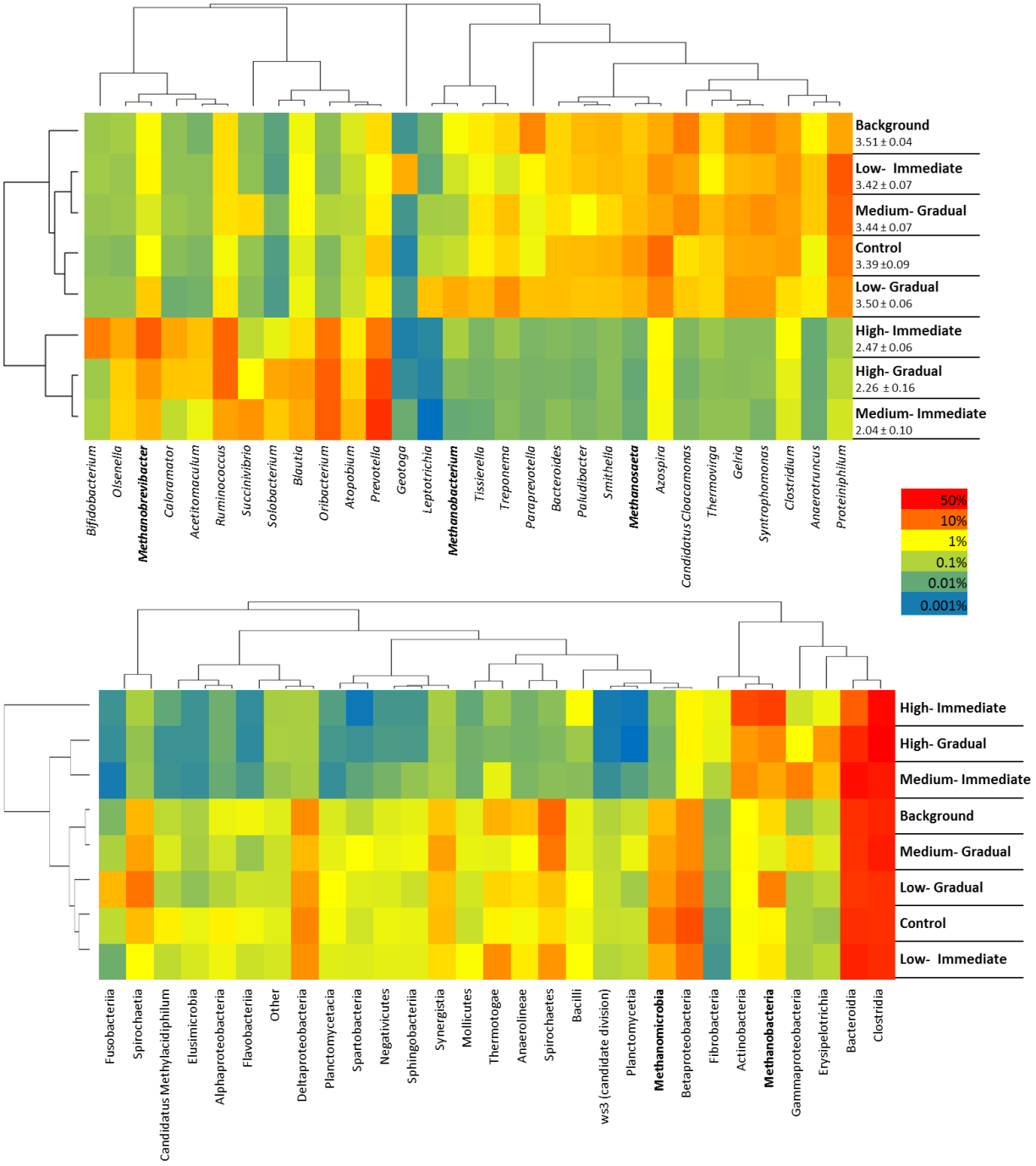

Figure 3.4 Dual hierarchal clustering of averaged communities on Day 110 (n=3 for triplicate digesters) for class (bottom) and 30 most abundant genera (top). Coloring indicates the relative abundance of the class or genera within the digesters. Each group is evaluated using Krushkal-Wallis analysis of variance and cosine distances. Archaea are

shown in bold. Shannon diversity index calculated using all genera for each sample is reported under each digester label (average ± average deviation).

In a dual hierarchal clustering of the 30 most abundant genera, digesters that continuously produced methane grouped together, and were different than inhibited digesters in which methane production nearly ceased (see Figure 3.4). The Shannon-diversity indexes were greater in all of the functioning digester sets compared to the digester sets where methane production decreased (Figure 3.4). Specifically, the genera *Prevotella* was highly selected for in the inhibited digesters. *Prevotella* are common members of the vaginal and ruminal microbiome and some species been shown to display resistance to antibiotics (Russell and Rychlik, 2001; Boskey et al., 1999; Boyanova et al 2010). *Prevotella* are found abundantly in digesters which include a pre-acidification step and were likely selected in this study because of their tolerance to low pH, and perhaps because of previously acquired resistance mechanisms (Bouallagui et al., 2004). In the uninhibited digesters, *Proteiniphilum* was detected at higher abundance. This genera encompasses acetate producing organisms which have been found in anaerobic digesters that treat protein-rich brewery waste; it is suspected these organisms were enriched because the dog food feed was high in protein (Chen and Dong, 2005).

Microbial community shifts may be responsible for adaption to TCC and increased resistance in functioning digesters. An nMDS plot that includes all digester sets can be found in Appendix H; the differences in community structure between the functioning and failing digesters is so stark that differences among the functioning digesters cannot be distinguished. The community structures of functioning digesters were further analyzed by nMDS (Figure 3.5). On Day 45, when digesters had not yet

received increased TCC loadings above background levels, the communities were very similar based on heavy overlap of 95% confidence ellipses (Figure 3.5a). By Day 110, when communities had received TCC at different levels for over 6 SRT values, the communities diverged (Figure 3.5b). The control digesters were different than all digester sets at 95^{th} percent confidence interval except for the TCC background digester set. The low TCC digesters and the medium-gradual digesters all shifted away from the background digester set, and thus were distinctly different from the control and background digesters. These shifts in community structures suggest that the microbial communities shifted towards bacteria that were more resistant to TCC. In general, communities shifted further away from the control as the TCC concentration increased.

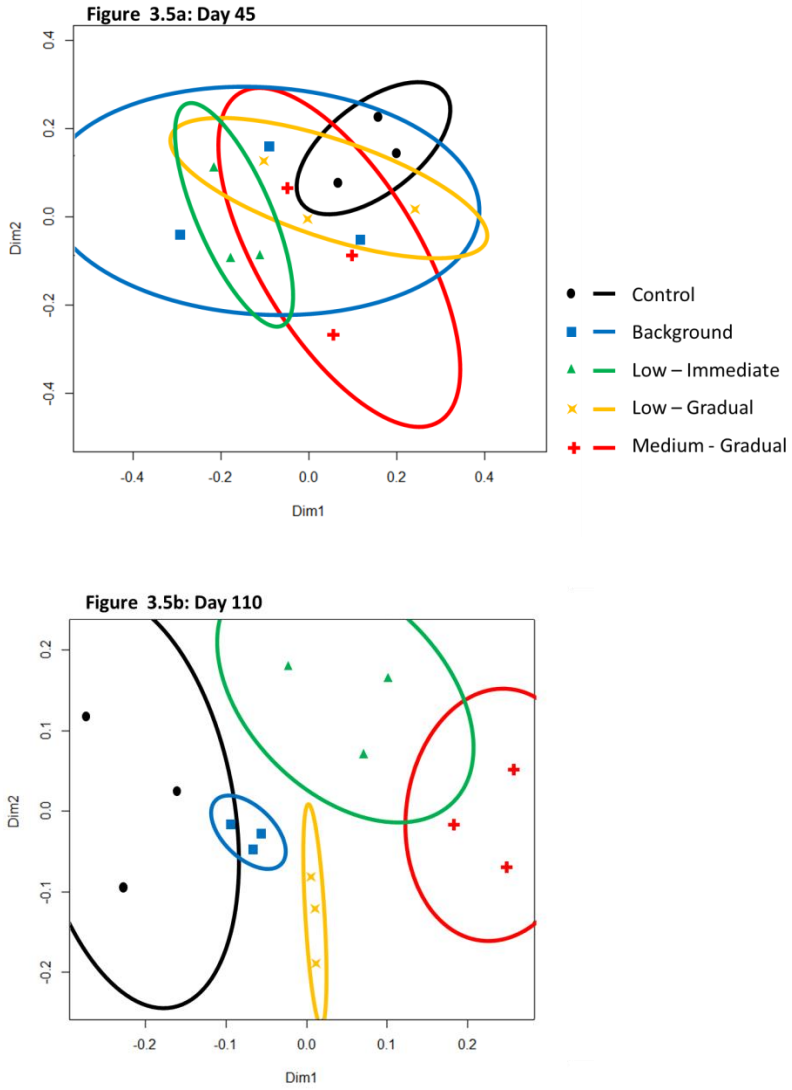

Figure 3.5 nMDS of all genera data generated for digesters that maintained methane production. Data from Day 45 are shown on top in Figure 3.5a, data from Day 110 are shown in Figure 3.5b. Ellipses represent 95% confidence intervals for the three points (each group represents the three triplicate digesters).

When considering the nMDS plot along with sequencing results, the shifts in the TCC-communities away from the control communities stemmed from changes in several genera. A Kruskal-Wallis test revealed that 52 genera had significant differences among

these digesters ($p<0.05$), but only 7 genera were represented by more than 1% of the population (47 genera made up a total of 1.1% of the average population of functioning digesters). The genera which represented more than 1% of the average population were: *Candidatus cloacamonas* (5.7%), *Proteiniphilum* (12.4%), *Methanobacterium* (1.1%), *Paraprevotella* (3.0%), *Bacteroidales* (1.1%), *Azospira* (7.9%), and *Thermovirga* (2.2%). These genera may represent some of the major genera which TCC selects for (*Methanobacterium, Candidatus cloacamonas*) or against (*Bacteroidales, Azospira),* and may contribute to TCC resistance in a digester. The functioning TCC digesters also all had a greater fraction of *Bifidobacterium*, *Olsenella*, *Methanobrevibacter*, *Oribacterium*, *Atopobium, Ruminococcus,* and *Blautia* relative to the control. Conversely, the functioning TCC digesters had lower fractions of *Clostridum, Proteiniphilum, Paludibacter, Smithella, Thermovirga,* and *Methanosaeta* relative to the control.

3.5 Implications

To better understand the impacts of TCC on public health and engineered systems, TCC needs to be further investigated for its role in impacting antibiotic resistance and microbial community structure, specifically in anaerobic digesters where TCC often resides. The results of this research suggest TCC is already present in anaerobic digesters at concentrations that act as a selective pressure for or against antibiotic resistance. The abundance of the multidrug efflux pump encoded by the *mexB* gene was at least an order of magnitude higher in all lab-scale anaerobic digesters that received TCC when compared to a control. The selection for *mexB* occurred at a TCC concentration (30 mg/kg) which is the same order of magnitude as the national median (22 mg/kg) and mean (39 mg/kg) concentrations (USEPA, 2009). This is the first

research to show TCC can select for a multidrug resistance gene in a mixed anaerobic microbial community. Further research using metagenomics needs to be conducted to determine if *mexB* is the only ARG for which TCC enriches. Additionally, research should be conducted to determine if removing TCC as a stressor can reduce the abundance of the *mexB* gene to better understand how changes in consumer usage can alter ARG profiles in digesters.

In the lab-scale digesters where high concentrations of TCC resulted in high levels of VFAs, decreased pH, and decreased methane production, the ratio of *tet*(L) genes to 16S rRNA gene copies increased by three orders of magnitude. Concentrations of 680 mg/kg of TCC resulted in a 90% decrease in methane production under the gradual loading conditions used in this study; concentrations as high as 441 mg/kg were found in a nationwide biosolids survey (USEPA, 2009). A doubling of the environmental maximum TCC concentrations could cause digester failure; however, the concentration of TCC in the majority of anaerobic digesters is well below toxic concentrations. Important questions to answer are i) in which environments (*e.g.*, anaerobic digesters, soils, sediments) and how many environments is TCC selecting for antibiotic resistance ii) is this resistance reversible, iii) how is TCC altering the dynamics of microbial communities in full-scale digesters and other real-world environments, and iv) do TCC, TCS, and antibiotics have synergistic effects on antibiotic resistance in anaerobic digesters?

3.6 References

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4 THE INFLUENCE OF TRICLOSAN ON ANAEROBIC DIGESTION: FUNCTION, ANTIBIOTIC RESISTANCE AND MICROBIAL COMMUNITY STRUCTURE

This chapter has been submitted to *Environmental Science: Processes and Impacts*:

Carey, D.E, Zitomer, D.H., Hristova, K.R., Kappell, A.D., Choi, M., & McNamara, P.J. Chronic exposure to triclosan alters microbial community and sustains increased *mexB* levels in anaerobic digesters. *In-Review*.

4.1 Introduction

Antibiotic resistance has been recognized as a major threat to public health (CDC, 2013) and can be stimulated by use of antibiotics, which creates an opportunity for bacteria to become resistant (Alanis, 2005). In addition to concern stemming from the overuse of antibiotics, antimicrobials are a concern with regard to proliferation of antibiotic resistance (Oggioni et al., 2013). Antibiotics have specific inhibition mechanisms towards certain bacteria which make them useful for medical treatments; in contrast, antimicrobial is a general term used for chemicals which kill or inhibit microorganisms. Antimicrobials in personal care products are generally thought to be broad-spectrum inhibitory chemicals.

Triclosan (TCS) is an antimicrobial chemical found in multiple consumer products, including liquid hand soaps, lotions, toothpaste, plastics and many other personal care products (Yazdankhah et al., 2006). Resistance to TCS has been well documented in pathogenic bacteria (Saleh et al., 2010; Yazdankhah et al., 2006). TCS has specific genetic targets within cells which inhibit fatty acid synthesis at low concentrations (McMurry et al., 1998). Perhaps because of this specific inhibition, multiple species have developed resistance to TCS. Common resistance mechanisms to TCS include FabI modification, membrane alteration, or active efflux (See Chapter 2; Brenwald and Fraise, 2003; Levy, 2002; Champlin et al., 2005; Massengo-Tiassé and Cronan, 2009).

TCS is an especially concerning antimicrobial because resistance to TCS can also result in cross- resistance to antibiotics (Saleh et al., 2010; Schweizer, 2001). Multiple studies covering various species have shown exposure to TCS can result in increased

resistance to chloramphenicol and tetracycline. The cross-resistance for chloramphenicol developed from TCS exposure has been found in *E. coli* (Braoudaki and Hilton, 2004), *P. aeruginosa* (Chuanchuen et al., 2001), *S. maltophilia* (Sanchez et al., 2005), and *S. enterica* (Birosová and Mikulásová, 2009; Karatzas et al., 2007).

TCS is widely detected in the environment and ubiquitous in wastewater treatment plant influent. It has further been linked to resistance in bacteria found in pipes, sinks, wastewater treatment effluent, activated sludge, anaerobic digestion, and streams (Nietch and Quinlan, 2013, Middleton and Salierno, 2013, Son et al., 2010, Mcbain et al., 2003). The majority of TCS that enters a treatment plant sorbs to solids and passes through anaerobic digestion. TCS is persistent under anaerobic conditions (Heidler and Halden, 2007, Pycke et al., 2014; Ying et al., 2007). Additionally, TCS has been shown to alter microbial community structures in anaerobic environments (McNamara, Lapara, and Novak 2014). Previous research demonstrated that TCS could select for *mexB*, a component of a multidrug efflux pump, in mixed anaerobic communities seeded with manure, but no research describes the impact of long-term chronic exposure to TCS in anaerobic communities seeded with municipal anaerobic digester sludge.

The objective of this study was to determine if long-term exposure to TCS resulted in sustained increases in antibiotic resistance genes (ARGs) and altered microbial community structure. Lab-scale digesters were seeded with municipal biosolids from anaerobic digesters, and the digesters were acclimated to various elevated levels of TCS. Digesters were operated under steady-state conditions for 6.5 solid retention time (SRT) values before being sampled for ARGs. Quasi steady-state samples were taken on 3 different days from triplicate digesters after the 6.5 SRT values and analyzed for the

relative abundance of *mexB*, *intI1*, *tet*(L), and *erm*(F). Samples for microbial community analysis were taken after 6.5 SRT values as well.

4.2 Experimental

4.2.1 Setup

Lab-scale anaerobic digesters (160 mL serum bottles with 50 mL working volume) were operated for 110 days. The digesters were fed synthetic primary sludge (3.6 g COD/LR-d, 10 day SRT) daily with a syringe. Synthetic sludge was ground, sieved (40 mesh) dog food (Nutro- Natural Choice, Franklin, TN, USA) in a nutrient medium (See Appendix B). The digesters were seeded with municipal anaerobic digester biomass from South Shore Water Reclamation Facility (Oak Creek, WI, USA).

4.2.2 TCS Digester Concentrations

A total of 15 digesters (5 sets in triplicate) were operated for 45 days and fed the background TCS concentration measured in the biomass (30 mg/kg) with the exception of the control which received no TCS. On Day 45 three sets of digesters were fed ‘low’, ‘medium’, and ‘high’ concentrations of TCS (See Figure 4.1). The low concentration (100 mg/kg) was between the 95th percentile (62 mg/kg) and 98th percentile (124 mg/kg) TCS biosolids concentration observed during an EPA survey of municipal biosolids (USEPA, 2009). Medium (850 mg/kg) and high (2500 mg/kg) concentrations correlated to the concentrations of TCS which inhibited methane production rate by 10 and 50%, respectively, based on a previous anaerobic toxicity assay using the seed biomass (Appendix J). The ‘background’ set of digesters was maintained at 30 mg/kg throughout

the entire experiment. All concentrations in the biosolids were confirmed by Liquid Chromatography-Mass Spectroscopy (Appendix K). TCS was added to the synthetic primary sludge by mixing an appropriate amount of TCS dissolved in methanol to dog food which was then evaporated to dryness to remove methanol.

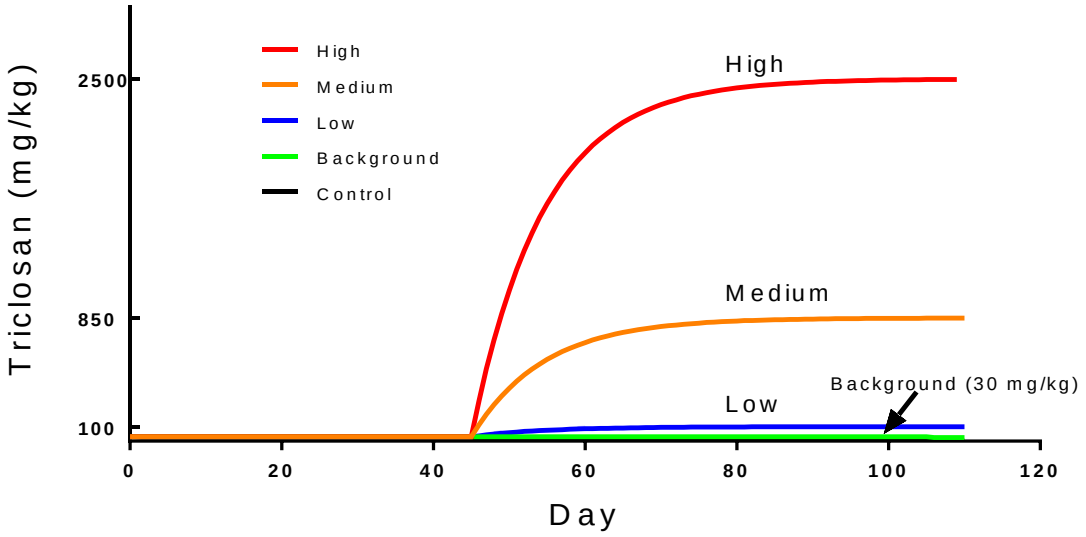

Figure 4.1 Concentration of TCS in digester sets over the duration of the study. All digester sets (except for the control set operated at 0 mg/kg for the total 110 days), were operated at 30 mg/kg for the first 45 days.

4.2.3 Analytical Methods

The pH was measured using a probe and meter (Orion 4 Star, Thermo, Waltham, MA, USA). Volatile fatty acids (VFAs) and methane percent of biogas were measured by gas chromatography (7890A, Angilent Technologies, Irving, TX, USA) (Schauer-Gimenez et al., 2010). Carbon dioxide content was estimated by calculating (100% - Methane %).

4.2.4 DNA extraction

DNA was extracted using a commercial kit (MP Fast DNA SPIN kits, Solon, Ohio) modified with freeze thaw cycling to improve yield (McNamara et al., 2014). Extraction was performed on biomass samples collected on Day 45, 105, 107, and 110 from each digester. Approximately 2 mL of biomass suspension was used for extraction.

4.2.5 qPCR for Resistance Genes and *intI1* Quantification

Quantitative polymerase chain reaction (qPCR) analysis was performed for select resistance genes. The *mexB* gene, associated with a multidrug efflux pump, has been previously associated with resistance to TCS and cross resistance to antibiotics (Mcnamara et al., 2014; Pycke et al., 2010). A tetracycline resistance gene, *tet*(L), was also quantified as it encodes for an efflux pump (Jin et al., 2002). As a control, *erm*(F) was quantified, as TCS concentration was not suspected to influence abundance of this gene because the gene specifically confers resistance macrolides, licosamides, and streptogramin by mutating the target of these drugs, rRNA(Rasmussen et al., 1986). Finally, *intI1*, which is associated with class 1 integrons that facilitate the horizontal exchange of resistance genes, was quantified (Mazel, 2006). Specific primer sets, annealing temperatures, efficiencies and quantification limits are described in Appendix D.

4.2.5 16S rRNA Gene Sequencing

The microbial community of each digester was determined by partial sequencing of the 16S rRNA genes of samples from Day 45 and 110 using the methods outlined in

Chapter 3, section 3.2.2.3 (performed by MRDNA Molecular Research LP, Shallowater, TX). Briefly, amplification of the V4 region of the 16S rRNA gene was performed prior to Illumina sequencing. Approximately, 20,000 sequences were identified per digester per time point, denoised sequences were binned in operational taxonomic units which had more than 97% similarity and classified using a database derived from GreenGenes, RDPII, and NCBI.

