THESIS

IMPACTS OF OPERATING CONDITIONS ON AEROBIC REMOVAL OF EMERGING CONTAMINANTS FROM ANIMAL WASTE: COMPOSTING POST ANAEROBIC DIGESTION

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ABSTRACT

IMPACTS OF OPERATING CONDITIONS ON AEROBIC REMOVAL OF EMERGING CONTAMINANTS FROM ANIMAL WASTE: COMPOSTING POST ANAEROBIC DIGESTION

The shift in the U.S. livestock industry from small farms to large-scale concentrated animal feeding operations has given rise to two challenges: how to cost effectively dispose of the large quantities of waste generated, and how to minimize the environmental impact of the growing use of veterinary pharmaceuticals. In dry states such as Colorado, livestock industries of all sizes also face a growing need for the advancement and application of sustainable and low water agriculture practices to help with the management of livestock waste. A common method of livestock waste disposal is through application to cropland as a fertilizer. However, this method is becoming less cost-effective due to higher transportation costs as generated waste overwhelms the capacity of adjacent land. In addition, the land application of manure creates a pathway for veterinary pharmaceuticals and other emerging contaminants (ECs) to be released into the environment through runoff into surface waters or by leaching into groundwater. Recent studies have found ECs can negatively impact soil microorganisms, crop growth, and aquatic wildlife. ECs also pose an unknown risk to human health due to routine consumption of low-levels of ECs in drinking water and could potentially be ingested from food crops if they have taken up ECs from the soil in cases where manure is land applied. In addition, there is a growing concern that the release of antibiotics and antibiotic resistant genes into the environment will lead to an increase in antibiotic resistant pathogens. Therefore, low water usage technologies

that can reduce the concentrations of these ECs from animal wastes prior to the release into the environment are needed to protect croplands and human health.

Manure management technologies such as composting and anaerobic digestion (AD) of manure or manure combined with food waste have been shown to reduce waste volume, reduce odor, kill pathogens, and reduce the concentrations of some ECs. In addition, AD systems have potential cost benefits due to energy production, which is enhanced further when manure and food waste are co-digested. A technology known as multi-stage anaerobic digestion (MSAD) combined with leachate recirculation has the benefits of AD and allows for reduced water usage, especially if reclaimed municipal wastewater is used as a water source. Recent testing on the MSAD system demonstrated it was possible to convert the system to an in-vessel composter after the AD phase was completed to further stabilize waste prior to land application. The addition of composting to the MSAD system has the potential to further degrade ECs that are known to be persistent (e.g., estrone) or only partially degraded (e.g., chlortetracycline) during AD. However, there has been little work to address the degradation of ECs in a combined AD/ composting system and few have identified specific operating conditions (such as temperature, pH, or retention time) that can optimize EC removal during aerobic composting (AC). Also, little is known about how well microbes originating from an AD system will perform when the system is changed to AC conditions and if ECs can be removed as well as in traditional composting methods. Thus, identifying operating conditions that optimize EC removal during the aerobic phase of a combined MSAD/AC system is critical to guide system design and operation.

The objectives of this project were to determine which operating conditions lead to the greatest biotic and abiotic degradation rates of ECs and to characterize microbial communities that originate from AD digestate that have the ability to degrade ECs under aerobic conditions. To achieve these objectives, three operational conditions were tested, which included: temperature (35°C, 45°C, and 55°C), pH (7, 8, 9), and carbon composition that varied protein, carbohydrates (easily biodegradable carbon), and cellulose (more recalcitrant carbon) concentrations. Five ECs were selected for this study either because of their wide usage with livestock (antibiotics chlortetracycline, oxytetracycline, sulfamethoxazole, and the hormone estrone) or because they could be present in reclaimed municipal water (naproxen, a non-steroidal anti-inflammatory drug) if used as a water source for a combined MSAD/AC system. Inocula were digested cattle manure and woodchips obtained from a combined MSAD/AC system, which was transitioning from the anaerobic phase to the aerobic phase. In advance of the microbial degradation studies, microbial communities were pre-acclimated to a synthetic manure/food digestate growth media under test conditions. Concentrations of ECs spiked into bench scale reactors were collected over 29 days and measured using an ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method developed as part of this work. Initial microbial community structures were analyzed for all reactor conditions and analyzed by next-generation sequencing of 16S rRNA genes.