4.2.6 Statistics

The R Project for Statistical Computing program (V 3.1.2, Vienna, Austria) was used to produce Non-Parametric Multidimensional Scaled (nMDS) plots using the VEGAN package. Dual hierarchal clustering (using Kruskal-Wallis analysis of variance and cosine distances), heat mapping and Shannon diversity indices were also calculated using R-scripts. GraphPad Prism (V 6.04, La Jolla, CA) was utilized to perform ANOVA and t-tests.

4.3 Results and Discussion

4.3.1 Digester Conditions

Methane production of the digesters receiving high concentrations of TCS substantially decreased; approximately 80% of methane production was lost by Day 71 (Figure 4.2). At this time, the average digester concentration of TCS was 2340 mg/kg. All other digesters continued to produce 67 ± 8.7 mL methane per day (>90% COD conversion). For the first 45 days, all digesters performed similarly and produced an

average of 68 mL ± 6.8 mL of methane per day with the total biogas being 32 ± 3.6 % CO_2.

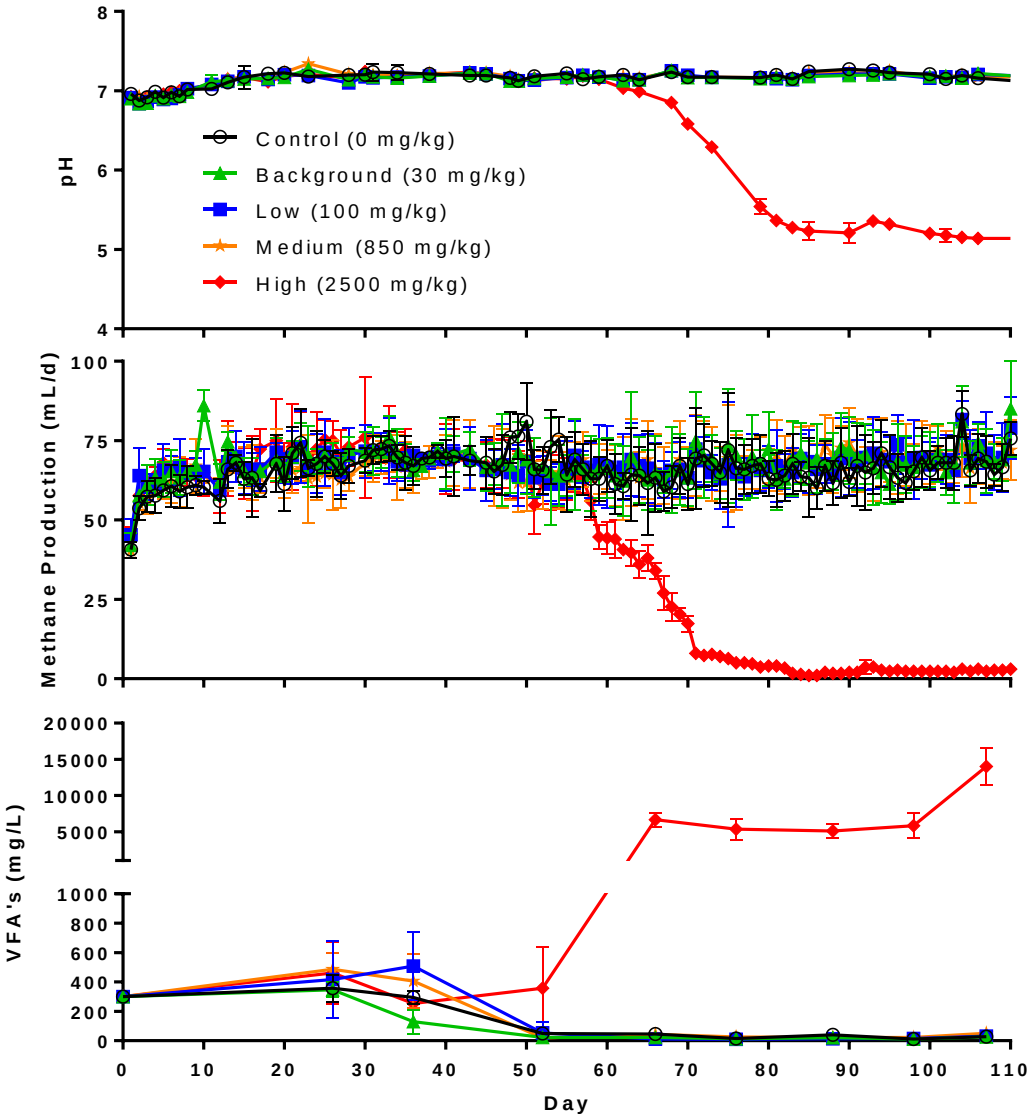

Figure 4.2 pH, methane production and VFA concentrations. Error bars represent standard deviations (n=3 for all points), and some error bars are small and not visible.

VFAs included acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid, and iso-valeric acid.

For the digesters with high concentrations of TCS, a key acid-utilizing bacterial (or perhaps archaeal) group was likely inhibited, resulting in a buildup of VFAs (Figure 4.2). It should be noted that this toxic concentration of TCS is much higher than that observed in full-scale digesters; the maximum TCS concentration found in the EPA biosolids survey was 133 mg/kg (USEPA, 2009). Environmental concentrations of TCS are unlikely to pose a threat to the functioning of full-scale anaerobic digesters.

4.3.2 Resistance Genes

The *mexB* gene relative abundance was statistically higher in every digester that received TCS compared to that of the control (Figure 4.3). However, higher TCS feed concentrations did not correlate with higher relative *mexB* abundance; the relative abundance of *mexB* was not statistically different among the TCS-amended digesters (ANOVA, $p= 0.79$). The *mexB* gene is of concern because it has been associated with resistance to TCS in more than one species (Chuanchuen et al., 2001; Pycke et al., 2010). Furthermore, bacteria that have increased resistance to TCS through the MexAB efflux protein have cross-resistance to other antibiotics, including tetracycline, ciprofloxacin, trimethoprim, erythromycin and gentamycin (See Chapter 2). It should be noted that all digesters were seeded with municipal anaerobic biosolids used to treat municipal wastewater primary sludge; therefore, background levels of all resistance genes were observed in the control.

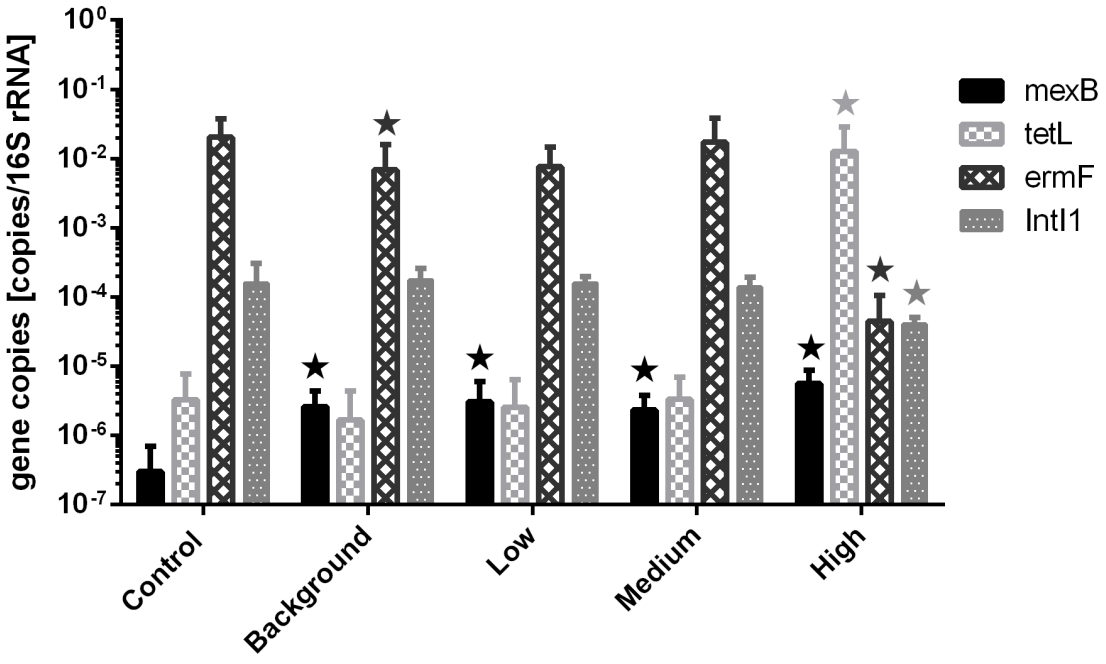

Figure 4.3 Gene abundance on Day 110 normalized to 16S rRNA concentration (triplicate days from triplicate digesters, n=9). Error bars represent standard deviation. Statistical differences from the control ($p < 0.05$) are indicated with a star. Note concentrations of 16S rRNA were not found to be statistically different between treatments (ANOVA, p= 0.46, n=9, see Appendix M).

To date, one other study investigated the impact of TCS on resistance genes in mixed anaerobic cultures; the researchers discovered that TCS was able to select for *mexB* in the anaerobic cultures (McNamara et al., 2014). The results from that study, however, were not pertinent to full-scale municipal digester scenarios for multiple reasons. First, *mexB* was observed after 17 days of digester operation and steady-state was not achieved; therefore, no data were generated to determine if this increase in *mexB* would be sustained through steady-state. Additionally, the selection for *mexB* occurred at a TCS concentration of 500 mg/kg, approximately 4 times the environmental maximum

detection (USEPA, 2009). Finally, the selection was observed in anaerobic digesters inoculated with biosolids from a manure-fed anaerobic digester that ostensibly was not previously exposed to TCS. While that study proved that TCS can select for *mexB* in mixed anaerobic communities, the current study shows that TCS can select for this multidrug resistance gene during steady-state operation of municipal anaerobic digesters at concentrations less than 100 mg/kg that have been observed in full-scale, operating anaerobic digesters. The research described in this manuscript demonstrates that sustained concentrations of TCS in municipal biosolids have a lasting impact on the abundance of *mexB*. The wide-spread use of TCS and its ubiquitous detection in biosolids indicates that TCS is a continuous selective pressure in anaerobic digesters. The minimal threshold concentration of TCS that selects for *mexB* in anaerobic digesters is yet to be determined

The relative abundance of the *tet*(L) gene was statistically similar for the control, background, low and medium digesters (ANOVA, p=0.75). The concentrations of *tet*(L) in the high digesters were over three orders of magnitude greater than in the other digesters. The high-TCS digesters functionally failed, ceasing to convert COD efficiently (COD conversion <5% to methane. It is suspected that the acidic conditions selected for bacteria that harbored *tet*(L) (the 16S abundance were not statistically different from other digesters on a volume basis; see Appendix M). Some efflux pumps are capable of expelling small molecules (such as dyes and detergents) from within bacteria (Piddock, 2006); likewise, the Tet(L) pump may be able to expel toxic molecules which are produced under acidic conditions. Tet(L) may also be intrinsic to a phyla that was highly selected for in the high-TCS digesters, that can survive at low pH conditions. In either

case, TCS did not select for *tet*(L) under in digesters which maintained greater than 90% COD conversion.

The digester with high concentrations of TCS had a relative abundance of approximately 2 orders of magnitude less *erm*(F) than other digesters. The control, background, low and medium digesters had statistically similar relative abundance of *erm*(F) (ANOVA, p-value = 0.31). The *erm*(F) gene was not expected to be influenced by TCS because this resistance mechanism specifically resists macrolide compounds by methylating rRNA (the target of macrolide drugs) (Rasmussen et al., 1986). Similar to the *tet*(L) observations, the acidic conditions in the high-TCS digesters were suspected to be selecting against organisms containing *erm*(F).

The relative abundance of the integrase gene of the class 1 integron is independent of TCS concentration and bacterial population composition in these functioning digesters. No statistical difference was seen in the relative abundance of *intI1* between the medium, low, background and control digesters (ANOVA, p=0.86). The high-TCS digesters had a statistically lower concentration of *intI1* when compared to the control (t-test, $p<0.05$). These results suggest that the concentration of TCS (2500 mg/kg), or the low pH, selected against bacteria with Class I integrons, possibly indicating that resistance to TCS was not integron based. Feasibly, the concentration of integrons could be sufficiently high in all digesters for significant horizontal gene transfer to occur in all digester conditions.

4.3.3 Community Structure

On Day 110, when digesters had reached quasi steady-state (i.e., operating under the same TCS-loading conditions for > 3 SRT values), the functioning TCS-amended

communities, including the background level TCS communities, had diverged from the control (Figure 4.4). Earlier, however, on Day 45, the TCS-amended communities were not statistically different from the control, as indicated by 95th percentile confidence intervals overlapping. The control communities at Day 45 were significantly different from the control communities on Day 110; this variation in community structure over time is common in biological systems and highlights the importance of maintaining a control (Drury et al., 2013). Microbial communiesin the background, low, medium, and high digesters were also different from themselves between Day 45 and 110, but remained in overlapping clusters on each day. The fact that the TCS-amended communities did not overlap with the control communities on Day 110 indicates that TCS impacts microbial community structure even when the digesters maintain function.

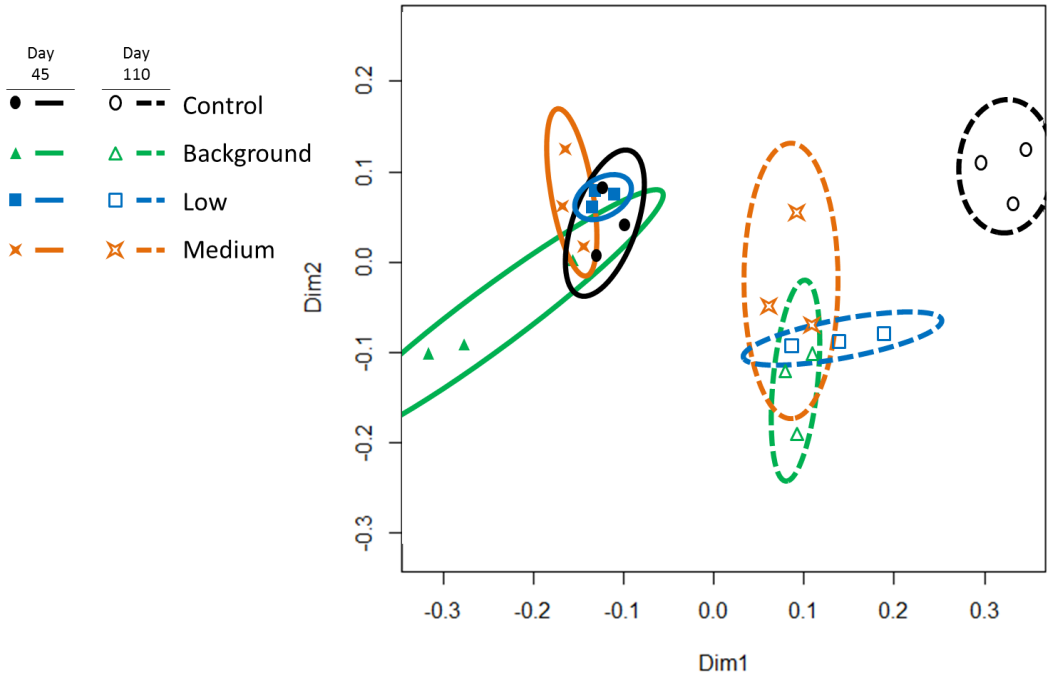

Figure 4.4 nMDS ordination of genus level data at Day 45 and Day 110. The community structure data was gathered from Illumina partial sequencing 16S rRNA gene. Ellipses represent 95% confidence intervals as calculated by the VEGAN package in R. High TCS treatment was not included because the community is starkly different and reduces plot resolution. An nMDS plot including the 'high' digesters can be found in Appendix L.

The community composition data indicate that TCS may be selecting for phyla and genera which contain pathogens and commensal organisms in functioning anaerobic digesters (Figure 4.5). Pathogens and commensal organism are more likely to have been previously exposed to TCS (or other resistance stressors) due to their association with people; therefore, these organisms may have previously gained resistance mechanisms. It should be noted the seed biomass for these digesters came from a real world treatment

plant which was exposed to relatively lower levels of TCS and many other organic chemicals, which may impact previously gained resistance mechanisms. In the control digesters, the relative abundance of the phyla Tenericutes, Fusobacteria and Spirochaetes was less than half of the relative abundance in the TCS-amended functioning digesters (Figure 4.5 [Left]). Pathogens and commensal organisms are found in each of these 3 phyla, suggesting live TCS digesters enrich for organisms which were previously exposed to high concentrations of TCS (Huang et al., 2001; Lis et al., 2015; Aliyu et al., 2004; Redford et al., 2005). Conversely, the control digesters had a higher relative abundance (approximately 2 fold higher) of the phyla Proteobacteria, Euryachaeota, Acidobacteria, Thermotogae, and Elusimicrobia. These phyla may be sensitive to TCS. With the exception of Proteobacteria, these phyla are largely environmental Bacteria or Archaea and are not typically commensal organisms (Aminov, 2013; Nesbø et al., 2010). The functioning digesters which contained TCS selected for several genera compared to the control, including *Cadidatus cloacomonas, Leptotrichia, Bacteroidales, Atopobium, Crocinitomix, Dermatophilus, Flavinofractor* and others which were less abundant (Figure 4.5 [Right]). *Leptotrichia, Bacteroidales, Atopobium, Dermatophilus, Flavinofractor* are major genera containing organisms which or pathogenic and commensal (Baldacchino et al., 2013; Eribe and Olsen, 2008; McLellan and Eren, 2014; Takagaki et al., 2014; White et al., 2011). *Candidatus cloacomonas* is suspected to be a syntrophic organism which is mainly found in anaerobic digesters (Pelletier et al., 2008). The community shift towards these clades could account for the selection of resistance genes, like *mexB*.

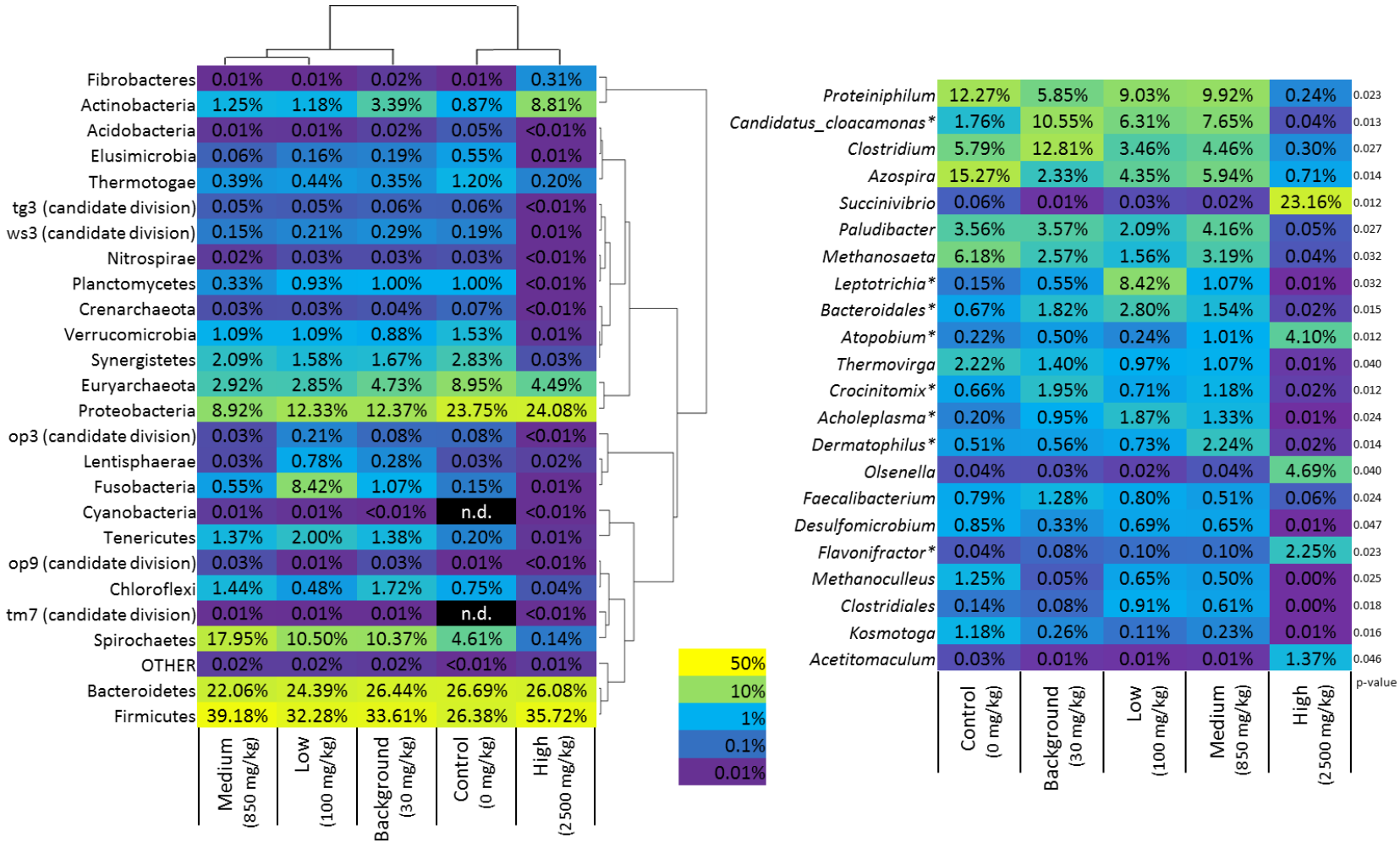

Figure 4.5 [Left] Dual hierarchal clustering of phyla (average of the 3 digesters). Black boxes represent no detection (n.d.). [Right] Genera that show significant differences between digester sets based on a Kruskal-Wallis test, and represent at least 1% of community in at least one digester set (average of 3 digesters). The p-value from the statistical test is shown or the right. Genera with star next to the name represent genera which were selected for in the live digesters which contained TCS (i.e., selected in background, low and medium digesters).

The functioning digesters had similar dominant phyla as observed in metagenomics analysis of full scale municipal digesters (Guo et al., 2015; Yang et al., 2014). These studies report Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria as the dominant phyla. Spirochates was more abundant than Actinobacteria in the current study, but detects were relatively high in the other studies as well. Further, TCS selects against two abundant genera of methanogens (*Methanocelleus* and *Methanosaeta*), but not to the extent that methane production ceased in functioning digesters. Major syntrophic bacteria were not significantly affected (*Smithella, Syntrophus, Syntrophomosos*; data not shown) (Smith et al., 2015).