Results indicated that temperature has an impact on EC removal for sulfamethoxazole (SMX), estrone, and chlortetracycline (CTC). SMX and estrone had near complete removal at 35°C, with less removal at higher temperatures. By contrast, CTC showed increased removal at higher temperatures; however, concentrations dropped to

below detection limit at all temperatures after 29 days. The pH test was run at pH 7, pH 8, and pH 9; however, results indicated that changes to pH over this range had little impact on EC degradation. SMX was the only EC to be impacted by pH, with near complete removal at pH 7, but only 77% removal and 68% removal at pH 8 & 9, respectively. Carbon source composition had a significant impact on SMX, estrone, and CTC removals. In media with high concentrations of easily biodegradable carbon, SMX dropped to below detection limits, while estrone had the highest removal percent in the media with least carbon concentration. CTC showed increased removal in media with low concentrations of easily degradable carbon concentration, but again concentrations dropped to below the detection limit for all carbon source composition reactors after 29 days.

SMX and estrone were likely degraded by microbes, while CTC and oxytetracycline (OTC) appeared to degrade through abiotic processes. At 35°C, results indicate that Naproxen was degraded by microbes, however at 45°C and 55°C live and killed control reactors had similar removal indicating abiotic degradation. The microbial community structure was determined for test inocula, and three potential EC degrading microbial phylotypes were identified via comparison to literature. *Pseudomonas* has been identified as an estrone degrader in literature. For this research, *Pseudomonas* was only found in reactors that had microbial degradation of estrone (the 35°C reactors and the reactors with low levels of easily degradable carbon), and was therefore a likely estrone degrader. *Bacillus* has been found in literature to degrade numerous ECs including SMX, OTC, estrone, and naproxen. Since SMX had some level of microbial degradation under all test conditions, and *Bacillus* was present in all reactors for this research, *Bacillus* was a likely degrader of SMX.

Successful removal of ECs from full-scale systems will require an understanding of the contaminant and media chemical properties specific to that system. However, the results from this experiment indicate that operational conditions such as temperature and carbon composition can potentially be optimized for EC removals and that microbes are present at the end of AD that have the ability to further degrade ECs such as sulfamethoxazole and estrone under aerobic conditions.

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1.1 Research Motivation

Livestock industries in dry states such as Colorado face a growing need for the advancement and application of sustainable agriculture practices to help with the management of livestock waste. Practices such as anaerobic digestion (AD) and aerobic composting (AC) of manure or of manure mixed with food waste have the benefits of generating bioenergy and reducing waste, while treated water from municipal wastewater treatment plants can be used as a water source for AD or for crop irrigation. However, these practices can also lead to the release of emerging contaminants (ECs) into the environment as animal waste and treated water has been shown to include high levels of endocrine disrupting compounds, antibiotics, and other pharmaceutical compounds (see Figure 1). Recent studies have found these ECs can negatively impact soil microorganisms, can be taken up into crop roots or plant tissues, and can leach out of the soil and migrate to ground or surface waters. The negative impacts on terrestrial and aquatic ecosystems have been widely reported, and there is a growing concern that antibiotics in the environment will lead to an increase in antibiotic resistant bacteria. In addition, while contaminants have been found at low levels, the potential negative impact on human health from routinely consuming these contaminants in food and in water raises concerns. Therefore, technologies that can reduce the concentrations of these ECs from animal wastes prior to the release into the environment are needed to protect croplands and human health.

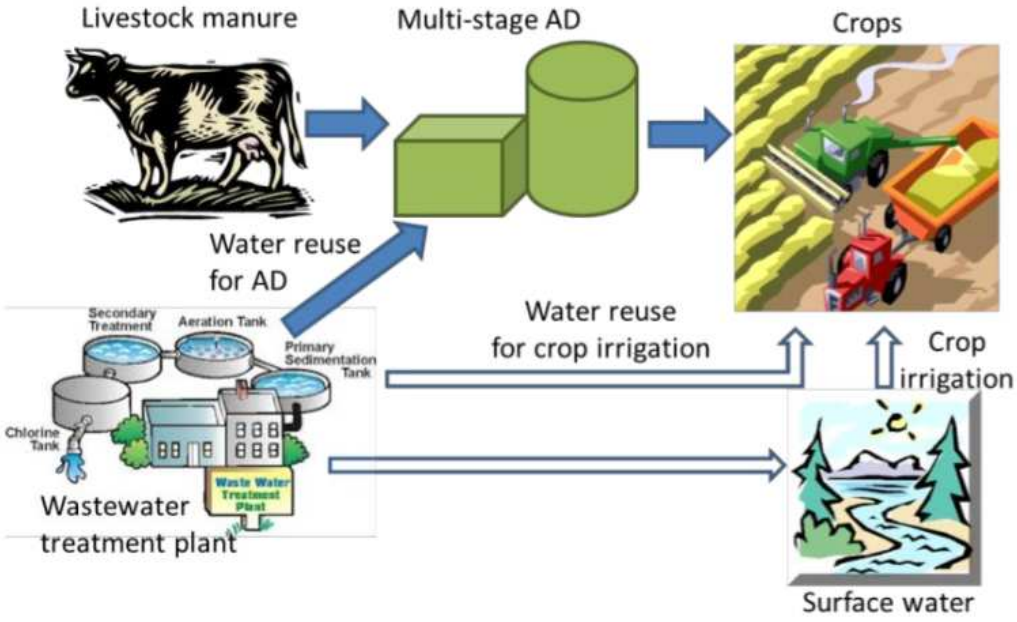


Figure 1: Potential pathways of crop exposure to emerging contaminants.
Pathways shown in solid arrows are the focus of this research.

One solution involves advanced biological treatment of manure and food waste materials and recycled water prior to field application. Recent studies have shown that a broad range of emerging contaminants is biodegradable. Although AD already has been shown to reduce concentrations of some emerging contaminants (e.g., chlortetracycline), some contaminants such as estrone degrade little or even accumulate. However, composting (an aerobic biological process) has been shown to remove estrone and other contaminants not removed during AD. Therefore, the combination of AD and AC could still have the benefits of converting wastes into bioenergy and marketable soil amendments while adding the potential of removing a broad range of contaminants during the composting phase of operations. Much research has been done to look at degradation in either an AD or an AC system. However, there has been little work to address the degradation of ECs in a combined system and few have identified specific operating

conditions (such as temperature, pH, or retention time) that can optimize EC removal during AC. Also, little is known about how well microbes from an AD system will perform when the system is changed to AC conditions and if those microbes will be able to remove ECs as well as in traditional composting methods. Thus, identifying operating conditions that optimize EC removal during the aerobic phase of a combined AD/AC system is critical to guide system design.

1.2 Research Objectives

The objectives of this project were to determine: 1) which operating conditions lead to the greatest biotic and abiotic degradation rates of five ECs, and 2) identify microbial communities that originate from AD digestate that have the ability to degrade ECs under aerobic conditions. To achieve these objectives, inocula were obtained from a combined AD/AC system as it was transitioning from the anaerobic phase to the aerobic phase. In advance of the microbial degradation studies, microbial communities were pre-acclimated to a synthetic manure/food co-digestate feedstock in growth media under test conditions. EC concentrations were then monitored for 29 days by using an ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method developed as part of this work. Initial microbial community structures were analyzed for all reactor conditions and tested by sequencing of 16S rRNA genes.

1.3 Thesis Overview

Chapter 2 is a literature review covering the usage and release of emerging contaminants (EC) into the environment. Topics covered include: manure management challenges faced by the livestock industry, benefits of advanced treatment technologies such as AD and AC, and previous studies on the degradation of ECs in AD and AC systems.

Current analytical tools used for measuring EC levels in animal waste and for the identification of microbes in treatment systems are also summarized. Chapter 3 is structured as a manuscript for publication about the impact of operational conditions on the aerobic degradation of ECs. The appendices include the methods for growth media preparation, analytical chemistry method development, tables with statistical analysis results, and an analysis of chlortetracycline transformation products.

2: BACKGROUND AND LITERATURE REVIEW

2.1 Livestock Manure Production and Disposal Challenges

Each year the U.S. livestock industry produces and needs to dispose of large volumes of manure. The USDA estimated that in 1997 there was a combined total of 132 million dry tons of manure production. This breaks down to 8.5 million dry tons of swine manure, 16.2 million dry tons of poultry litter, and 107.4 million dry tons of cattle waste (Kumar, Gupta, Chander, & Singh, 2005).