The high-TCS digesters were significantly different from the functioning digesters (not included in figure 4.4, see Appendix L). Microbial diversity in the high-TCS digesters was lower than in the functioning digester sets, yet the overall abundance of total bacteria was similar (see Appendix M for 16S rRNA concentrations). The Shannon-diversity index (performed with genus level data) for the high-TCS digesters was 2.04 ± 0.12, which is significantly lower than the index for control, background, low, and medium concentrations (all statistically similar, 3.49 ± 0.14). The high-TCS digesters selected for the phyla Fibrobacteres, Actinobacteria, and Firmicutes. Firimicutes were the most abundant phylum in the TCS containing digesters. The *tet*(L) gene is common to Gram positive organisms; given that Firimicutes and Actinobacteria are gram positive, the increase in these phyla could explain the increase in the relative abundance of *tet*(L). Other phyla which had over 10-times lower concentrations in the high-TCS digesters than the functioning sets include ws3 (candidate division), Plantomycetes, Verrucomicrobia, Synergistetes, Fusobacteria, Tenericutes, Chloroflexi, and Spirochaetes. Furthermore, at

the genus level, *Succinivibrio, Atopobium*, *Olsenella*, *Flavonifractor*, and *Acetitomaculum* are enriched in these same digesters. All of these genera are known commensal organisms with humans, cows, sheep and pigs (Le Van et al., 1998; Petri et al., 2013; Stevenson and Weimer, 2007). While all of these genera are known to be acid tolerant, four of the five are found in the ruminal or digestive tract (*Atopbium* is not associated with the rumen or digestive tract). The heightened VFA concentrations in the high-TCS digesters provide conditions in which these clades can thrive (Mao et al., 2012).

4.4 Conclusions

This research demonstrated that increased *mexB* concentrations are sustained in anaerobic digesters seeded with municipal biosolids when chronically exposed to TCS. This selection occurred at environmentally relevant levels, indicating that selection is likely occurring in full-scale digesters. In addition, other genes are selected for (*tet*(L)) or against (*erm*(F)) if TCS inhibits the digesters. TCS has little or no effect on the abundance of class 1 integrons. This research revealed that TCS selects for clades which contain pathogenic and commensal bacteria. It is suspected that these clades may have previous exposure to antibiotics or antimicrobials, which affords the bacteria the opportunity to gain resistance mechanisms. Moreover, it is concerning that resistant and commensal/pathogenic organisms could be dispersed into the environment from full scale anaerobic digesters where they once again can come into contact with humans.

TCS should be included with antibiotics in studies which address risk assessment of antibiotic resistance. Given its ubiquity and relatively high concentration in biological wastewater treatment operations, TCS should not be ignored as a chemical stressor of

resistance in the environment. Impacts of other stressors (e.g., antibiotics, antimicrobials, metals, etc.) need to be established to quantitatively determine the relative magnitude of TCS to stimulate antibiotic resistance. Understanding the stressors for antibiotic resistance in each environmental compartment allows research to focus treatment technologies and potential policy in areas of greatest concern.

For future research, a metagenomics approach would be appropriate for this type of study to identify a broader spectrum of resistance genes which might be affected by TCS. In addition, isolating the role of mixed antibiotic and antimicrobials to determine synergistic or antagonistic effects could prove useful to determine synergistic or antagonistic effects.

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5 WASHOUT OF ANTIMICROBIALS FROM ANAEROBIC DIGESTERS: EFFECT ON COMMUNITY STRUCTURE AND ANTIBIOTIC RESISTANCE

5.1 Introduction

Multiple environmental concerns related to triclosan (TCS) and triclocarban (TCC) have been identified, including formation of dioxins and harmful impacts on animals, aquatic life, and microorganisms (Anger et al., 2013; Chalew and Halden, 2009; Cherednichenko et al., 2012). In previous chapters, antibiotic resistance in anaerobic digesters was influenced by TCC and TCS, which may facilitate spread of resistance genes into the environment. Furthermore, TCS has been shown to directly stimulate antibiotic resistance in pathogenic bacteria and influences bacterial communities in the environment (Braoudaki and Hilton, 2004; Chuanchuen et al., 2001; Sanchez et al., 2005). Both chemicals are suspected to be endocrine disrupting compounds in humans and wildlife (Ahn et al., 2008). TCC and TCS have been found to be persistent in the environment and yield degradation products that have potentially toxic effects (Miller et al., 2008; Sanchez-Prado et al., 2006).

Given these implications, some jurisdictions have imposed bans or have pending bans on TCS in consumer products. Although previously banned from packaging containers, in 2015 the European Union mandated that TCS be phased out of hygiene products (European Chemical Agency, 2015; European Commission, 2010). According to state legislature imparted in 2014, personal care products sold in Minnesota cannot contain TCS starting January 1st, 2017 (State of Minnesota, 2014). Other governmental entities, including those in Canada and other US states, have suggested removing TCS from consumer products. Private entities, like the retail store Wal-Mart and the manufacturer Procter & Gamble, have announced efforts to phase out potentially harmful chemicals including TCS (USA Today, 2013).

As far as the authors are aware, TCC has not appeared in legislation nor been addressed by private industry for removal from consumer products. It is, however, the opinion of the Canadian Environmental Law Association that TCC should be removed from consumer products because of impacts on aquatic toxicity, persistence in the environment and potential reproductive toxicity in animals (Canadian Environmental Law Association, 2014). Researchers also share the sentiment that TCC has adverse environmental effects and regulation may be appropriate (Halden, 2014).

However, it is unknown if removing TCC or TCS from consumer products will actually have a distinct impact on wastewater treatment systems and specifically on anaerobic digesters that have already been chronically exposed to these chemicals. Removing chemical stressors from microbial systems may or may not allow the systems to revert back to pre-perturbed conditions. For example, in some experiments designed to perturb microbial communities in anaerobic digesters and other syntrophic communities, the resulting stable communities differed from the original community (Ferris et al., 1997; Tale et al., 2015); in other experiments, microbial communities recovered to a similar community composition as they were previous to perturbation (Hong et al., 2013). Anaerobic digesters are known to be genetically diverse and contain functional redundancies, which makes either outcome a possibility (Briones and Raskin, 2003). Regardless of the specific community structure, maintaining efficient COD conversion to methane is the most important aspect from a functional stand point. With respect to antibiotic resistance genes, another question is whether or not resistance genes return to pre-perturbation levels once the stressor is removed.

The objective of this work was to determine the impact of removing antimicrobial stressors on the abundance of antibiotic resistance genes, community structure, and functional performance in anaerobic digesters. Digesters were acclimated for 110 days to various levels of TCS and TCC. Following quasi steady-state operation TCC and TCS were removed from the feed and the washout effects were observed after seven solid retention time (SRT) values. With mounting pressure to remove TCS from the consumer market, understanding how removal of TCS or TCC will affect microbial communities may impact judicial decisions. This research is the first work to evaluate how an anaerobic digester community responds following the removal of an antimicrobial selective pressure.

5.2 Materials and Methods

5.2.1 Anaerobic Digesters

Anaerobic digesters (50mL serum bottles) were seeded with biomass from full-scale anaerobic municipal digesters and maintained for 180 days. The digesters described in this chapter are some of the same digesters described in Chapter 3 (TCC) and Chapter 4 (TCS). Operation was continued for an additional 70 days for control, background, low, and medium digesters without continuing the addition of antimicrobials. For continuity, the naming (control, background, low and medium) are maintained in this chapter. The digesters were previously acclimatized to background levels of TCC or TCS for 45 days, then an increased concentration of TCC or TCS was fed to some digesters until day 110. After day 110, TCC or TCS was no longer included in the feed so the antimicrobials were washed out of the digesters (see figure 5.1 for nominal loading and table 5.1 for

concentrations). Digesters were fed at a rate of 3.6 g COD/L-d with ground, sieved dog food as substrate. All digester conditions were operated in triplicate.

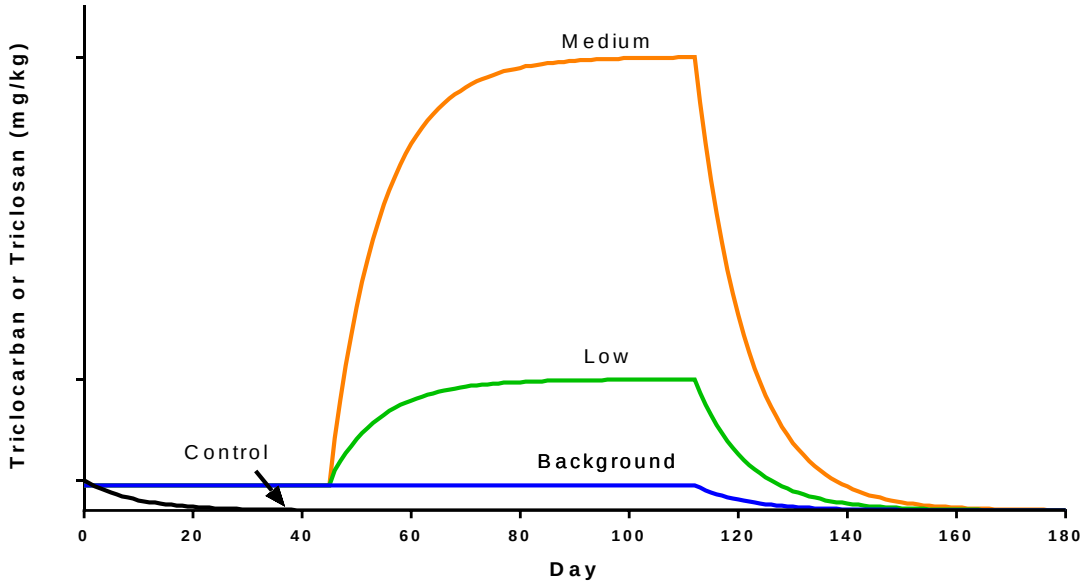

Figure 5.1 Nominal concentration of TCC or TCS in digesters over 180 days. Each condition was tested in triplicate. Concentrations of TCC or TCS are indicated in table 5.1.

Table 5.1 Quasi steady-state concentrations in digesters prior to washout

Triclosan (mg/kg total solids)	**Concentration**	**Triclocarban (mg/kg total solids)**
0	Control	0
30	Background	30
100	Low	130
850	Medium	450

Starting on day 111, TCC or TCS was removed from the feed, allowing TCC and TCS to washout of the system. Digesters were operated until day 180 when concentrations were near zero as predicted and confirmed by LC/MS (see figure 5.1). Digester health and function was monitored using the same methods as the first 110 days. Biogas volume was measured daily with a wetted syringe; methane percentage in biogas and volatile fatty acid (VFA) concentration was measured approximately every 10 days by Gas Chromatography (Schauer-Gimenez et al., 2010), and pH was measured 2-3 times a week (Orion 4 Star, Thermo, Waltham, MA, USA).

5.2.2 Molecular Methods

Biomass samples were collected for microbial analysis during quasi steady-state of antimicrobial feeding (day 105, 107, 110) and following antimicrobial washout when digesters had concentrations of antimicrobials near zero (day 175, 177, and 180). DNA extraction was performed as described in Chapter 3, section 3.2.2.1. To understand how the removal of antimicrobials affects antibiotic resistance, quantitative Polymerase Chain Reaction (qPCR) was performed on resistance genes (*mexB*, *erm*(F), *tet*(L)) and the class 1 integrase gene (*intI1*), which were also assessed in chapters 3 and 4. The *mexB* gene, associated with a multidrug efflux pump, has been previously associated with TCS (McNamara et al., 2014; Pycke et al., 2010). A tetracycline resistance gene, *tet*(L), was quantified because it also encodes for an efflux pump (Jin et al., 2002). The *erm*(F) gene was quantified as a control i.e., TCS and TCC were not suspected to influence abundance of this gene because it specifically targets macrolides, licosamides, and streptogramin (Rasmussen et al., 1986). Finally, *intI1* was targeted because it is the integrase of class 1 integrons which facilitate the horizontal exchange of resistance genes (Mazel, 2006). To

understand how removal of TCC and TCS impacts Bacterial and Archaeal populations, microbial community analysis was performed by partial sequencing of 16S rRNA genes (v4 region). Microbial community analysis and qPCR were performed by the same procedures as described in Chapter 3, section 3.2.2.3.

5.2.3 Statistics

Average, standard deviation, ANOVA, two-way ANOVA, and t-tests were performed on GraphPad Prism (V 6.04, LA Jolla, CA) to determine if microbial communities were different between day 110 and 180, and to determine if antimicrobial-amended communities were different from control communities at day 180 after washout was complete. A non-parametric multidimensional scaling (nMDS) graphical method (overlapping confidence intervals displayed in 2D space) was used to determine if microbial communities were similar to the control digesters. Analysis by nMDS was performed in the R program utilizing vegan and MASS packages with the isoMDS and ordiellipse command. ANOSIM was also performed in R using the vegan package.

5.4 Results and discussion

5.4.1 Digester Function

All digesters maintained efficient COD conversion after the removal of the antimicrobials. Each set of digesters maintained methane production rate and neutral pH over the duration of the study (Figure 5.2). The digester sets produced 69 ± 9 mL of methane per day at 35^{o}C and 1 atm (corresponding to a COD conversion rate of 90 ± 15%) during quasi steady-state operation, as seen in Figure 5.2 and were not statistically

different from each other (ANOVA, $p > 0.05$). The methane concentration in the biogas was 69. Total individual VFA concentrations were below 50 mg/L for all digesters after day 110. Overall, these data indicated that these concentrations of TCC and TCS do not inhibit methane production in full-scale anaerobic digesters, and removing the antimicrobials would have negligible overall effect on functional performance.

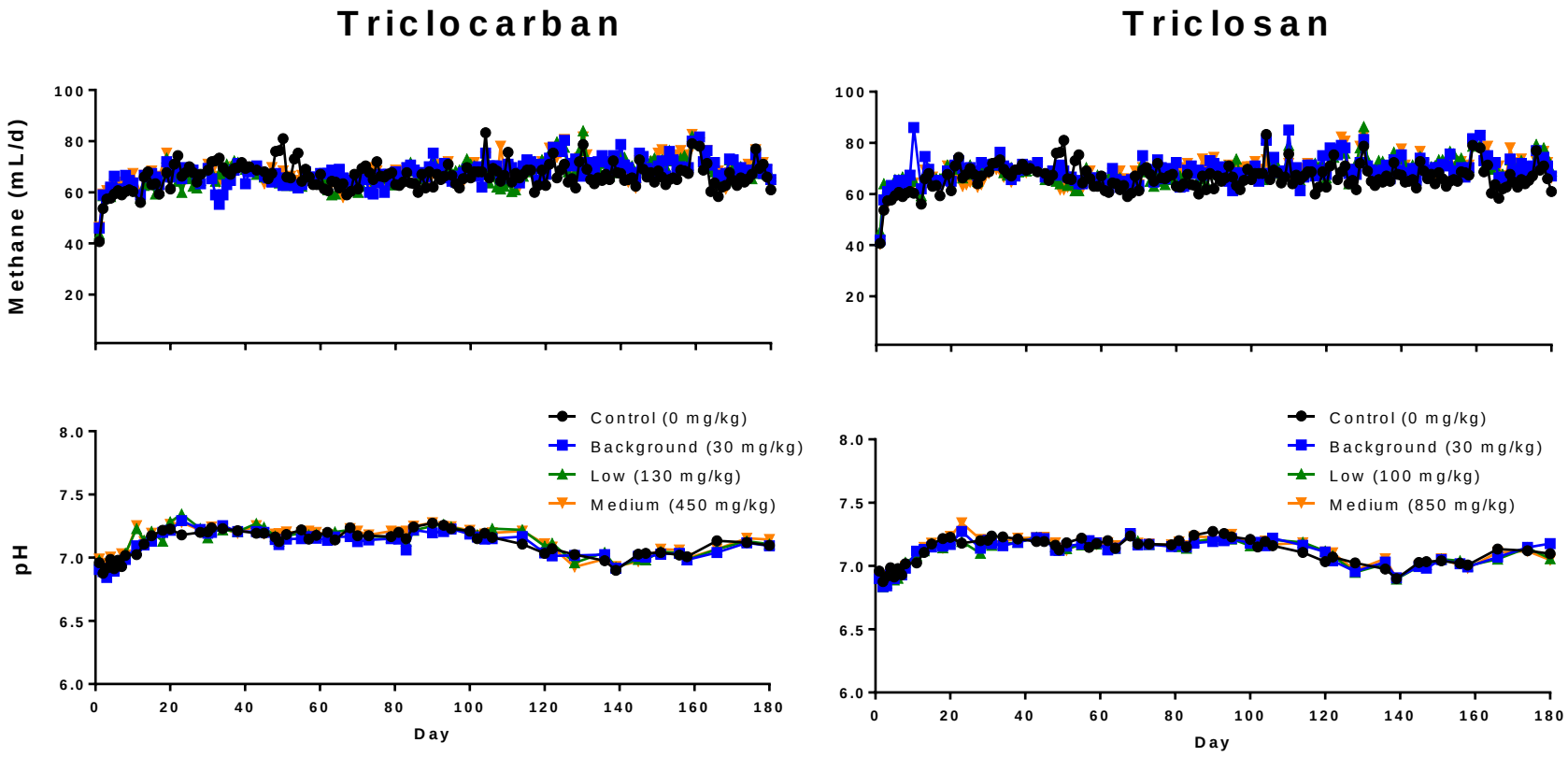

Figure 5.2 Average (n=3) methane production (measured daily) and pH. A figure with error bars that represent the standard deviation can be found in Appendix N.

5.4.2 Community Analysis: Comparison Between Day 110 and 180

All antimicrobial containing digesters had statistically different communities between day 110 and day 180 after the removal of TCC or TCS (ANOVA, $p < 0.05$); the control had no statistical difference between day 110 and 180 ($p = 0.48$), as shown in Figure 5.3. Digesters which had TCC or TCS removed had a universal drop in relative abundance of Firmicutes and Actinobacteria. This drop suggests that both TCC and TCS enriched for these phyla at all concentrations employed. The relative abundance of Proteobacteria increased in all digesters that had TCS removed, but no consistent effect of removing TCC was observed on Proteobacteria. Many other changes were observed; however, no other universal trends were gleaned from this information.

Several studies reveal Proteobacteria, Bacteroidetes and Firmicutes as the most abundant phyla within municipal anaerobic digesters (Guo et al., 2015; Yang et al., 2014). Firmicutes were the most abundant phyla on day 110 in digesters containing TCC and TCS. The phylum Firmicutes contains well known fermenters, such as *Clostridia*, which are associated with VFA fermentation. After removing TCC or TCS from the influent, the detection of Firmicutes dropped and the relative abundance of Proteobacteria or Bacteroidetes increased.

Proteobacteria represent a phylum of Bacteria which appear to be sensitive to triclosan and become more abundant after TCS is removed on day 180. On the other hand, TCC digesters had an increase in Bacteroidetes on day 180 which offset the decrease of Firmicutes. Proteobacteria were the most abundant in digesters treating municipal waste (10-40%) (Guo et al., 2015; Yang et al., 2014). Proteobacteria contain major classes of Alpha-, Beta-, Delta-, and Gamma-Proteobacteria classes; Alpha- and

Delta- were the most abundant in this study. Bacteroidetes are also well known fermenters and produce CO_2, H_2, and organic acids during anaerobic digestion.

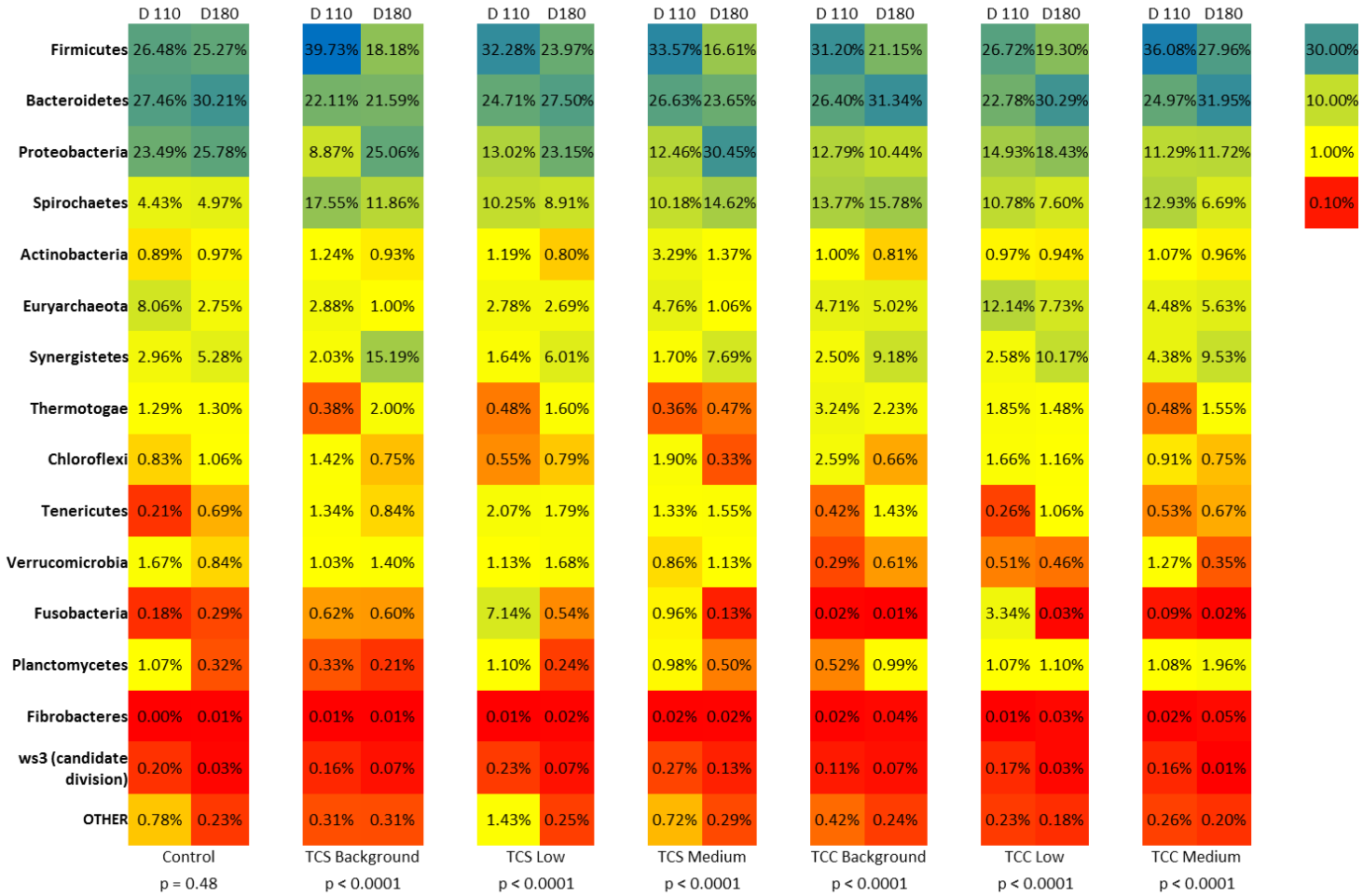

Figure 5.3 Heat map to compare digesters on day 110 (D110) and day 180 (D180) side by side at phyla level. The p-values shown below are results from a 2-way ANOVA test where independent variables are time and phyla; note only the control had no significant differences between day 110 and 180. The OTHER category includes all phyla which, on average, were represented by less than 0.1% of the community population.

5.4.2 Community Analysis: Comparison Between Washout Communities and Control Communities

On day 110 when microbial communities were at steady-state with antimicrobial amendment, TCS-amended communities were distinct from the control communities (Figure 5.4). After TCS washout, the background digester community converged with the control on day 180. The low and medium digesters were similar to the background but not the control. These results indicate that digesters which have sustained levels of TCS below 30 mg/kg can recover, or reconverge, to that of the control after TCS is removed from the influent. An analysis with ANOSIM confirms that digesters communities are more similar on day 180 than on day 110 (performed at Operation Taxonomical Unit [OTU] level, grouped by loading level, significance 0.001 for both). On day 110, ANOSIM yields an R statistic of 0.61. Performing this same analysis on data from day 180 yields an R statistic of 0.41 indicating that the antimicrobials had a greater effect on the population prior to washout. These ANOSIM results are reflected in Figure 5.4 where the communities at day 180 are grouped closer together than they are at day 110.

The nMDS results indicate that the microbial communities show resilience to TCS at 30 mg/kg. Conversely, digesters with concentrations of TCS above 100 mg/kg do not recover and converge to the control after 7 SRT values when TCS is washed out, thus the perturbation by the concentrations of TCS was significant. It should be noted that the digesters were seeded with biomass which had approximately 30 mg/kg of TCS and control digesters were made by letting the original TCS (and other adulterants) wash out over 18 SRT values. In a similar setup, the background digesters maintained the background concentration for 11 SRT values and then had TCS washout over 7 SRT

values. This similarity in operation whereby TCS was never increased could be an explanation as to why the background and control digester sets were similar. While the control used is not ideal because it has been previously exposed to TCS, a biomass that has never contained TCS and treats municipal waste likely cannot be found because TCS is ubiquitous in municipal waste flows (USEPA, 2009). Anaerobic digesters that treat industrial wastes may have never been exposed to TCS, but the nature of the waste probably creates a significantly different microbial community, and seeding a digester with non- municipal biomass would yield different results that are less pertinent to municipal anaerobic digesters.

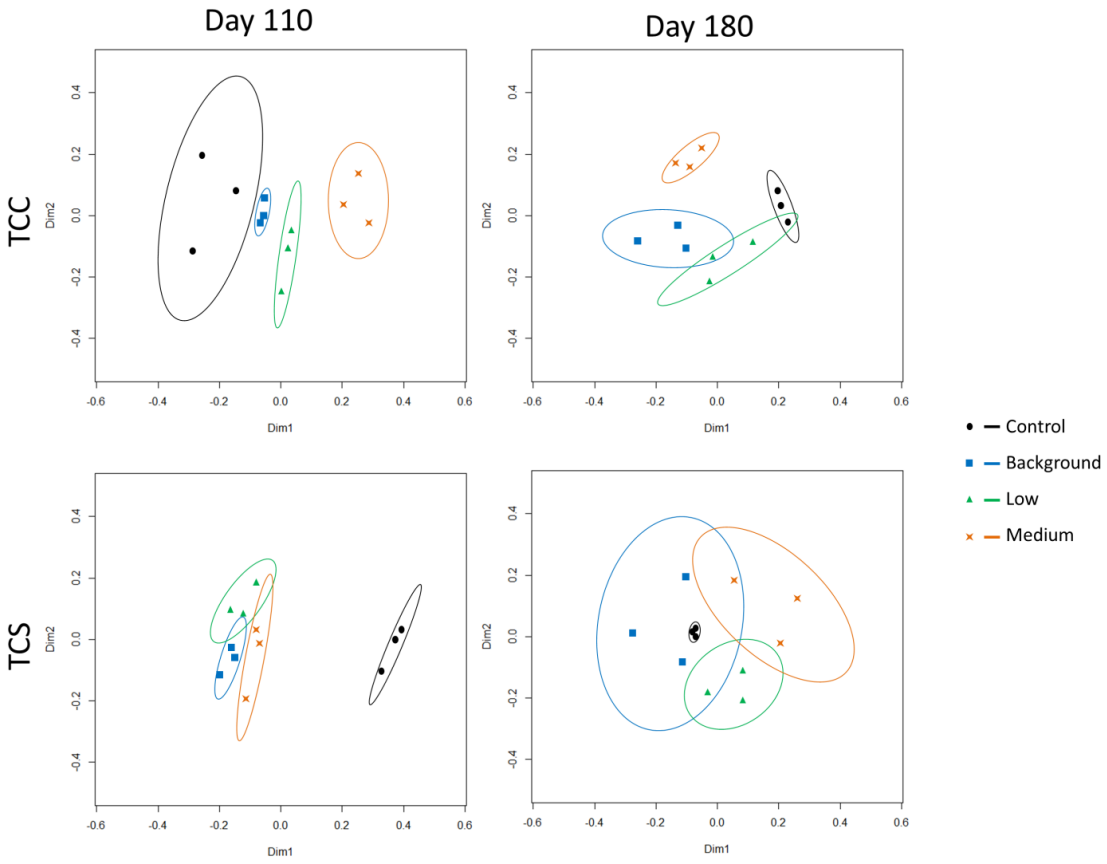

Figure 5.4 nMDS of genus level data on day 110 and 180. Ovals represent the 95% confidence interval as determined by the ordiellipse command in the R program. When interpreting this nMDS plot, communities which do not have overlapping ovals can be distinguished as statistically different. Conversely, those which have overlapping ovals are considered not statistically different.

On day 110, TCC-amended communities were distinct from the control communities (figure 5.4). On day 180 in the TCC digesters, the low-TCC digester set had a similar community to the control. This result is surprising: after removing 30 mg/kg and 850 mg/kg of TCC the communities were different from the control, yet after removing

450 mg/kg of TCC the communities were not statistically different from the control. Upon close inspection however, only the fringe of the background oval overlaps with the control and the community data points representing background are in a distinct location from community data points representing the control. The apparent overlap is likely due to inherent uncertainty in this analysis. The general separation between TCC communities and control communities even after washout indicates that TCC irreversibly alters microbial communities. The R statistic produced from ANOSIM for day 110 and 180 were 0.94 and 0.95 respectively (grouped by loading level, OUT level analyzed, significance 0.001 for both). These R statistics indicate that the loading level had a very strong effect on the differences between the digester communities both during antimicrobial loading and after washout

At the genus level, the relative proportion of clades differed between TCC, TCS, and control digesters on day 180 (figure 5.5). In all digesters, 84-89% of each community was composed of the same thirty genera. The three most abundant genera were *Proteiniphilum, Azospira,* and *Thermovirga*. Other studies report *Proteiniphilum* as highly abundant organisms in municipal digesters (Guo et al., 2015; Yang et al., 2014). TCS amended communities had different distributions of these genera than TCC amended communities. As reflected by the nMDS plot in Figure 5.4, the TCS exposed communities showed more similar distributions to the control than the TCC exposed communities.

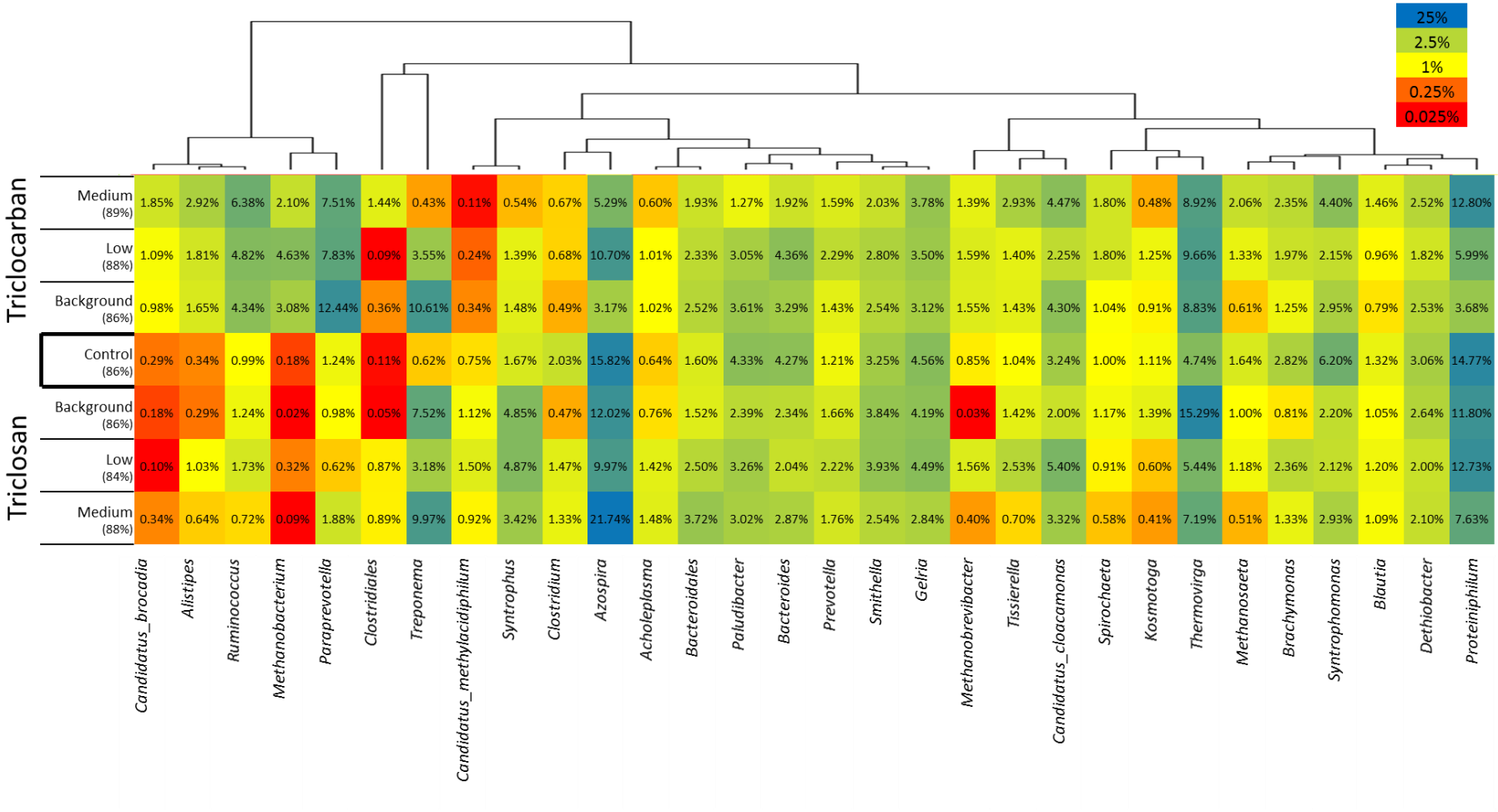

Figure 5.5 Thirty most abundant genera in each digester on day 180 represented in a heat map. Genera are clustered in a hierarchal according to cosine distances of relative abundances. The percentage under the digester condition on the vertical axis (left side) show the coverage (e.g., 86% of the Control digesters are represented by these 30 genera)

5.4.3 Resistance Genes

Overall, the removal of the antimicrobials did not substantially increase or decrease the relative abundance of resistance genes surveyed. While the relative abundance of *mexB* was higher than the control on day 110 in all digesters, the relative abundance was similar or lower compared to the control on day 180 after the removal of TCC or TCS in every instance (Figure 5.6). The increase in *mexB* in the control between day 110 and 180 is partially responsible for this result. For this reason, t-tests were performed to compare the change in resistance genes within each digester set between day 110 and 180. With this comparison, no statistical difference was observed from day 110 to day 180 (except that the concentration of *mexB* in the control was statistically higher at day 180). In the control, the lack of a chemical stressor could allow the bacterial population to genetically drift. Dynamic microbial communities in functionally stable digesters have been observed in other studies (Fernández et al., 1999). In mixed microbial communities, chemical stressors can deterministically select for a niche population which tolerate the selective environment. Conversely, in populations that lack a selective pressure, functional redundancy within the community allows functional stability with stochastic fluctuations in the microbial population. This is likely the explanation for temporal variation in the relative abundance of *mexB* in the control between day 110 and 180. The abundance of *mexB* genes did not increase in digesters that had TCC and TCS washout by any comparison. Based on the results presented herein, the washout digesters no longer had statistically higher relative abundances of *mexB* relative to the control digesters at day 180, indicating that removing antimicrobials returns *mexB* to control levels. However, the relative abundance did not decrease in the washout digesters, but

rather the relative abundance in the control increased. Thus it is difficult to conclude the impacts of washout on antibiotic resistance genes without having operated a suitable positive control, i.e., a digester set with background concentrations of TCC and TCS should have been maintained for 180 days to compare results to the washout digesters.

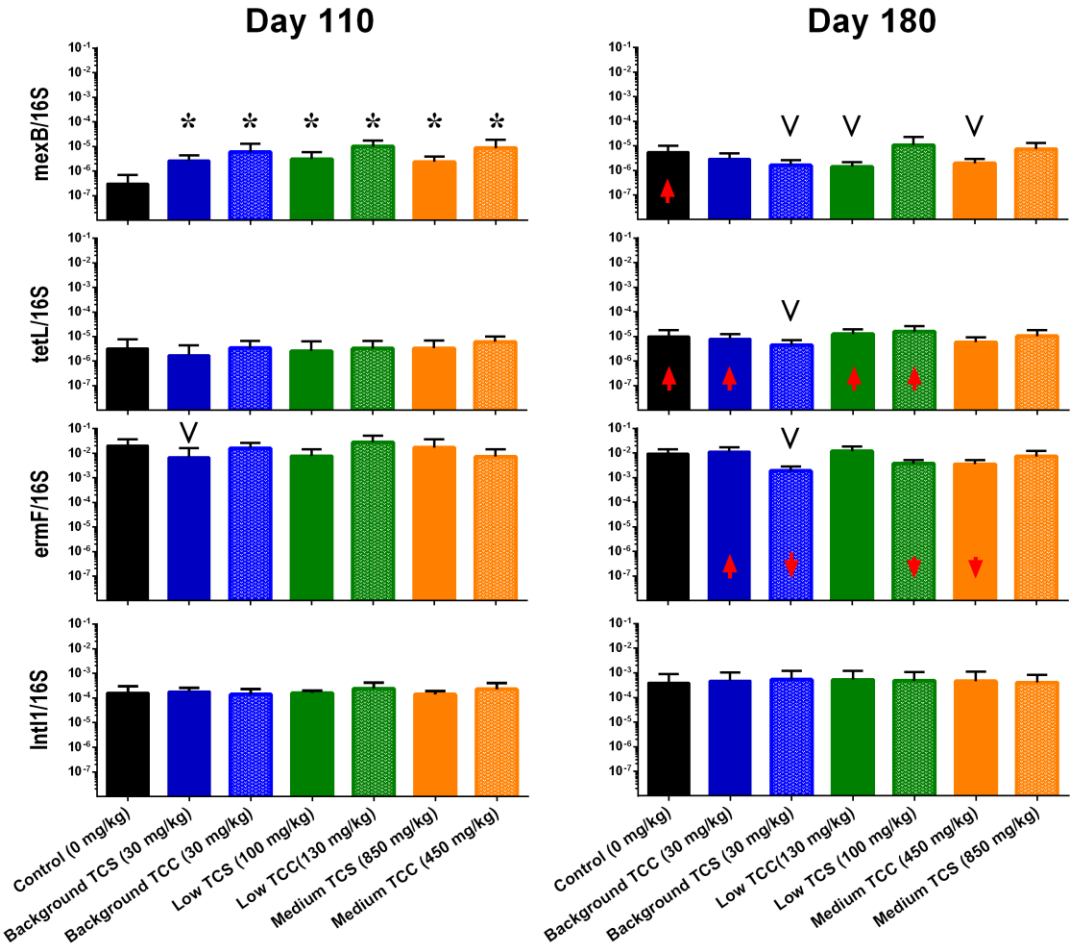

Figure 5.6 Gene copies normalized to the 16S rRNA gene. For all bars, n=9 and error bar depicts standard deviation. An asterisk (*) demonstrates statistically higher relative gene

abundance than the control, while (V) is statistically lower than the control. Statistically significant differences between day 110 and day 180 are shown with red arrows on the right hand graphs: An upward pointing arrow indicates a statistically higher relative abundance on day 180 compared to 110, and a downward pointing arrow represents a statistically lower abundance. A t-test was performed on the log values of relative abundance to compare to make these comparisons (comparison to control or comparison between day 110 and 180) and was considered to be statistically significance with $p<0.05$.

While *tet*(L) was statistically similar in all digesters on day 110, only the background TCS digester showed an actual decrease in the abundance of *tet*(L) on day 180 compared to the control. Some concentrations of *tet*(L) increased between day 110 and 180 (control, background TCC, low TCC and low TCS). Although statistically significant between the two time points, they did not change in comparison to the control. Previously, the abundance of *tet*(L) increased significantly in digesters which lost function and experienced a drop in pH. Since all digesters here are functional, no change was expected.

The abundance of *erm*(F) had some differences among samples between day 110 and 180. The background TCC had an increase in concentration between the two time points, but the relative abundance was not higher than the control on day 180. Other samples statistically decreased over the 70 day time period (including the background and low TCS digesters). The TCS background had a statistically lower concentration of *erm*(F) when compared to the control on day 180. Despite minor differences, TCC and

TCS washout has little to no impact on *erm*(F) abundance for the concentrations used in this study.

The relative abundances of *intI1* were all statistically similar to the control at day 110 and 180. Further, no statistical differences existed in concentrations between day 110 and day 180. The class 1 integron abundance was not impacted by concentrations of TCC or TCS employed in this study. One interpretation is that TCC and TCS do not stimulate horizontal transfer of resistance genes by this mechanism, or at the very least, do not increase the abundance of resistance gene transfer vectors.

5.5 Implications and Conclusions

This research demonstrates that removing TCC or TCS from municipal waste streams elicits no functional harm on digesters. The removal of the antimicrobials never yielded a clear net increase in the resistances gene surveyed, nor did the abundance of antibiotic resistance genes always decrease. The microbial community structures will not necessarily return to that of a control in the timeframe of this study (70 days) and the previous steady-state concentration of the antimicrobial impacts the changes. Only washing out background levels of TCS produces communities which are similar to control communities; however, there seems to be a "point of no return" concentration of TCS. This research suggests that, if concentrations of TCS are 100 mg/kg or higher in an anaerobic digester, then the microbial community may not be able to recover a community similar to the control. Perhaps a longer recovery period (greater than 7 SRT's) would allow for a more substantial shift towards the control communities. Removal of antimicrobials did not decrease the clades which were previously observed to increase following antimicrobial amendment (Chapter 4, section 4.3.3), thus indicating

TCC or TCS can have long-lasting effects on a microbial community even after removal. Banning or removing TCS from consumer products before concentrations increase in wastewater could be important to maintain microbial communities. Research from chapter 4 suggests that TCS might select for pathogenic and commensal bacteria. Given that the community selection seems to be irreversible after certain thresholds of TCS, preventing the rise in TCS concentrations may be imperative. The implications of removing TCC are less clear cut. However, it appears that digesters exposed to TCC over several SRTs may not return to a community structure that matches the control, at least not over the duration studied in this experiment. Functionality, though, was maintained regardless of community structure.

Removing TCC and TCS does not have adverse effects on the relative abundance of antibiotic resistance genes. The relative abundance of resistance genes was similar to or lower than the control in all digesters after washout of antimicrobials; however, this result is likely partially a result of a genetic drift in the control. Removing TCS could have positive implications for antibiotic resistance, or at the very least no negative response. Banning TCS before concentrations reach higher than 30 mg/kg in the majority of digesters could help reduce the perpetuation of antibiotic resistant organisms.

More robust testing is required to understand real world implications. For example: What are the repercussions if TCS is no longer in wastewater but other antimicrobials are still at current concentrations? Are the impacts of antibiotics on antibiotic resistance in anaerobic digesters far greater than TCC or TCS? This research provides a basis to begin gathering data of this nature. The data gathered here also helps

answer key questions about washout of these chemicals which may occur due to policy or legislation.

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6 ALTERED TOLERANCE TO ANTIBIOTICS IN ANAEROBIC COMMUNITIES FOLLOWING EXPOSURE TO ANTIMICROBIALS

6.1 Introduction

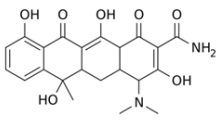
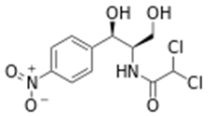
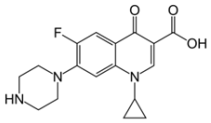
Antibiotic resistance is influenced and stimulated by many types of stressors (e.g., antibiotics, antimicrobials, metals) in a variety of different environments (Alanis, 2005). In some cases, resistance to one stressor can result in resistance to another stressor (Sefton, 2002); this phenomenon is referred to as cross-resistance and is well documented in literature. Cross-resistance to antibiotics stimulated by the antimicrobial triclosan (TCS) has been investigated in many pathogenic bacteria (Giuliano and Rybak, 2015; Saleh et al., 2011; see Chapter 2, section 2.3). Another antimicrobial with similar structure and function, triclocarban (TCC), remains largely uninvestigated for its impact on cross-resistance in pathogens. In the previous chapters, TCS and TCC have been shown to impact antibiotic resistance in anaerobic digestion.