Land application of manure to croplands is a manure disposal method commonly used in the U.S. There are many benefits to this method such as low disposal costs and the addition of valuable nutrients to the soil (Kumar, Gupta, Chander, et al., 2005). However, there are limits to the amount of nutrients that crops can utilize before accumulating in soil. (Kellogg, 2000) Excessive amounts of phosphorus can runoff into streams and cause algal blooms, while excessive amounts of nitrogen can pose health risks if it seeps into water supplies.

Contributing to the manure management challenges was the shift the U.S. livestock industry made during the last half of the 20th century from small and medium-sized operations to large-scale confined operations. Even though the number of livestock operations decreased by roughly 25% from 1982 to 1997, the number of animals units remained constant between 91 to 95 million(Kellogg, 2000). With large numbers of animals confined in relatively small areas, the amount of manure produced exceeds the assimilation capacity of land within economic transport distances (EPA-NRMRL, 2004). The USDA estimated that the amount of excess nutrients produced each year that could not be

applied to the land on the same farm the waste was produced increased from 899 million lbs in 1982 to 1,472 million lbs in 1997 (Kellogg, 2000). This has led to an increasing need for technologies that can decrease the volume of waste and that can add value to the waste to offset disposal costs.

2.2 Common Technologies Used for Manure Management

Manure water content is one of the key parameters that determine which manure management technologies are available to livestock producers. Stockpiling and composting are more commonly used for drier waste with high solids content. Lagoon storage and AD are commonly used for waste with low solids content, although dry AD technologies have also been developed for wastes with high solids content.

2.2.1 Composting and Stockpiling

Composting is a process where bacteria, and other microorganisms, break down organic materials to stable, usable organic substances. One of the main benefits of composting is waste volume reduction, which can be as high as 30 to 50% (L. Chen, de Haro Mari, Moore, & Falen, 2011). Other benefits include reduction in odor, and the killing of pathogens and weeds. The composting process goes through four phases which include: mesophilic, thermophilic, cooling and maturation (Figure 2). For the first couple of days after a compost pile is started mesophilic microbes (optimal growth between 25 °C and 40 °C) are dominant and quickly increase the temperature. Once temperatures rise above 40 °C thermophilic microbes take over. This phase can last for a few days to several weeks depending on compost pile properties and has the greatest rate of decomposition and stabilization of organic matter (L. Chen et al., 2011). Weeds and pathogens are destroyed if temperatures greater than 55 °C last for more than three days (Onwosi et al., 2017).

Microbial activity begins to slow and temperatures decrease during the cooling phase until they stabilize around 35 °C at the start of the maturation phase. The maturation phase lasts a couple of months up to a year and is responsible for stabilizing organic acids and other resistant compounds.