Determining the functional impact of antibiotic cross-resistance might be best approached by testing of specific antibiotics in microbial cultures that have been conditioned to tolerate another chemical stressor. As performed in previous chapters, quantitative polymerase chain reaction (qPCR) can be utilized to look at the relative abundance or expression (with Reverse Transcription qPCR) of specific resistance genes as stimulated by given stressors (Guarddon et al., 2011; Holzem et al., 2014). While this approach gives concise enumeration of relative gene abundance or expression, qPCR is somewhat narrow in focus in that only one gene can be targeted per reaction. A metagenomics approach could give a broader view of resistance gene selection by broadening the field of genes quantified (Zhang et al., 2011). However, metagenomics relies on a database for comparison; it is naïve to assume that all resistance genes would be within any given database. Further, neither a qPCR or metagenomics approach can

predict how a bacterium or microbial community will directly respond to another chemical stressor. Both methods lack specific contextualization with a quantitative basis for determining cross-resistance.

Many antibiotics have been affected by cross resistance forming from TCS exposure. Chemical properties of the antibiotics used in this study can be found in Table 6.1.

Table 6.1 Chemical properties of the 3 antibiotics used in this study

	Tetracycline	Chloramphenicol	Ciprofloxacin
			
Log K_{ow}	-1.37	1.14	0.28
Water Solubility	231 mg/L @ 25°C	2500 mg/L @ 25°C	30000 mg/L @20°C
pKa	3.3	5.5	6.1

Tetracycline is a polyketide class of antibiotics which inhibits protein synthesis. Tetracycline is widely prescribed to treat bacterial pathogens in people and animals such as *Chlamydia*, *Mycoplasma* and *Rickettsia* (Chopra and Roberts, 2001). Tetracycline resistance is seen in many species of bacteria and occurs most commonly by efflux or ribosomal protection by a protein (Auerbach et al., 2007). In some bacteria the MexAB-OprM protein can increase resistance to tetracycline, and this protein can also be selected by TCS (Chopra and Roberts, 2001; McNamara et al., 2014). Cross-resistance to tetracycline forms from TCS exposure in pathogens (Braoudaki and Hilton, 2004; Chuanchuen et al., 2001; Kappell et al., 2015; Sanchez et al., 2005).

Chloramphenicol is also a protein synthesis inhibiting drug. It is considered an essential medicine by the World Health Organization because of its effectiveness against typhoid, cholera and meningitis (WHO, 2010). Resistance to chloramphenicol occurs by outer envelope mutation, mutation of the gene target, and enzymatic inactivation (Li et al., 1994). Specific chloramphenicol resistance genes are plasmid borne and can occur on a plasmid carrying resistance genes specific to many classes of antibiotic, including tetracycline (Schwarz et al., 2000). Chloramphenicol resistance is stimulated by exposure to TCS in pathogenic bacteria (Birosová and Mikulásová, 2009; Braoudaki and Hilton, 2004; Karatzas et al., 2007).

Ciprofloxacin is a fluoroquinolone antibiotic which inhibits nucleic acid synthesis (Kümmerer et al., 2000). This antibiotic is used to treat respiratory and urinary tract infections, among others. Like many antibiotics, resistance has occurred because of its wide use. Resistance mechanisms include target site mutation and active efflux (Jacoby, 2005). Mechanisms associated with TCS resistance (e.g., efflux by AcrAB) are also associated with ciprofloxacin resistance (Piddock, 2006). Indeed, cross-resistance to these antibiotics after exposure to TCS has been documented, but these previous studies investigated pure-cultures. Little information is available regarding cross-resistance in mixed environmental communities, i.e. does TCS or TCC exposure in anaerobic digesters make the communities more resistant to other antibiotics?

The objective of this research was to determine if long-term TCC or TCS exposure in anaerobic digesters impacts functional resistance/resilience (as measured by methane production) to three antibiotics (tetracycline, ciprofloxacin, and chloramphenicol). To meet this objective three 4-L digesters were operated: a control

with no antimicrobial, a TCC-amended digester, and a TCS-amended digester. These digesters are referred to as ‘mother digesters’ throughout this manuscript because the biomass from these digesters was used for inoculum for the experiments that tested antibiotic toxicity. It was hypothesized that TCC or TCS-amended biomass would tolerate higher concentrations of antibiotics (relative to the control biomass) due to cross-resistance imparted by the antimicrobials.

6.2 Materials and Methods

6.2.1 Acclimatizing Mother Digesters to Antimicrobials

Three mother digesters were established as a biomass source for testing antibiotic toxicity against antimicrobial acclimatized anaerobic biomass: a control digester, a TCS-amended digester, and a TCC-amended digester. Biomass from these digesters was used to determine the concentration of antibiotics required to inhibit 50% of methane production during batch methanogenic assays.

Each mother digester had four liters of working volume and was seeded with biomass from a full-scale mesophilic anaerobic digester at South Shore Wastewater reclamation facility (Oak Creek, Wisconsin). Biomass from this facility was previously measured to have TCC and TCS concentrations of approximately 30 mg/kg for both antimicrobials in March of 2014. The solid retention time of the mother digesters was 15 days and each digester was given 6 g of ground and sieved (40 mesh) dog food daily (1.8 g COD/L-d) in nutrient medium to simulate primary sludge. Digesters were operated for a total of 210 days. Qausi steady-state operation was established over the first 100 days

(>6 SRT's, based on steady methane production), and biomass was collected for toxicity testing over the remaining 110 days.

The control digester was not fed any antimicrobials. For the TCC and TCS amended digesters, antimicrobials were added to an aliquot of dog food prior to mixing the feed. A calculated mass of TCC or TCS was first dissolved in acetone or methanol, respectively. The solvent solution was applied to 6 g of ground dog food and allowed to dry for at least 24 hours. The dog food was then mixed with the nutrient solution (Appendix B) immediately prior to feeding. Quasi steady-state concentrations within the digester biomass were 150 mg/kg for the TCC digester and 850 mg/kg for the TCS digester. These concentrations were chosen because it was previously determined that biomass could tolerate these concentrations without digester failure. After 100 days of operation, antibiotic toxicity testing was performed with waste biomass.

6.2.2 bacteria Anaerobic Toxicity Assay (bATA)

Anaerobic toxicity assay (ATA) style tests were performed to test the toxicity of three antibiotics (Stuckey et al., 1980). ATAs require three main components: anaerobic biomass, carbon source (acetate), and a toxicant. An ATA measures methane production as a surrogate for activity at different doses of a toxicant in a batch test. Because acetate is mainly a substrate for methanogens (Archaea), the assay specifically measures the impact of the toxicant on methanogens. The experiments performed in this chapter differ from traditional ATAs in that a more complex feed carbon source was utilized (dog food or propionate). Dog food was used because degradation to produce methane flows through all trophic groups (Bacterial and Archaeal) in an anaerobic digester. Propionate was used to more narrowly focus on inhibition of syntrophs (Bacteria) and methanogens

(Archaea). The toxicant used in the modified ATAs was one of 3 antibiotics. Given that trophic groups from Bacteria or Archaea were potentially inhibited based on the substrate fed, the modified assays that are performed in this work are referred to as "bacterial anaerobic toxicity assays" (bATAs), as bacteria refers to all prokaryotes, i.e. Bacteria and Archaea.

6.2.3 bATA Setup

Waste biomass was collected from the mother digesters over a five day period. The biomass was allowed to degas for an additional 3 days before testing. For a given bATA test, a constant volume of biomass (50 mL) and a constant COD load (3.5 g COD/L) was employed for each bottle. Glass serum bottles (160 mL) were utilized as batch digesters. A spectrum of toxicant doses was employed ranging from no toxicant to inhibitory concentrations (inhibitory concentration was based on a preliminary test right before performing the bATA and maximum dosage with as high as 50,000 μg antibiotic per g total solids). For these experiments, seven toxicant (antibiotic) doses were used in triplicate to span several orders of magnitude. Antibiotics were added in 1 mL of dimethyl sulfoxide or water. For each biomass, three antibiotics were chosen to test toxicity: chloramphenicol, tetracycline, and ciprofloxacin. Each antibiotic was acquired from Sigma Aldrich (St. Louis, MO).

After each bottle was loaded with biomass, substrate, and toxicant, the bottles were sparged with a 70/30 ratio of N_2/CO_2 gas and capped with an airtight butyl-rubber stopper. Biogas volume was measured every 6-24 hours for approximately 10 days. When approximately 100 mL of biogas was produced in any given bottle, the methane percentage was determined with the GC method as outlined in 6.2.4. Methane production

rate was then determined over a period of approximately 10 days. Results were interpreted as described in section 6.2.5; ultimately, the concentrations of antibiotic which reduced methane production rate by 50% (IC_{50}) were determined.

6.2.4 Analytical Methods

The pH of the mother digesters was monitored approximately every other day (Orion 4 Star, Thermo, Waltham, MA, USA). The methane content was measured by GC-FID approximately every 10 days (Schauer-Gimenez et al., 2010). Volatile fatty acid (VFA) concentrations of acetic, propionic, butyric, iso-butyric, valeric, and iso-valeric acid and total solids concentration were measured approximately every 20 days as described by Schauer-Gimenez et al., 2010.

6.2.5 Statistical Interpretation

During the bATA, methane was constantly produced over time, and methane production data were analyzed to determine the concentration of antibiotic which inhibited 50% of methane production (IC_{50}). An example of methane production rate is shown in figure 6.1. These curves were made by recording biogas volume over the duration of the study. Before graphing, the biogas volume was multiplied by the methane percent measured. Maximum production rate was determined by taking a 2 day average (approximately 4-5 sample points) surrounding the highest production rate.

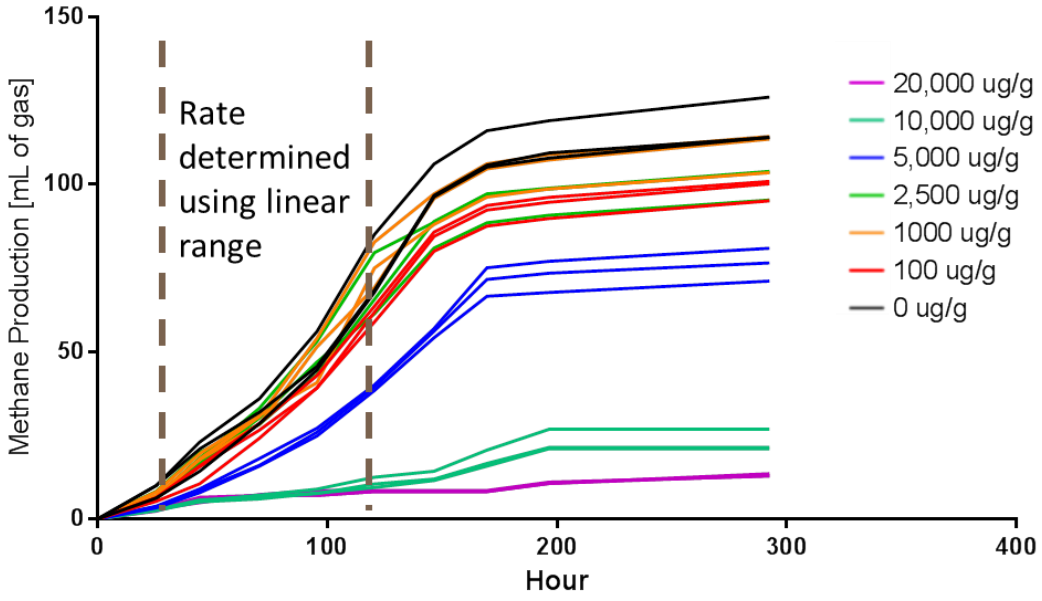


Figure 6.1 Methane production in anaerobic digesters after the addition of 7 concentrations of antibiotics for one biomass (performed in triplicate, 21 curves). Each color represents a given concentration of an antibiotic.

As the concentration of antibiotic increased, the rate of methane production eventually decreased. The linear methane production rates were first determined using Excel (Microsoft, 2013) and the rates were then used to determine the IC_{50}. Prism ® (GraphPad Software, La Jolla, California) was used to determine the IC_{50} and 95% confidence intervals. Briefly, the program interpolates a toxicant concentration that coincides with 50% inhibition of methane production based on the dose response. A visual example is given in Figure 6.2 where maximum methane production is on the y-axis and toxicant dose is on the x-axis. Further, with triplicate data the program can determine a confidence interval from variation between replicates. When comparing the IC_{50} of two biomasses, a higher IC_{50} indicates more resistance to the toxicant.

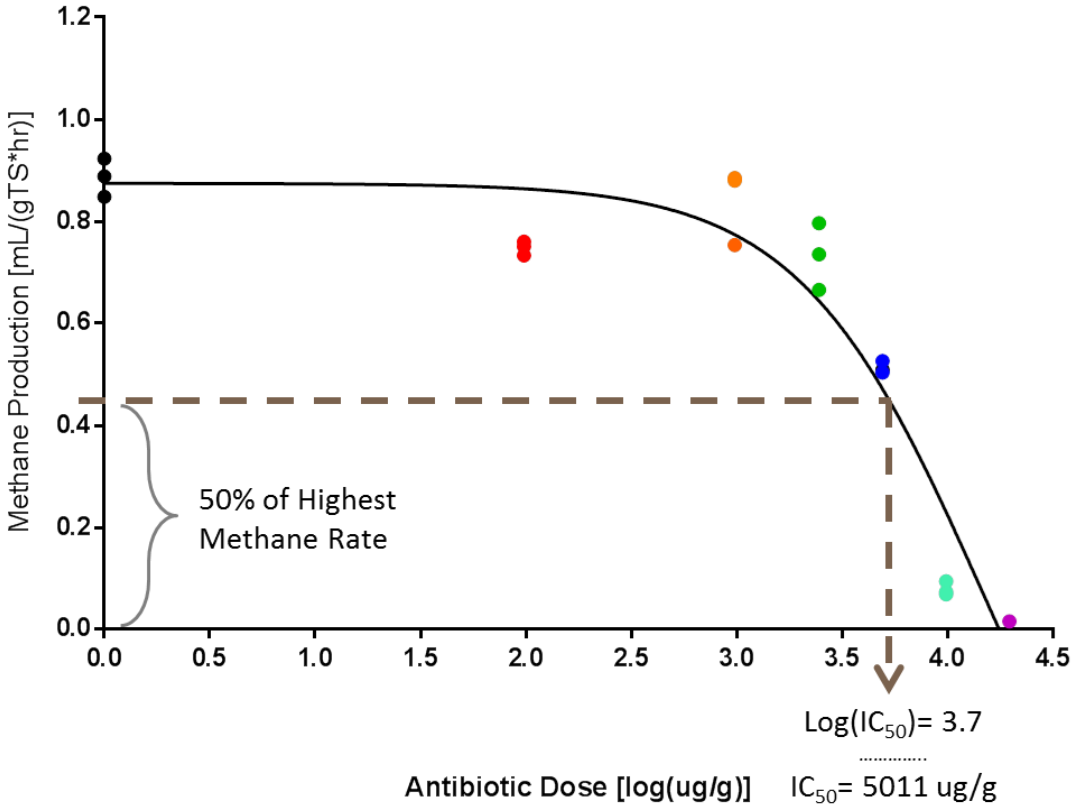


Figure 6.2 Example of a dose response curve showing the impact of antibiotic concentration on methanogenic activity. The points correlate to the slope determined from the activity data shown in Figure 6.1. The color of the points corresponds to the concentrations marked by the same color in Figure 6.1 (this concentration is also shown on the x-axis in log-scale).

6.3 Results and Discussion

6.3.1 Mother Digester Operation

All three mother digesters (control, TCC-amended, and TCS-amended digesters) maintained function, a pH of approximately 7-7.5 and VFA levels less than 60 mg/L (see figure 6.3). Biogas production was similar among all digesters, with average biogas production of 3.6 ± 0.6 L/day. Methane concentration in biogas was 68 ± 3.8% in control digesters, 66 ± 4.4% in TCC digesters, and 64 ± 5.0% in TCS digesters. These values correlate to 86%, 84% and 81% COD conversion in the control, TCC, and TCS digesters respectively. Solids concentration in the digesters was at 9.5 ± 0.1 g/L after day 100 and was constant for all bATA tests. The biomass for each assay was collected for 5 consecutive days for each test. In total, five bATA tests were performed with initial biomass draws occurring on day 101 (chloramphenicol and propionate), 124 (tetracycline and propionate), 146(tetracycline and dog food), 177(chloramphenicol and dog food), and 199 (ciprofloxacin and dog food).

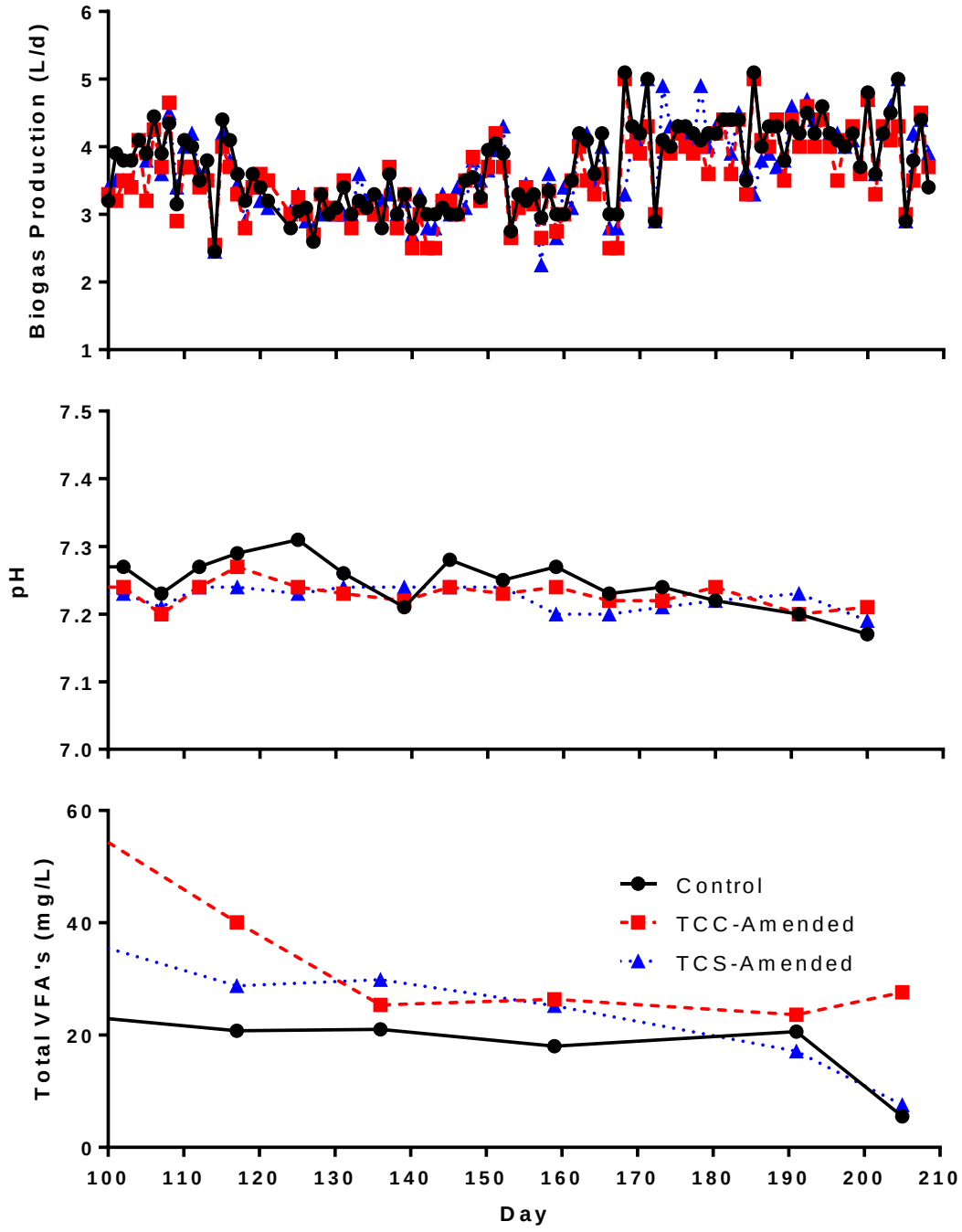


Figure 6.3 Biogas, pH and total VFA concentration from day 100 to 210. Total VFAs is the sum of acetic, propionic, butyric, iso-butyric, valeric, and iso-valeric acid. Control digester contained no antimicrobial, while TCC-amended contained 150 mg TCC/ kg solids, and TCS-amended contained 850 mg TCS/ kg solids.

6.3.2 Tetracycline bATA

The TCC-amended biomass was more susceptible to inhibition by tetracycline relative to the control biomass (Figure 6.4, p-value < 0.05). The IC_{50} was statistically lower than the control in test sets which received either dog food (control IC_{50} = 5700 mg/kg, TCC IC_{50} = 780 mg/kg) or propionate (control IC_{50} = 4700, TCC IC_{50} = 1800). Some antibiotics have been shown to have synergistic inhibition effects on anaerobic digestion (Cetecioglu, 2014; Ozbayram et al., 2015). For example, tetracycline has greater inhibition of methanogenesis when used in combination with sulfamethoxazole or erythromycin (Aydin et al., 2015)

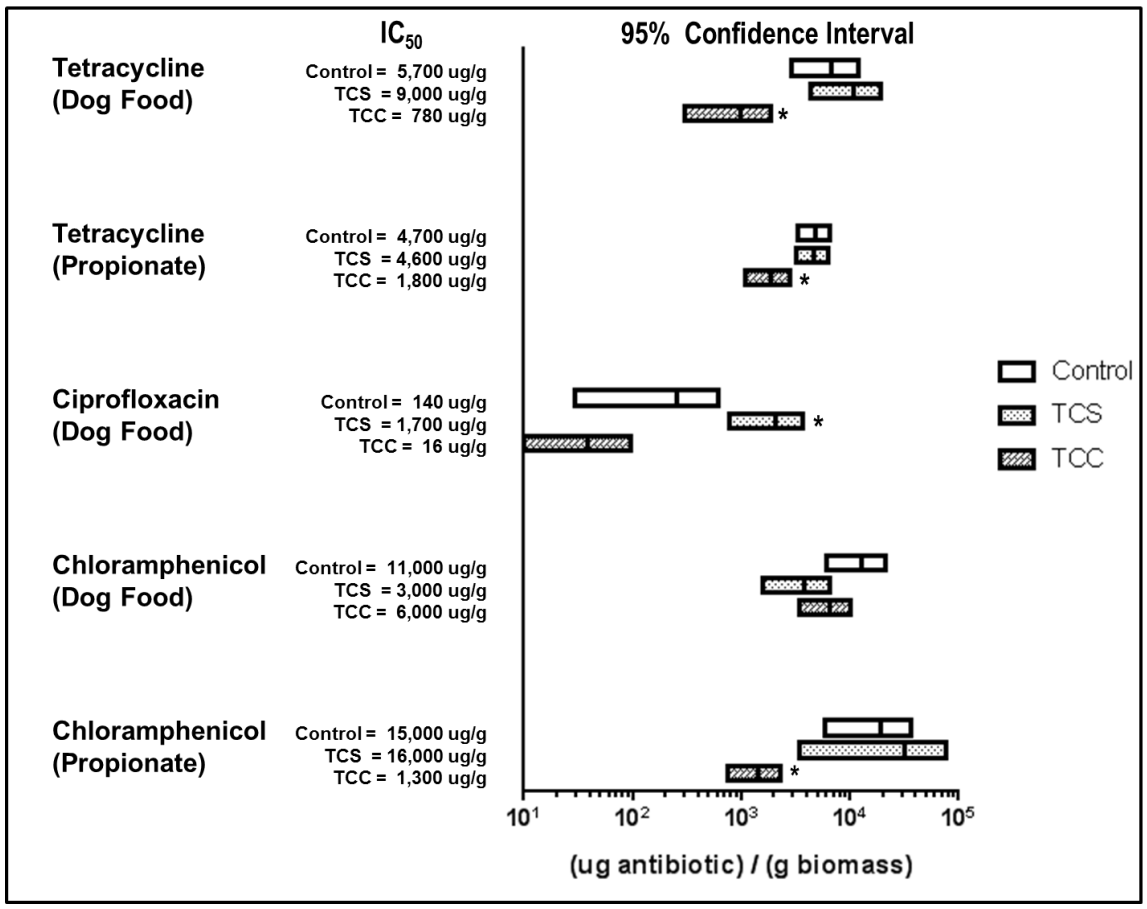


Figure 6.4 IC_{50} determination with plotted 95 % confidence intervals. The bars represent the mean value with flanking confidence intervals. The mean is specified on the left side of the graph. Asterisks (*) to the right of the bar indicate statistically significant difference from the control, i.e. $p < 0.05$. When control bars are at higher concentrations and do not overlap with the antimicrobial bars, the biomass became more sensitive to antibiotics after exposure to antimicrobials (such as TCC: chloramphenicol/propionate and tetracycline). When control bars are at lower concentrations and the 95% confidence interval does not overlap with the 95% confidence interval of the treatment group , the biomass became more resistant to antibiotics after exposure to antimicrobials (such as TCS: Ciprofloxacin/dogfood). Raw data can be seen in Appendix P.

The mother digester amended with TCC may have been operated at a threshold concentration of TCC. Thus, when this biomass was introduced to another chemical stressor (tetracycline in this case), the biomass was more readily inhibited. Tetracycline is known to work by inhibiting protein synthesis, whereas TCC does not yet have known intracellular mechanisms. Given that TCC is thought to intercalate within the cellular membrane, it is possible that TCC made cell membranes more porous and allowed tetracycline to enter into cells more easily. Tetracycline is a hydrophilic chemical that may easily pass through newly formed pores in the aqueous phase. It is difficult to parse the mechanism because there is a paucity of research regarding the mechanism of TCC inhibition. Research from previous chapters suggests that anaerobic communities can diverge after 60 days of acclimatization to TCC (Chapter 4 and 5). Perhaps TCC selected for organisms which maintained the function of the anaerobic digester, yet were intrinsically more sensitive to tetracycline.

Tetracycline impacted the TCC-amended biomass and the TCS-amended biomass in distinctly differently ways. For TCS-amended biomass, no statistical difference was observed from the control with either substrate. This result suggests that TCS and tetracycline inhibit cells by independent manners and the chemicals do not have synergistic inhibitory nor additive cross-resistance effects.

6.3.3 Ciprofloxacin bATA

The IC_{50} tests indicated that TCS-amended digesters gained cross-resistance to ciprofloxacin. The TCS-amended digesters had a statistically higher IC_{50} than the control digesters. This result suggests that resistance mechanisms which allow bacteria to tolerate

TCS also allow bacteria to tolerate higher concentrations of ciprofloxacin. It is likely that TCS shifted the microbial community towards members that were more resistant to tetracycline than the initial biomass microbial community.

Fluoroquinolones (the family of antibiotics to which ciprofloxacin belongs) have known resistance mechanisms in Bacteria. While many of the resistance mechanisms rely on target mutation, efflux is also a known resistance mechanism against fluoroquinolones (Jacoby, 2005). In fact, some of the exact same efflux resistance mechanisms that resist ciprofloxacin have been found to resist TCS in pure culture experiments (McMurry et al., 1998). Previous experiments showed than *Salmonella enterica* (a pathogenic bacterium) exposed to 0.5 mg/L of TCS had increased resistance to ciprofloxacin, and it was concluded that an efflux system (AcrAB) was responsible for this cross-resistance (Birosová and Mikulásová, 2009). Alternatively, TCS could have shifted the microbial community such that the digester still operated, but community members were intrinsically more tolerant to ciprofloxacin. Either scenario could result in an overall increase in total ciprofloxacin resistance genes.

TCC-amended biomass did not have a statistically different IC_{50} from the control. Cross-resistance to TCC has not been previously characterized nor was it found in this study. Perhaps TCC and ciprofloxacin inhibit bacteria through distinct pathways so that these toxicants had no interaction. Tetracycline is much more hydrophilic than ciprofloxacin; it is possible that TCC was able to aide passage of the hydrophilic chemical into the cell but had no effect on the hydrophobic chemical. Based on functional data alone it is not possible to know the exact mechanisms. Further research on these

samples including metagenomics analysis would provide more insight into the changes in the microbial community and how they might impact addition of antibiotics.

6.3.4 Chloramphenicol bATA

For the combination of chloramphenicol and propionate, the control and TCS-amended biomass had very similar IC_{50} values. This result indicates that TCS has no net effect on resistance to chloramphenicol under the conditions studied.

In contrast, TCC-amended biomass with propionate yielded a statistically lower IC_{50} than the control. TCC may act synergistically to inhibit methanogenesis with chloramphenicol or might make certain Bacteria more sensitive to antibiotics. Propionate degrading organisms, such as *Smithella* and *Syntrophamonas,* could be the key organisms in this cascade. These results indicate that, when Bacteria are exposed to TCC, they are more susceptible to chloramphenicol (McMahon et al., 2004).

For chloramphenicol, the bATA performed with dog food did not yield statistically significant differences for TCC-amended or TCS-amended biomass compared to the control. The IC_{50} for the control biomass is higher than the TCS-amended and TCC-amended biomass (by at least 5,000 μg/g); however, the 95% confidence intervals overlap heavily. The IC_{50} analysis was performed using log values and small deviations in log values can reflect very large differences in actual values, which may account for why this seemingly large difference was not statistically significant. Alternatively, it is possible that chloramphenicol was immobilized on the dog food, due to its hydrophobic nature, making the chemical less bioavailable when compared to just adding propionate to the matrix.

6.4 Conclusions and Implications

TCC increased the toxicity for 2 of the 3 antibiotics used in this study. TCC did not induce cross-resistance to any antibiotics, as hypothesized. Synergistic inhibitory effects between antibiotics have been previously observed in anaerobic digesters; likewise, TCC had synergistic inhibitory effects with antibiotics. In previous chapters, *mexB* was found to be selected for by TCC. Apparently, simply selecting for the MexAB pump cannot overcome the effects of chloramphenicol, tetracycline, or ciprofloxacin in an anaerobic digester.

TCS induced functional resistance to ciprofloxacin in bATA tests. While cross-resistance to ciprofloxacin induced by TCS has been directly observed in isolated pathogens, this is the first indication of TCS imparting cross-resistance to ciprofloxacin in a complex community. Given that TCS is highly prevalent in biosolids and wastewater treatment systems, full-scale anaerobic digesters could be serving as a "hotspot" for ciprofloxacin resistance gene proliferation. Increased resistance to certain classes of antibiotics in the environment may have clinical implications such as quicker development of antibiotic resistance in pathogens. Given that anaerobic digesters contain many clades of Bacteria and Archaea, resistance could have manifested from horizontal gene transfer or population selection. Either way, the total abundance of antibiotic resistance genes or organisms resistant to ciprofloxacin within the TCS-amended mother digester was presumably higher. Not all resistance genes that impact ciprofloxacin resistance are known, nor all environmental organisms which resist ciprofloxacin; the IC_{50} metric used in this study provides a basis to begin identifying these parameters by

indicating which environments are likely to have resistance genes and resistant organisms. Further, anaerobic biosolids are land applied and this practice could afford more opportunity for exchange of these resistance genes in the environment.

Determining which classes of antibiotics are the most susceptible to gaining cross-resistance to the most abundant chemical stressors can help guide further research. Indeed, these experiments demonstrated that cross-resistance cannot be expected between all chemical stressors. Quantitatively understanding the impacts of TCC or TCS antibiotics on cross-resistance can help the research field focus on areas of greatest concern. Quantification of cross-resistance should also be a focus of future research to identify whether resistance to certain antibiotics is more likely than others. Perhaps resistance may emerge to a class of antibiotics more quickly if cross-resistance is abundant in the environment, and if so, this class of antibiotics should be given special attention in medical use, research, and risk assessment.

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7 CONCLUSIONS

Triclosan (TCS) and triclocarban (TCC) are two antimicrobials which can serve as stressors for increased antibiotic resistance. Specifically, TCS is a known stressor for antibiotic resistance in isolated cultures (Yazdankhah et al., 2006), but the impact of TCS on antibiotic resistance in mixed anaerobic environmental cultures has only begun to be elucidated. While TCC is another widely used antimicrobial, research regarding its impacts on antibiotic resistance is scarce.

TCC and TCS are found in wastewater treatment systems because of the ubiquitous use of consumer products that contain these chemicals (USEPA, 2009). A substantial fraction of the influent mass of TCC and TCS sorbs to solids within the treatment plant because they are hydrophobic chemicals (Heidler and Halden, 2007). Solids in wastewater treatment are often anaerobically digested. Although TCC and TCS can be transformed under aerobic conditions, anaerobic conditions combined with digester retention times are not favorable for biological transformation (Veetil et al., 2012).

Anaerobic digestion is possibly a prime location for enrichment of antibiotic resistance because bacteria are exposed to relatively high concentrations of TCC and TCS for several days. The central goal of this dissertation was to understand the impact of TCC and TCS on the relative abundance of resistance genes, digester function, and microbial community structure in anaerobic digesters, and their role in selecting for cross-resistance to antibiotics.

7.1 Key Findings

This research was performed to further develop our understanding of antibiotic resistance as it relates TCS and TCC in anaerobic digestion. The first goal was to observe how TCC and TCS impact digester function, resistance gene concentrations, and community structure with different loading conditions of antimicrobials. The second study was aimed to elucidate the impacts of these antimicrobials on cross-resistance to antibiotics by amending digesters with antimicrobials and then testing toxicity of antibiotics.

The research conducted demonstrates that TCC increases the proliferation of antibiotic resistance genes and alters community structures in anaerobic digesters. If digesters are gradually acclimated to TCC, then anaerobic communities can adapt to concentrations which are otherwise toxic. Digesters which were acclimated to 450 mg/kg of TCC over 3 SRTs maintained function, whereas those which were immediately spiked with 450 mg/kg lost function. TCC significantly shifted community structure in both functional and inhibited digesters, suggesting some organisms are more sensitive to TCC than other organisms. With respect to resistance genes, concentrations of TCC ranging from 30 mg/kg to 850 mg/kg stimulated statistically higher concentrations of the resistance gene *mexB*. In digesters which were fully inhibited, the concentration of the *tet*(L) resistance gene increased by three orders of magnitude and the relative abundance of the resistance genes *erm*(F) statistically decreased. The abundance of the class 1 integrase gene was unaffected by TCC, suggesting that TCC neither stimulates nor inhibits the rate of horizontal resistance gene transfer through class 1 integrons.

TCS also influenced antibiotic resistance gene profiles and community structures. Digesters containing TCS selected for clades that include pathogenic and commensal organisms, suggesting that organisms which are commonly interacting with humans (and therefore exposed to TCS through use of personal care products) may have higher levels of tolerance to TCS. The resistance gene *mexB* was also selected for in all digesters containing TCS compared to the control. In inhibited digesters the *tet*(L) gene was selected for and *erm*(F) was selected against; the low pH from VFA build up and digester failure was likely the selective pressure for these genes. TCS was not found to affect the concentration of the class 1 integron.

Removing TCC and TCS from digesters did not yield a ubiquitous reduction of resistance genes nor did the microbial communities always shift back to that of the control in the timeframe studied. After washout of antimicrobials over 7 SRT values, the community structure of higher antimicrobial concentrations did not revert to that of the control. Instead, the community was significantly different than the control after washout of antimicrobials. However, after washout of background concentrations of TCS (30 mg/kg), the community structure was statistically similar to the control and a reduction in *mexB* was observed compared to the control. With respect to time, the relative abundance of *mexB* in other digesters did not decrease. However, the relative abundance of *mexB* was no longer statistically higher than the control digesters because the relative abundance in the control digesters increased.

When testing the toxicity of antibiotics on TCC or TCS amended biomass (biomass was amended for > 6 SRTs prior to testing), some antibiotics became more effective (i.e., toxic), and other antibiotics were less effective. Previous exposure to TCS

stimulated cross-resistance to ciprofloxacin, but cross-resistance was not observed to tetracycline and chloramphenicol. TCC did not lead to cross-resistance to antibiotics, but TCC made the anaerobic biomass more sensitive to tetracycline and chloramphenicol, i.e., TCC had synergistic effects when mixed with antibiotics.

The findings from this study can provide a scientific basis to better understand the impacts of TCC and TCS for product manufacturers and consumers. These results can contribute to policy making and consumer decisions regarding TCC and TCS.

7.2 Future Work Recommendations

Regarding anaerobic digestion and antimicrobials, a metagenomics approach should be used to understand the total resistome as impacted by TCC or TCS. Metagenomics could provide a more complete profiling of resistance genes which are increased or decreased following exposure to these antimicrobials. Mapping genes to specific organisms could be helpful to understand if pathogenic bacteria are impacted (i.e., understand which bacteria are most likely to affect humans). Furthermore, measuring expression of resistance genes through transcriptomics could help determine which resistance genes are functional and actively used to fight back against these antimicrobials.

Through research presented in this dissertation, it is now known that TCC and TCS have impacts on antibiotic resistance genes in mixed anaerobic communities. Further experiments should be performed with antibiotics and metals to quantify the relative impacts of TCC and TCS compared to antibiotics. Well-designed cross-resistance tests between antimicrobials, antibiotics and metals can also determine which antibiotics

are at the greatest risk of being resisted by bacteria, and therefore at the greatest risk of no longer being effective for public health medicine.

Resistance gene abundance data and transcriptomics data must be collected from people, animals, treatment plants, soils, and waterways which serve as reservoirs or locations of intense genetic exchange. Modeling of gene transfer between people, hospitals, water, and soils could be helpful for identifying areas of high risk. Furthermore, identifying resistance genes that are of higher threat (e.g., move quickly through the environment or confer resistance to many antibiotics) and prioritizing those which are most dangerous should be the focus of environmental-human resistome research. Technologies should be developed to reduce the transfer between compartments that pose greatest risk to humans. For example, biosolids handling by pyrolysis may reduce the abundance of antibiotic resistance released from treatment plants into the environment, but we need to better quantify the risk that genes leaving treatment plants pose to humans.

7.3 Broader Perspectives

TCS and TCC were originally placed into usage in consumer products to replace other antimicrobials that were deemed toxic. Hexachlorophene was formerly in consumer products which were widely used (Halden, 2014), and hexachlorophene is an antimicrobial which is quite similar in structure to TCS and TCC. It was formally found in soaps and toothpaste up until 1972. The FDA halted production and distribution of products containing more than 1% hexachlorophene because it was found to be toxic in a more traditional sense. Fifteen deaths were directly associated with the neural toxicity of

hexachlorophene. Triclosan was substituted into many of these products to maintain the label of “antimicrobial”.

Eliminating TCC and TCS from consumer products seems to have potentially positive impacts for slowing antibiotic resistance formation and dissemination. Antimicrobial alternatives which do not have implications with antibiotics certainly exist. For example, alcohol based hand sanitizer can be used on hands in lieu of TCS for reduction of microbes. The application is not exactly the same, as it would be found separate from hand soap, but the results are similar. Additionally, washing hands under warm water for 30 seconds using regular soap (i.e. no antimicrobial added) achieves required reduction of microbes (Aiello et al., 2007; Larson et al., 2004).

If TCC or TCS were banned, then either could be replaced by a chemical that has undiscovered or unknown properties. As we are now finding, triclosan does not have the direct toxic effects like hexachlorophene, but it does have unintended consequences of cross-resistance to antibiotics. Quaternary ammonium compounds serve a similar purpose as TCS, and have also been linked to resistance (Russell, 2002, 2000). Metal nanoparticles also have disinfection properties. Nanoparticles have been shown to affect the resistome, but the total impacts in the environment are still unknown (Miller et al., 2013). One possible outcome that a ban on these chemicals would have is slight chemical modification of existing antimicrobials to maintain similar functionality with the claim that the new chemical has no known-consequences because the specific structure has not been studied.

Overall, caution should be exercised in moving forward with regulation of TCC and TCS. Safe alternatives must be established for applications which heavily rely on

these chemicals. Additionally, it is important for consumers to understand when these chemicals are actually useful or necessary. These chemicals should only be applied for products that rely on an antimicrobial application to function with no alternatives. For instance, using triclosan in hospital settings at higher concentrations during an operation is much more important than having it be used non-discriminately in house-hold soaps. Continually monitoring of the impacts of TCC, TCS, and potential replacements should be employed if these chemicals are to be phased out. It is important to quantify risks thoroughly before proceeding with replacement so that even greater negative environmental impacts do not occur.

7.4 References

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APPENDICES

Appendix A- Quantifying Triclocarban in Biomass

TCC concentrations were measured by liquid chromatography-mass spectrometry (LC/MS). Briefly, 4 µg of ^{13}C-labed TCC (Cambridge Isotope Laboratories, Inc, Andover, MA, USA) were added to a 5 mL sample of wet biosolids. The sample was allowed to dry in a crucible for 72 hours at 35°C. The mass of the dried biomass was quantified and then extracted into approximately 20 mL of methanol by using an Accelerated Solvent Extraction System (Dionex ASE 350, Thermo Scientific, Sunnyvale, CA, USA). The extraction protocol was modified from Anger et al., to thoroughly remove TCC and TCS with methanol and acetone (Anger et al., 2013). For extraction, dried biosolids was placed into a clean ASE cell. The cell was heated to 60°C and held at a pressure of 1500 psi; it was heat cycled twice to this temperature and then flushed with 60% of the extraction cell volume.

Micropollutant concentrations from the ASE extracts were determined by injecting 20 µL into a Shimadzu LCMS-2020 (Shimadzu, Addison, IL, USA). Chromatography was performed with a Phenomenex Luna C18 column (3 µm particle size, 150 x 3 mm). The flow rate was 400 µL/min using mobile phase A of 100% HPLC grade water and mobile phase B of 100% methanol. The method began at 80% methanol and increased linearly over 13 minutes to 100% methanol. The mass to charge ratios used for detection of TCC and ^{13}C-TCC were 313 and 319, respectively. Concentrations were determined by using a seven-point standard curve.

Table A. TCC results and recoveries from extraction

Day	Sample	13-C TCC Recovery	Corrected TCC (mg/kg)	Difference from nominal concentration
0	Seed	58%	27	NA
33	Background	76%	25	18%
47	Control	57%	0.8	NA (Target conc = 0)
47	Low-Immediate	46%	126	3.1%
47	Medium-Immediate	44%	420	6.6%
47	High Immediate	50%	692	19%
110	Control	56%	0	NA (Target conc = 0)
110	Background	43%	31	3.3%
110	Low-Gradual	56%	131	0.8%
110	Medium- Gradual	45%	448	0.4%

Appendix B- Nutrient Media Fed to Anaerobic Digesters

Table B. Nutrient Feed Recipe

Constituent	**(mg/L)**
NH_4Cl	400
$MgSO_4.7H_2O$	195
KCl	400
$CaCl_2.2H_2O$	50
$(NH_4)_2HPO_4$	80
$FeCl_2.4H_2O$	*40
$CoCl_2.6H_2O$	*10
KI	10
$(NaPO_3)_6$	10
$NiCl_2.6H_2O$	1
$ZnCl_2$	1
$MnCl_2.4H_2O$	0.5
NH_4VO_3	0.5
$CuCl_2.2H_2O$	0.5
$AlCl_3.6H_2O$	0.5
$NaMoO_4.2H_2O$	0.5
H_3BO_3	0.5
$NaWO_4.2H_2O$	0.5
Na_2SeO_3	0.5
$NaHCO_3$	6000
$Na_2S.9H_20$	300
L-Cysteine	10
*Yeast Extract	*10
*Dog Food (seived >0.4 um)	*30000

*indicate deviations from (Speece, 2008)

Appendix C- Triclocarban Anaerobic Toxicity Tests

A dose response curve was constructed for TCC. Reactors (160-mL) were maintained with a 50 mL working volume. Triplicate digesters were given 7 distinct doses of TCC (Sigma-Aldrich, St. Louis, MO) based on previous observations (0, 1, 500, 1000, 2000, 5000, 10000, and 30000 mg/kg based on total solids) and 3.8 g/L_r of calcium propionate to ensure that substrate was not limiting. TCC was added to digesters in 50 µL of Dimethyl Sulfoxide. Biogas production rate was measured over 10 days. The maximum rate of biogas production was calculated for each dose of TCC. Dose response curves were constructed with these data and the concentrations which inhibit 50% of methane production (IC_{50}= 850 mg/kg) and 10% of methane production (IC_{10} = 450 mg/kg) were interpolated from the data using GraphPad Prism.