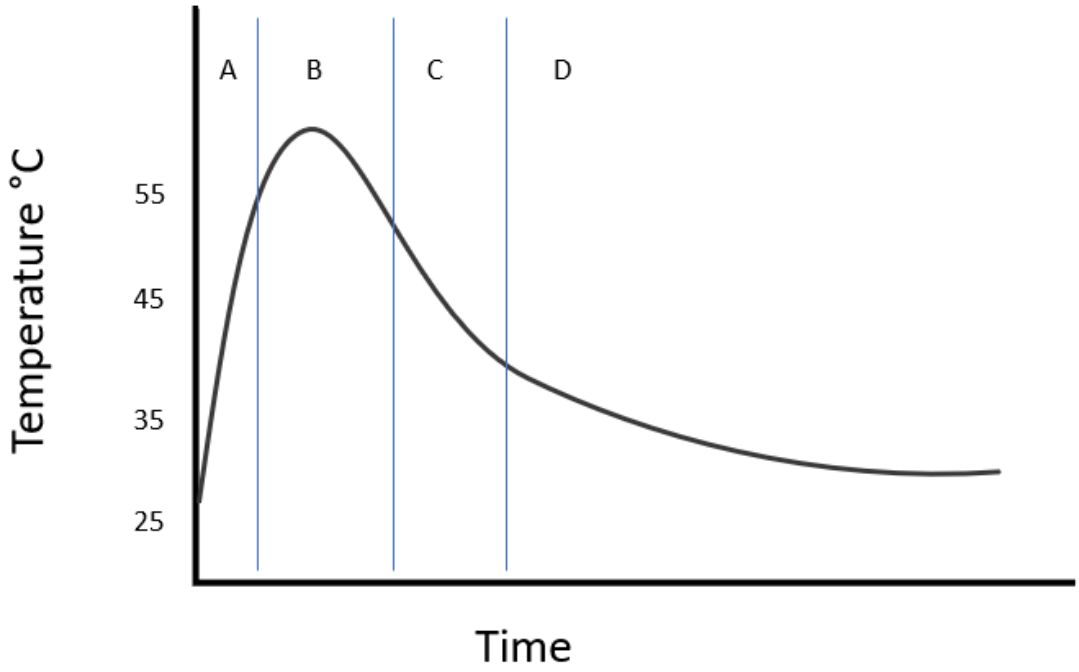


Figure 2: Thermal phases of composting. The four composting phases include A) mesophilic, B) thermophilic, C) cooling phase, and D) curing phase. Times vary depending on size, type, and management of compost system.

The quality and value of the finished compost varies depending on the intensity of the compost management methods. The least labor-intensive method is to simply stockpile the dry waste and bedding until it can be spread on fields. Depending on the stockpile size, there may be self-heating at the core of the pile that allows for some pathogen removal and thermophilic stabilization; however, the cooler outer layers are usually left unchanged (O. A. Arikan, Mulbry, & Rice, 2009). More labor-intensive methods

include the optimization of factors such as aeration, pH, the carbon to nitrogen (C/N) ratio, addition of bulking agents, and moisture content (Onwosi et al., 2017). Proper aeration is a key factor for composting because it controls the temperature, removes CO_2 and provides O_2 for biological processes. (Bernal, Alburquerque, & Moral, 2009) The most common method for aeration is through the turning and mixing of compost material. A pH of 6.7–9.0 supports good microbial activity during composting. Elemental sulphur (S_o) has been used as an amendment for avoiding excessively high pH values during composting (Bernal et al., 2009). Carbon and nitrogen are the two most crucial nutrients required by microorganisms. The optimal C/N ratio for composting is between 25 – 30:1. If there is inadequate nitrogen then microbial growth slows; if there is too much, then excess nitrogen is volatilized to ammonia gas resulting in compost with strong undesirable odors (Onwosi et al., 2017). The C/N ratio for raw manure is typically too low, however bedding material such as wheat straw, corncobs, sawdust or other bulking materials can increase the C/N ratio (EPA-NRMRL, 2004). Optimum water content levels vary depending on waste type, but generally compost mixtures should be maintained between 50 – 60%. Moisture content higher than 60% inhibits O_2 movement and the microbial processes can become anaerobic (Bernal et al., 2009). Maintaining compost piles at optimum operating conditions will result in a higher quality product that could be sold as a fertilizer, which can justify the additional operational costs (Bernal et al., 2009).

Composting with open-air windrow piles is one of the most commonly used methods because of its simplicity, but it requires large areas of land and can be impacted by weather conditions. In-vessel composting is an alternative that contains the compost in a large container that can have controlled temperatures, compost turning, and aeration

(Sandefur, 2017). One study of in-vessel composting was able to demonstrate that by maintaining a temperature of 60 °C in a pilot scale test the composting rate was accelerated and elimination of pathogens (*E. coli* and *Salmonella*) took place within 25 hours (Pandey et al., 2016).

2.2.2 Lagoons

Livestock operations that use lagoons for waste storage typically collect waste through slatted floors into subfloor channels. Floors are either scraped or washed with water or can be flushed with lagoon supernatant (EPA-NRMRL, 2004). Wastes can be transferred to lagoons by pumping or with gravity flow systems. These systems usually require solid content to be less than 10%, or less than 4% if pumping is required (EPA-NRMRL, 2004). Because lagoons are typically not mixed the lagoons tend to be anaerobic; as a result, strong odors can be released, which can lead to odor nuisance complaints. Covering lagoons with a synthetic material can prevent the release of odors and can allow for the recovery of methane for energy production (EPA-NRMRL, 2004).

2.2.3 Anaerobic Digestion

Anaerobic digestion (AD) is a biological method for treating animal waste that can reduce solids and stabilize waste while at the same time provide a source of renewable energy. The microbes in AD systems convert organic waste into biogas containing methane and carbon dioxide using the following biological processes: hydrolysis, acidogenesis/acetogenesis, and methanogenesis. During the hydrolysis phase complex polymers are converted into monomers like soluble sugars by extra-cellular enzymes. These monomers are then converted into volatile fatty acids and hydrogen during

acidogenesis/acetogenesis. Methanogenic bacteria use the fatty acids to form methane and carbon dioxide (Bernet & Beline, 2009).

Although covered lagoons are a simple, low-cost method of AD, there are more sophisticated methods available that may include temperature control, constant mixing, and biogas collection. While these systems can have higher operating costs, they tend to have shorter retention times and better methane production which can help to offset those costs through energy production (Bernet & Beline, 2009). Most of these systems are enclosed in structures that reduce the risk of leakage into groundwater, which is concern with lagoon storage. In addition, AD also has the benefit of decreased odor, pathogen reduction, reduced greenhouse gas emissions, improvement of non-point source pollution concerns, and production of end products that can potentially be land applied (Bernet & Beline, 2009; Sharvelle, 2012).

There are some challenges that livestock facilities may have with the implementation and use of AD technologies. Large operations might prefer to use shorter retention times to maximize throughput of large quantities of manure and to maintain high rates of methane production. However, with shorter retention times degradable organic compounds that could have been used for additional methane production are left in the digestate. Also, during storage these degradable organic compounds can produce strong odors and toxic compounds that can have deleterious effects on crops. Additional treatment of the digestate (such as dewatering and composting) might be required (Alburquerque, de la Fuente, Ferrer-Costa, et al., 2012). Co-digestion of animal waste with food waste has also been found to increase the methane production during AD. Zhang et al. (2013) ran semi-continuous lab-scale AD reactors for 24 days and found that methane

production more than doubled when food waste was co-digested with cattle manure at a ratio of 2:1.

Another challenge for the implementation of conventional, wet AD technologies is the requirement that waste have less than 17% total solids. This can be a challenge in arid states where solid contents can be as high as 90% when scraped from floors or dry lots (Sharvelle, 2012). Advancements in dry anaerobic technologies, such as multi-stage AD systems (Wasserbach, 2013; R. Wu, 2017) have made AD a more attractive option for dry states since they can operate with wastes that exceed 40% total solids (Angelonidi & Smith, 2015).

2.3 Multi-Stage Anaerobic Digestion Combined with Aerobic Composting

Multi-stage AD (MSAD) is a technology that isolates the AD phases into three separate reactors to optimize conditions for the different biological processes. As seen in Figure 3, in the leachate module low volumes of water are tricked through a bed of low moisture content waste. Hydrolysis converts particulate organic into soluble carbon compounds (fatty acids, amino acids, sugars, simple aromatics, etc.) and hydrogen. Leachate containing the soluble carbon compounds and hydrogen is then transferred to a leachate storage tank where acidogenic and acetogenic microbes convert the compounds into volatile fatty acids (VFAs). In the final stage, methanogens convert the VFAs into methane. Hydrogenotrophic methanogenesis, which converts hydrogen and carbon dioxide into methane, also occurs. Leachate is then recirculated back to the leachate module to be reused, making this a low water-usage process (Sandefur, 2017).