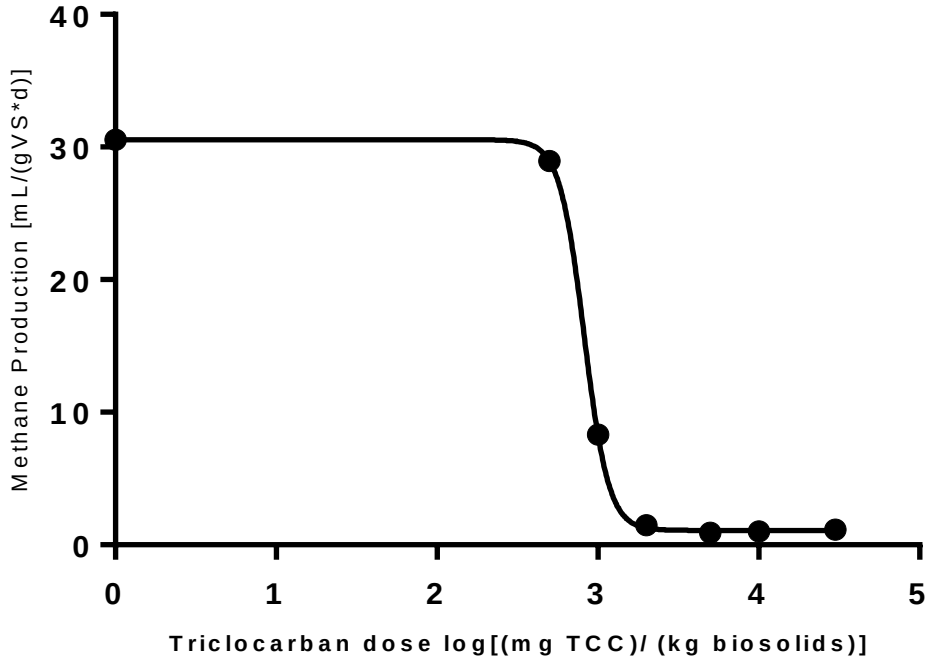

Figure C. Methane production at a given TCC dose (n=3). Error bars representing standard deviation of the mean are included, however they are occluded by the data points.

Appendix D- Primers and qPCR Conditions

Table D. qPCR details

	Forward & Reverse Primer	Annealing Temp (°C)	Average Efficiency (%)	Limit of Quantification (copies/µL)	Ref.
16S	F (5'-CCTACG GGAGGCAGCAG-3') R (5'-ATTACCGCGGCTGCTGG-3')	60	101.5%	10^4	(Muyzer et al, 1993)
mex(B)	F (5'-GTGTTCGGCTCGCAGTACTC-3') R (5'-AACCGTCGGGATTGACCTTG-3')	63	103.0%	$5x10^2$	(Yoneda et al., 2005
intI1	F (5'-CCTCCCGCACGATGATC-3') R (5'-TCCACGCATCGTCAGGC-3')	60	94.9%	$5x10^2$	(Goldstein et al., 2001)
tet(L)	F (5'-TCGTTAGCGTGCTGTCATTC-3') R (5'-GTATCCCACCAATGTAGCCG-3')	60	88.2%	$5x10^2$	(Ng et al., 2001)
erm(F)	F (5'-CAACCAAAGCTGTGTCGTTT-3') R (5'-TCGTTTTACGGGTCAGCACTT-3')	60	86.6%	$5x10^2$	(Patterson et al., 2007)

qPCR was performed on a BioRad CFX Connect Real Time System (Hercules, CA). Assays began with a 10 min initial denaturation at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s and combined annealing and extension at the primer-specific for 30 s. Reaction volumes of 20 µL consisted of 10 µL of BioRad iTaq SYBR Green Supermix (Life Science Research, Hercules, CA), 5 uL of diluted DNA and 5 uL of Ultrapure water with optimized quantities of forward and reverse primers (1 nM for resistance genes and intI1 and 2 nM for 16S rRNA gene). Approximately 50 ng and 0.25 ng of template DNA were required for resistance gene quantification and 16S rRNA quantification respectively.

Samples were diluted to be within the linear range of the standard curve and remove inhibitor substances. Data were only used if the the R^2 value was greater than 0.95. Resistance genes in the feed were below detection limits in all cases. Positive standards for PCR were generated as described elsewhere (LaPara et al., 2011; Kappell et al., 2015).

Appendix E- Digester pH in TCC digesters

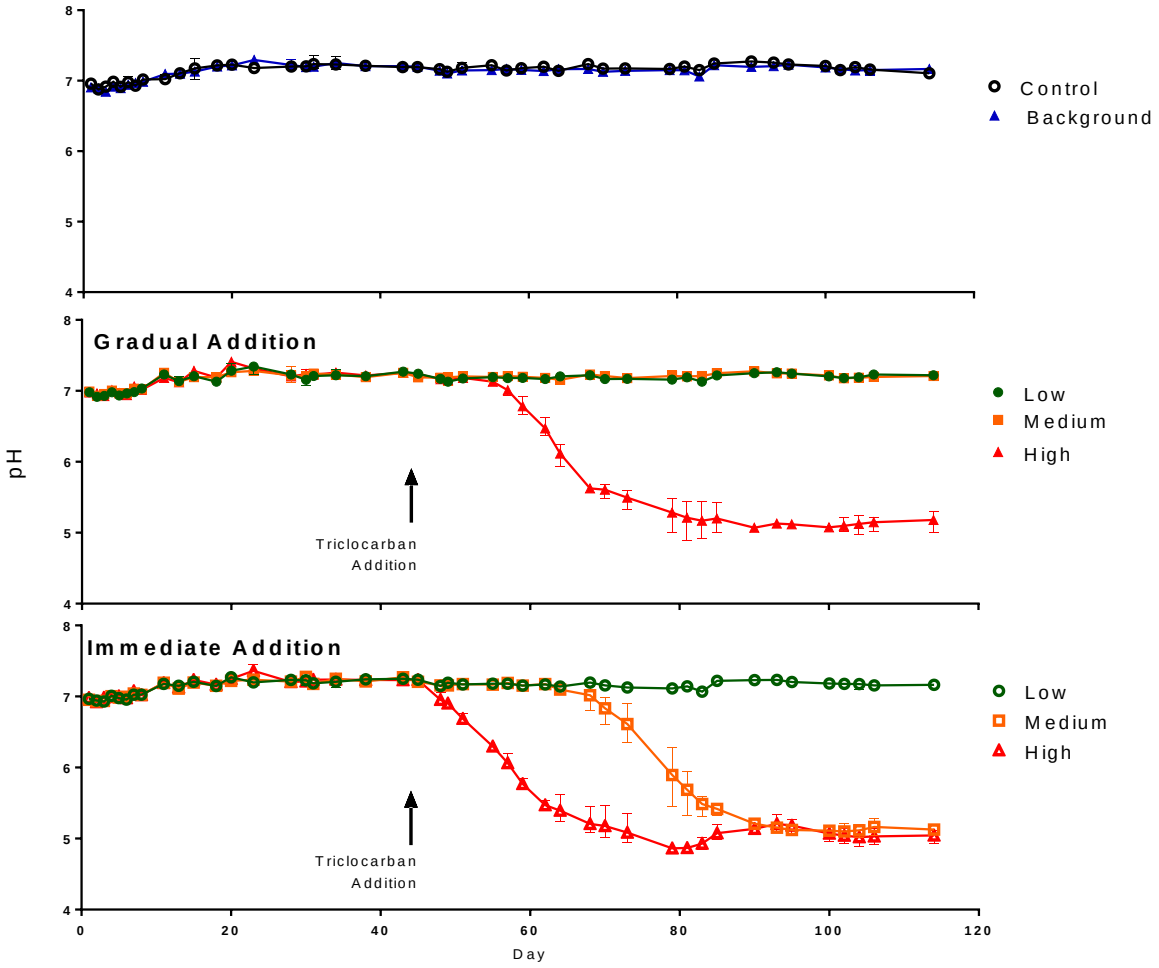

Figure E. Average digester pH over the duration of the study. Error bars represent the range of the data points.

Appendix F- Digester VFA Concentrations in TCC Digesters

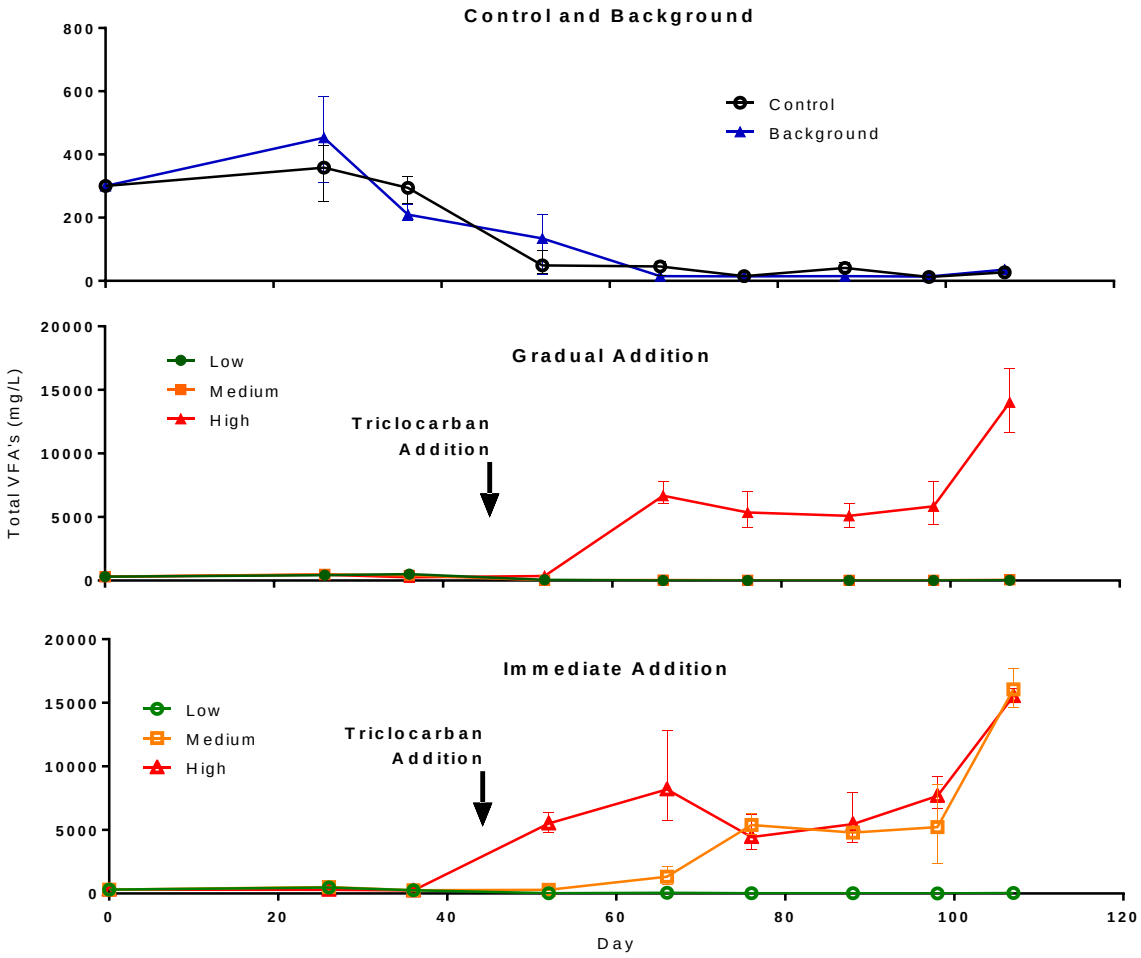

Figure F. Total VFA concentration in the bioreactors including acetic acid, proprionic acid, butyric acid, iso-butyric acid, valeric acid, and iso-valeric acid. Note the top graph is on a different Y-axis. Error bars represent the range of the data points.

Appendix G- Abundance of Genes Normalized to Digester Volume in TCC Digesters

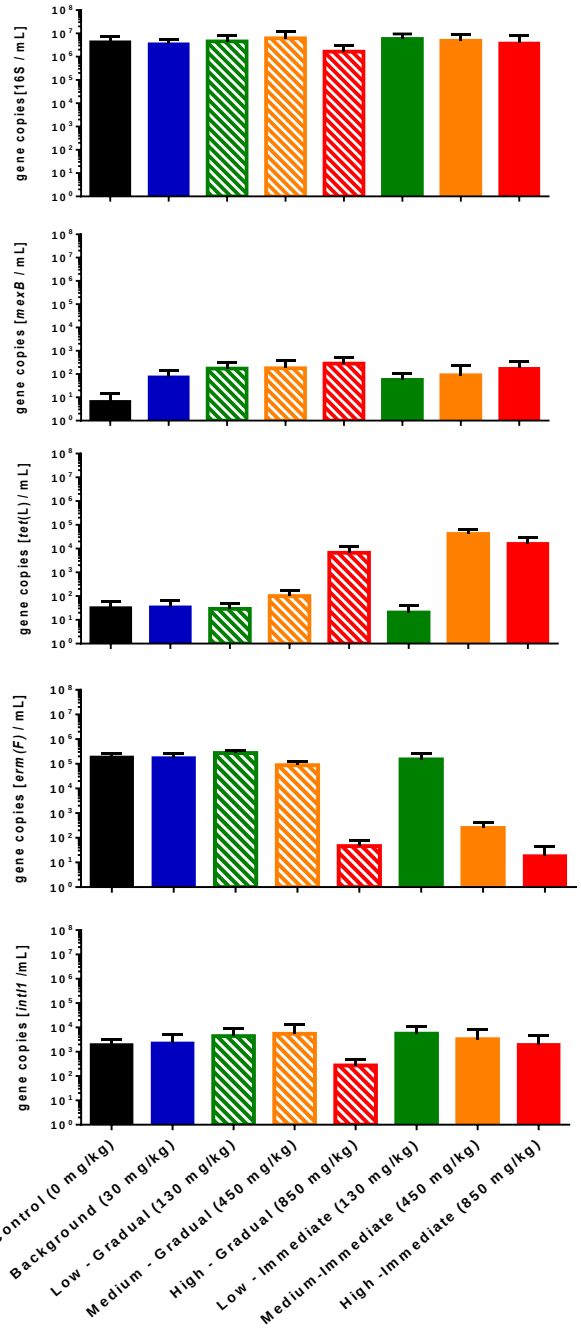

Figure G. Gene abundances normalized to mL of digester volume. Note no significant differences were found between concentrations of 16S rRNA with ANOVA testing (ANOVA, $p = 0.21$).

Appendix H- Total nMDS in TCC Digesters

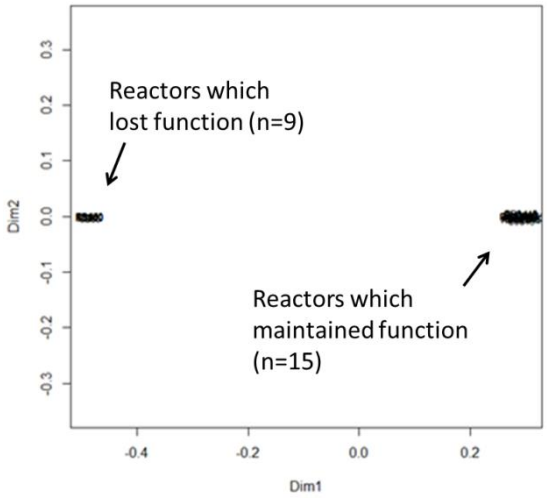

Figure H. nMDS plot of all digesters at Day 110. Differences between functioning and non-functioning digesters is at a level such that differences cannot be observed within these groups.

Appendix I- Digester Biogas Production

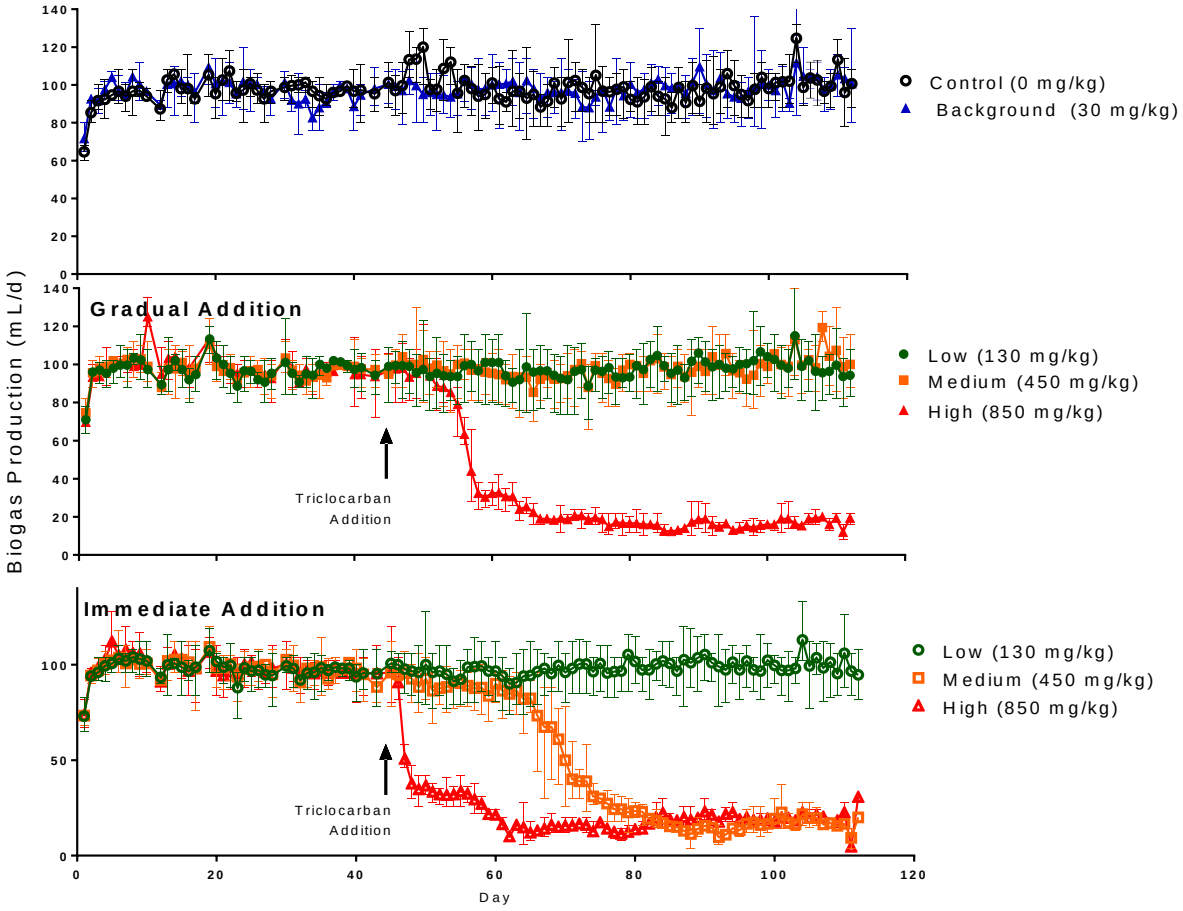

Figure I. Total biogas produced over the duration of the study. Error bars represent the range of the data.

Appendix J- Anaerobic Inhibition Testing of Triclosan

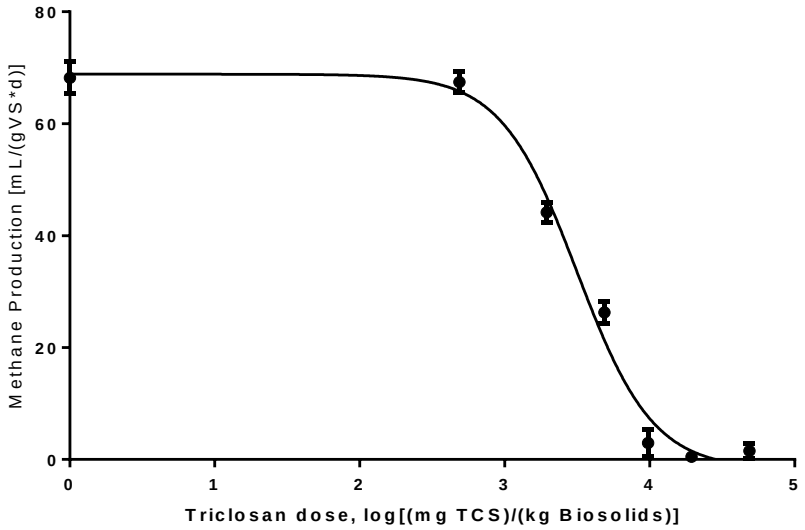

Figure J. Steady state methane production at various TCS concentrations (n=3).

Inhibition testing for TCS was carried out using an anaerobic toxicity assay style test. Seven triclosan doses (0, 500, 2000, 5000, 10000, 20000, and 50000 [mg TCS]/[kg total solids]) were delivered to anaerobic digesters and methane production rate was measured. Briefly, triplicate anaerobic digesters were prepared for each triclosan dose with a 50 mL working volume in a 160 mL serum vial. Each digester initially received 3.8 g/L_r of calcium propionate. The headspace was sparged with a 70/30 mix of N_2/CO_2 and sealed with a pressure containing rubber butyl stopper. TCS was then added to digesters in 50 µL of Dimethyl Sulfoxide. Biogas production was measured by displacement with a wetted gas syringe. Methane fraction in the biogas was measured after 10 days by gas chromatography (7890A, Angilent Technologies, Irving, TX, USA), when headspace gas was assumed to be equal to biogas produced by the biomass.

Appendix K- Triclosan Concentration Measured by LC/MS

Table K. Recoveries and concentrations of TCS in Biosolids

Sample	**13-C TCS Recovery** (%)	**Nominal TCS concentration** (mg/kg)	**Measured TCS concentration accounting for recovery** (mg/kg)
Seed	57%	N/A*	28
Control (Day 45)	78%	0	0.96
Background (Day 45)	64%	30	17
Control (Day 110)	87%	0	0
Background (Day 110)	73%	30	15
Low (Day 110)	60%	100	74
Medium (Day 110)	68%	850	770
High (Day 110)	73%	2500	2990

* Measured in the seed biomass, therefore no concentration is expected

Five mL samples were collected of waste biomass when TCS was quantified. The sample was placed in a crucible and allowed to dry for 72 hours at 35°C; total solids concentration was determined from mass measurements.

The dried biomass was scraped from the crucible, and a known mass was extracted using Accelerated Solvent Extraction System (Dionex 42 ASE, Thermo Scientific, Sunnyvale, CA, USA). Prior to extraction, the extraction cells were cleaned with a triple rinse of methanol, sonication in acetone, followed by another triple rinse with methanol (adapted from Anger et al.). The dried biomass was placed into the extraction cell and 2 µg of ^{13}C-labed TCS (Cambridge Isotope Laboratories, Inc, Andover, MA, USA) was added in a nonane solution and allowed to dry. The samples were then extracted by heating the cells to 60^{o}C while holding the pressure at 1500 psi using methanol as the solvent. The cells were heated through the cycle twice and 60% of the cell volume was collected after each cycle. The final extract volume was approximately 20 mL for each sample.

Liquid chromatography-mass spectrometry (LC/MS) was employed to measure the concentration of TCS and ^{13}C-TCS in biosolid extracts. Injection volumes of 20 µL were used on a Shimadzu LCMS-2020 (Shimadzu, Addison, IL, USA). A C18 column (Phenomenex Luna, 3 µm particle size, 150mm x 3mm) was used to perform chromatography. The mobile phase shifted linearly over a 13 minute runtime from 80/20 ratio of methanol/water to 100% methanol. The flow rate of the mobile phase was 400 µL/min. The M/Z ratios for detection on the mass spectrometer were 287 and 299 for TCS and ^{13}C-TCS, respectively. Peak interactions were accounted for when determining concentrations. TCS was assumed to be recovered at the same rate as ^{13}C-TCS and this recovery was applied in the calculation for TCS concentration in biosolids.

Appendix L- nMDS of All Triclosan Communities

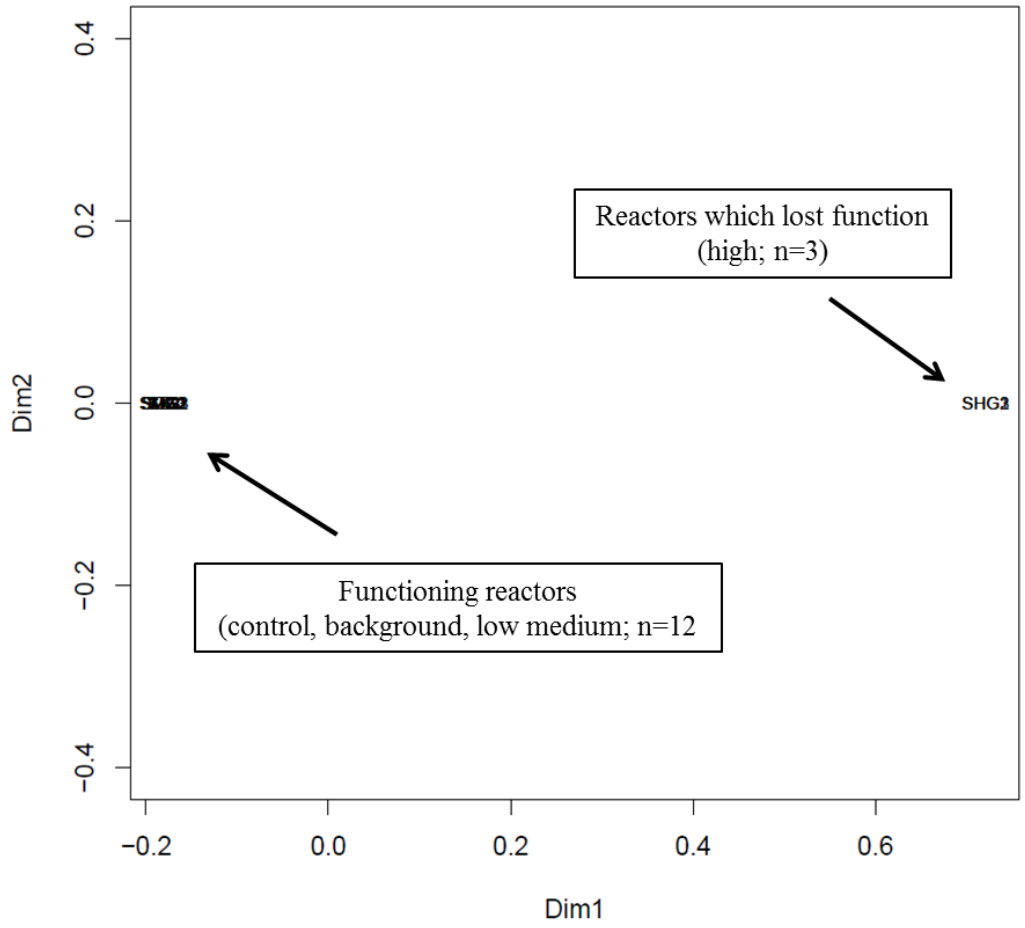

Figure L. nMDS of day 110 communities performed with genus level data including digesters which lost function. Resolution of functioning digesters is not appropriate to make conclusions.

Appendix M- 16S Gene Copies by Volume in Triclosan Digesters

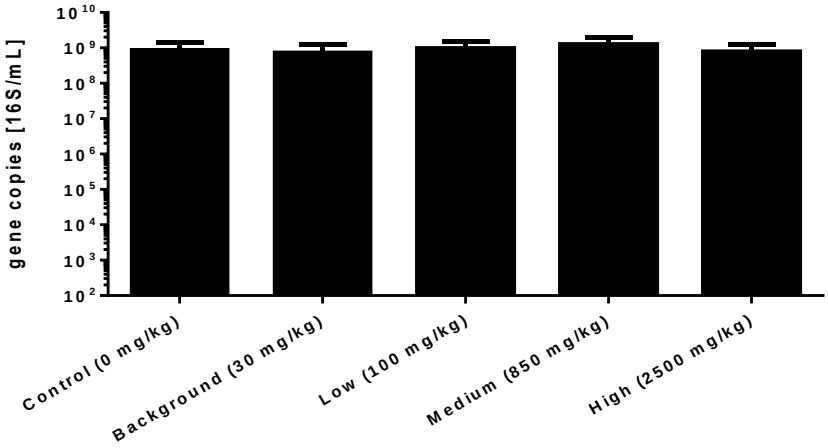

Figure M. 16S copies per mL of digester. ANOVA testing indicates that the concentrations are not statistically different (p= 0.46, n=9).

Appendix N- Methane Production and pH with Error Bars

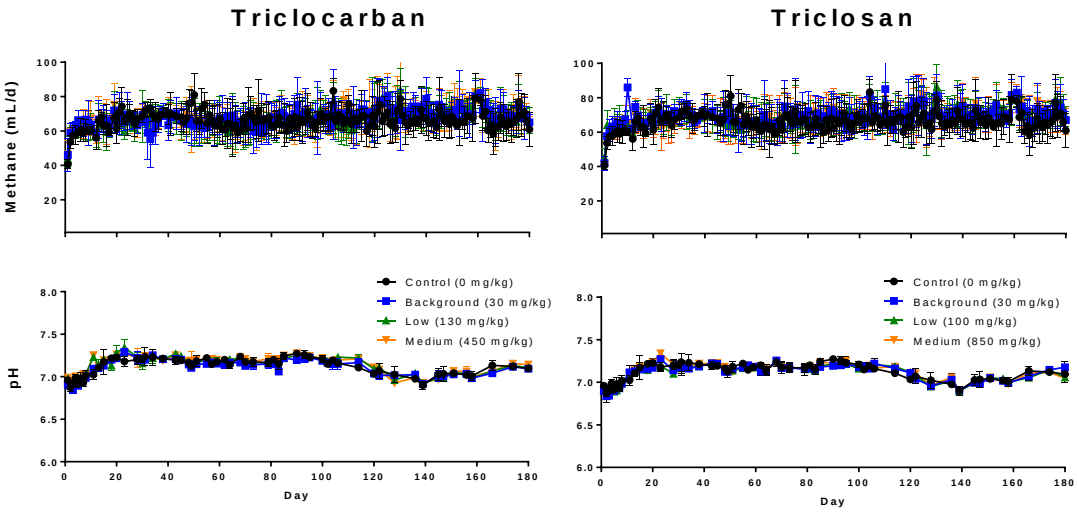

Figure N Methane production and pH with error bars for all gradual digesters

Appendix O- VFA Speciation in Digesters

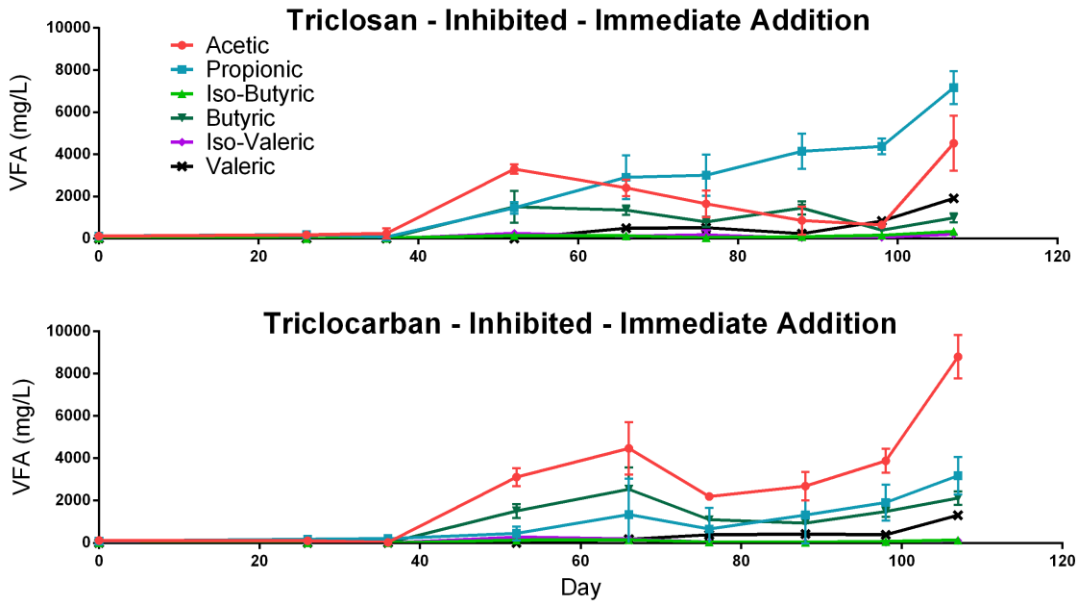

Figure O. Individual volatile fatty acids in inhibited digesters

Appendix P- IC_{50} Curves of Antibiotic and Substrate Combinations

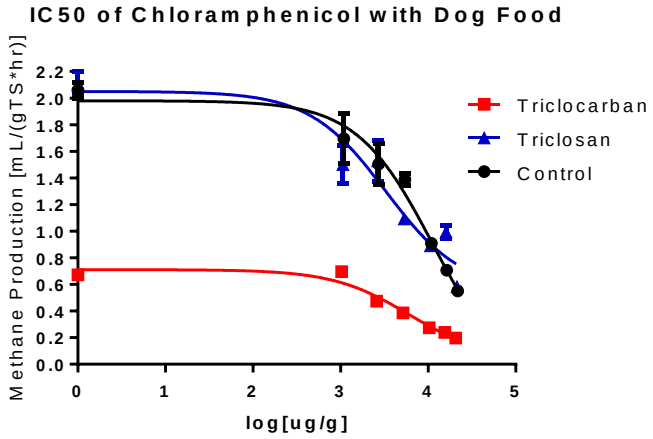

Figure P1. Chloramphenicol with Dog Food

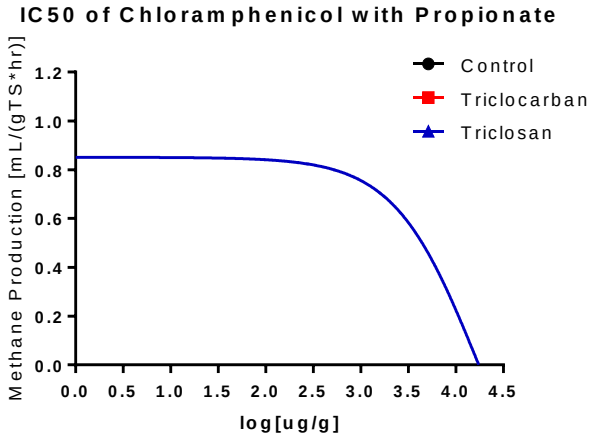

Figure P2. Chloramphenicol with Propionate

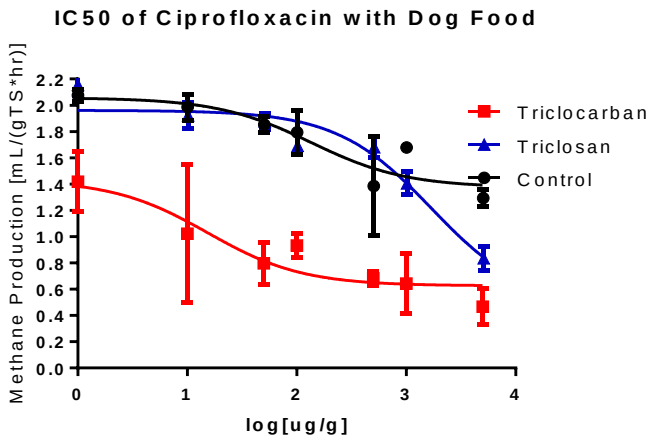

Figure P3. Ciprofloxacin with Dog Food

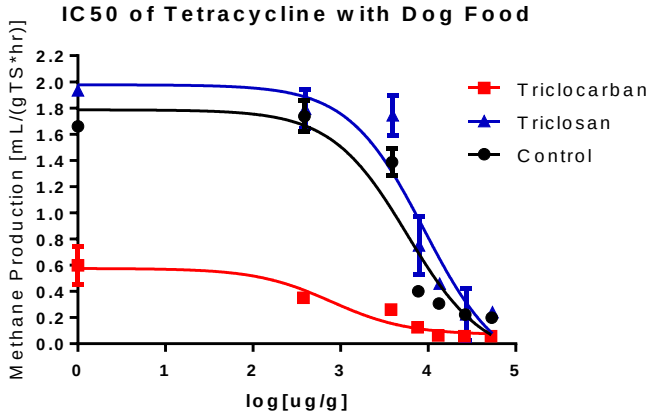

Figure P4. Tetracycline with Dog Food

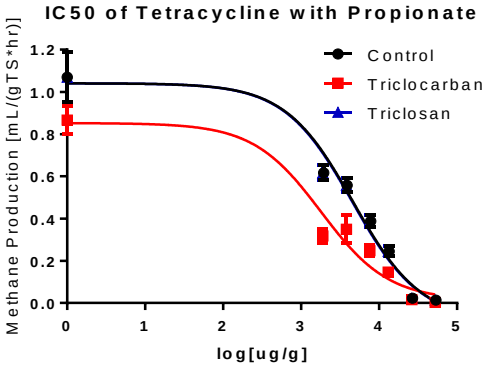

Figure P5. Tetracycline with Dog Food

Appendix Q- Canonical Correspondence Analysis (CCA) of Gradual Triclosan Digesters

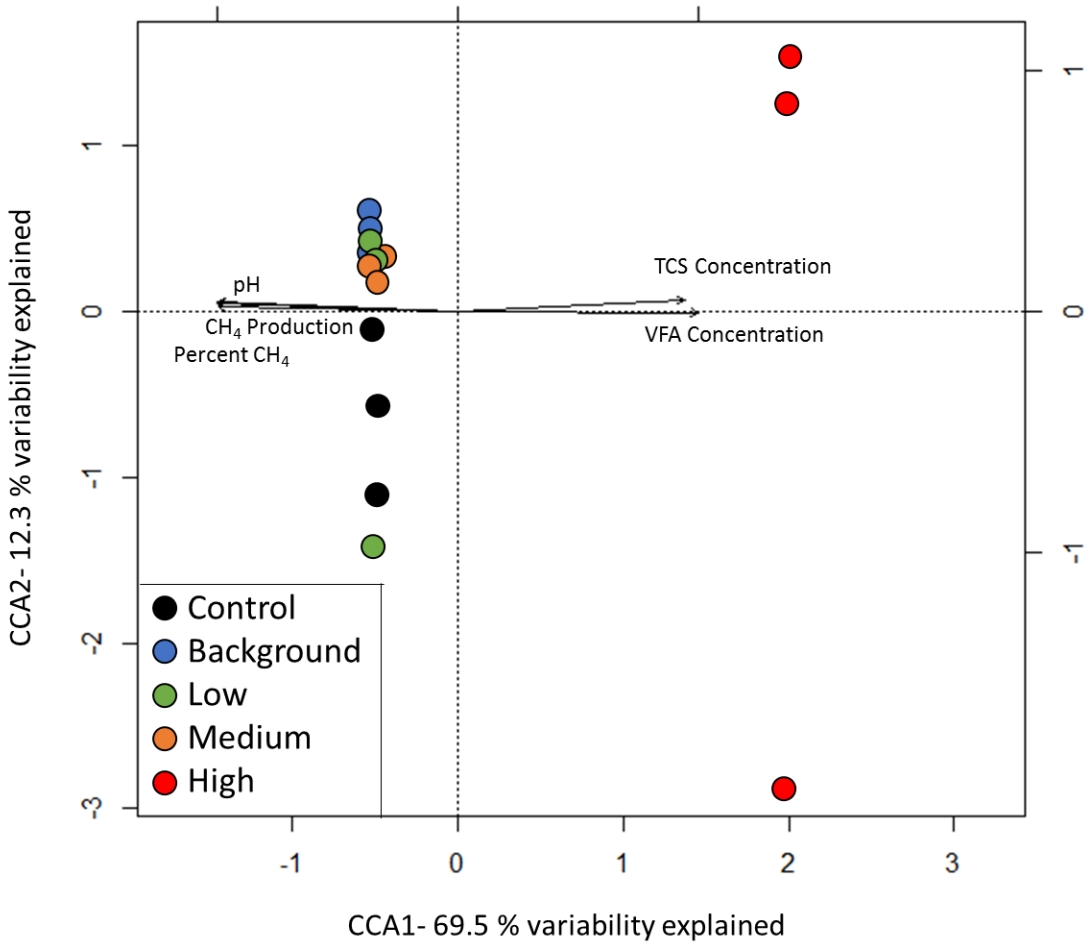

Figure Q. CCA of TCS digesters (OTU data) on day 110. Clear separation can be observed from the "High" digesters along the x-axis. Increased *TCS concentration* and increased *VFA concentration* were correlated with the High digesters. The high digester set had the highest TCS concentration (2500 mg/kg), VFA concentrations higher than 20,000 mg/L, a pH below 5, methane production of less than 5 mL/day, and less than 25% methane in the biogas. Conversely, increased *pH*, *CH_4 Production*, and *Percent CH_4* correlated with the Control, Background, Low, and Medium digesters. These digester sets had VFA concentrations < 50 mg/L, a pH near 7, biogas was near 70% methane, and methane production was near 70 mL/ day. The horizontal axis can explain 69.5% of the variability in the data and all of the continuous variables used to constrain the data set correlate along this axis. Further, *TCS concentration* and increased *VFA concentration* were anti-correlated to increased *pH*, *CH_4 Production*, and *Percent CH_4* which was expected.

Appendix R- Canonical Correspondence Analysis (CCA) of Gradual Digesters Before and After Removal of Antimicrobials

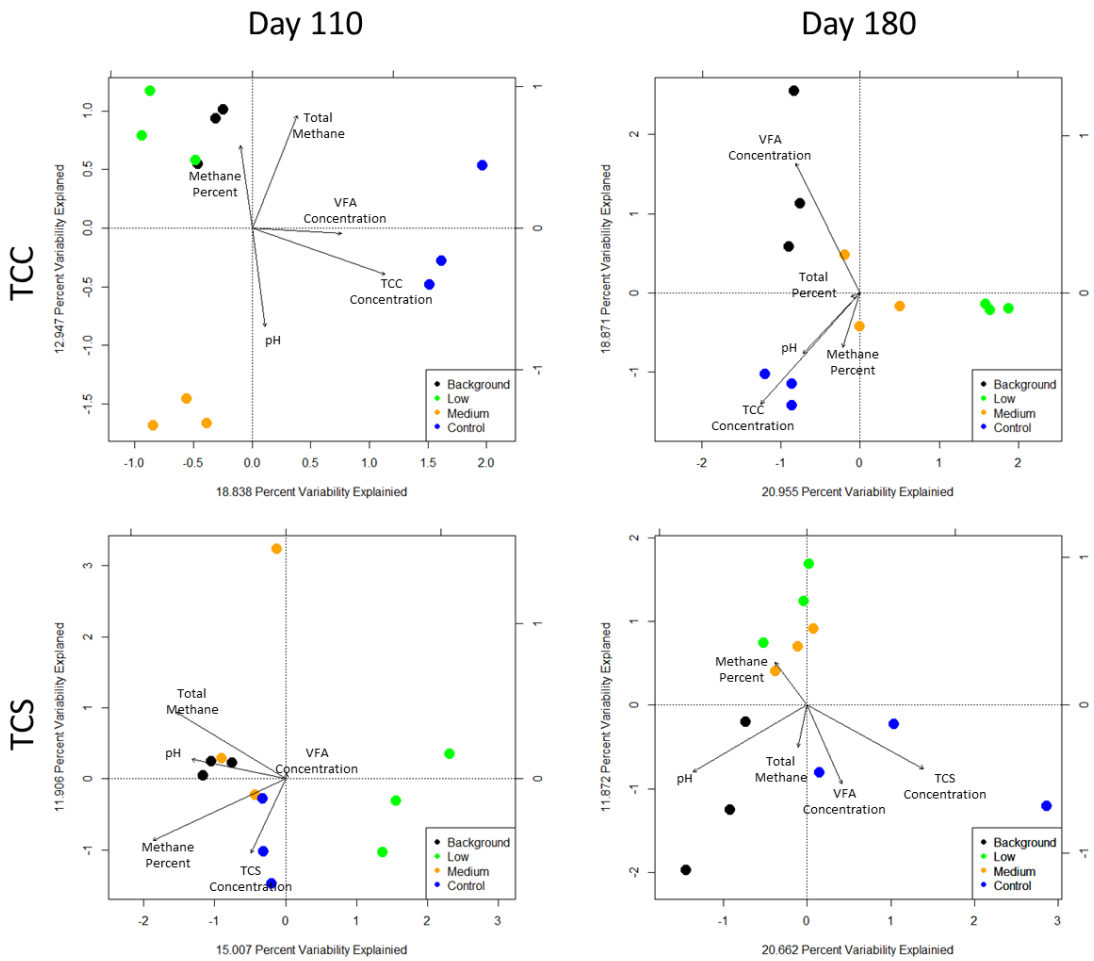

Figure R. CCA for biomass at day 110 and day 180 on OTU data. This CCA does not yield any information with seems to be helpful with interpretation of results. The randomness of the CCA information is ostensibly due to the clustering of the continuous variables used to constrain the CCA other than the concentration of the antimicrobial (ie. pH, total methane, methane percent, VFA concentration were similar in all samples).

Appendix S- References for Appendices

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