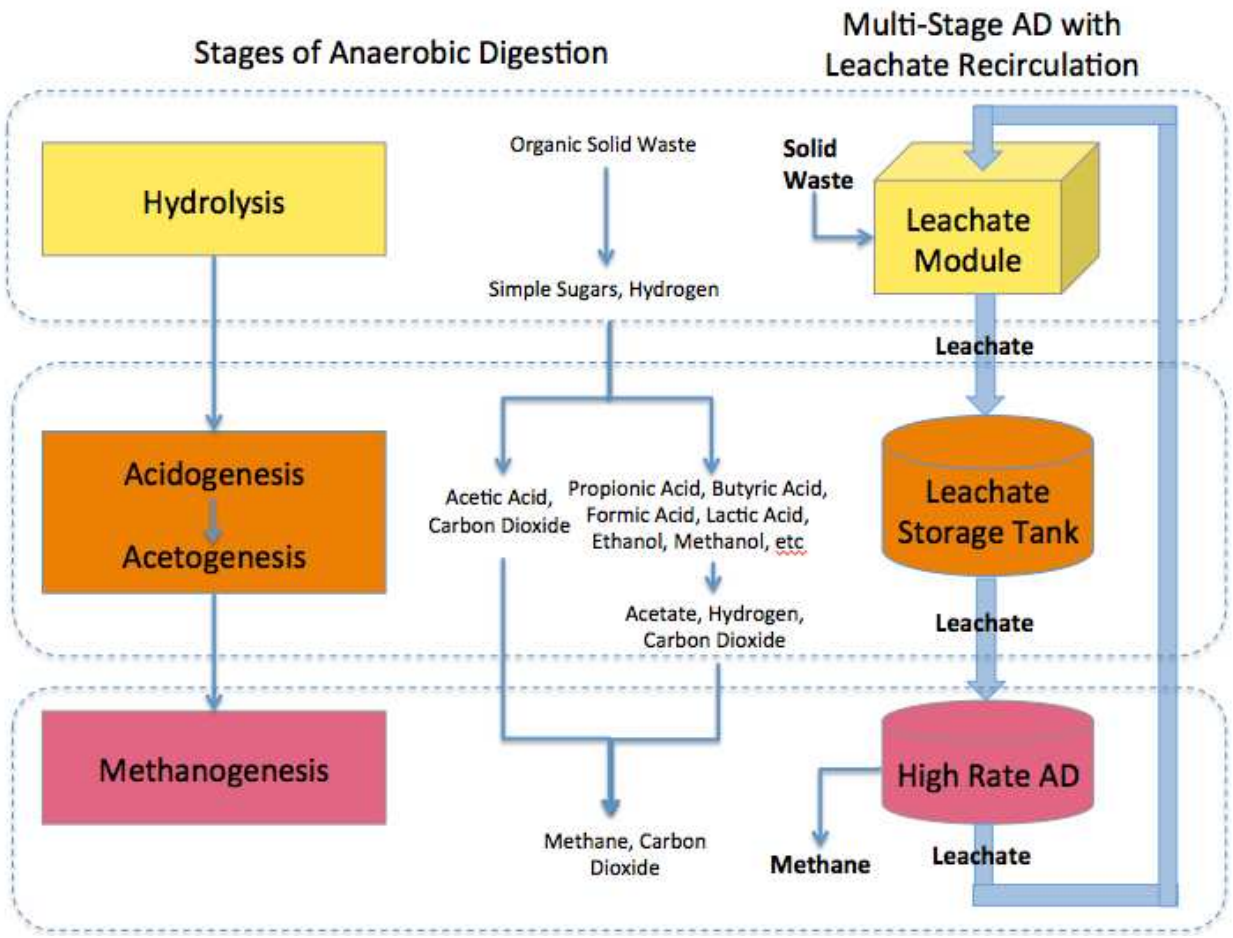


Figure 3: Stages of AD compared to MSAD system schematic. Figure is a modified version of schematic found in (Sandefur, 2017)

In MSAD systems, faster digestion rates and greater methane production are achieved since each stage can be optimized and controlled separately. This is particularly helpful in improving the reaction kinetics of the two rate-limiting processes: hydrolysis and methanogenesis (Fox & Pohland, 1994). The slow rate of leachate moving through the leachate module enhances hydrolysis by allowing more contact time between the enzymes and complex substrates. In the high rate AD reactor, methanogens are retained on biofilms or sludge granules and are able to thrive since there is no competition from faster-growing acidogenic organisms (Fox & Pohland, 1994). In addition, recirculating leachate through an

MSAD system provides advantages such as shorter digestion time, increased methane production, greater volatile solids reduction, and reduced water usage (Sandefur, 2017).

While anaerobic digestate has been directly applied to fields, there are disadvantages to this practice that include: increased transportation costs from higher water content waste, and the risk of odors and toxic compounds caused by digestate that is not completely exhausted of easily degradable organic compounds. Composting the material after digestion can solve those problems by producing a stabilized, easy-transferable end product (Sandefur, 2017). One study showed that a combined in-vessel AD/AC system produced fully matured compost more quickly than processing waste in an in-vessel composting-only system, (Walker, Charles, & Cord-Ruwisch, 2009). Recent testing on the MSAD system at CSU demonstrated it was possible to convert the leachate module to an in-vessel composting process by adding forced aeration after the AD phase was completed (Sandefur, 2017). This method was able to combine the benefits of in-vessel composting, such as faster composting rates and smaller land requirements, with the benefits of MSAD, such as low water usage and improved methane production.

2.4 Sources of Emerging Contaminants

A wide variety of veterinary pharmaceuticals are used by the livestock industry each year to treat diseases, prevent infections, increase weight gain, or to improve feed efficiency (Song, 2014). Common drugs include antiparasitics, antimicrobials, hormones, antifungals, anti-inflammatory drugs, sedatives, antacids, diuretics, and many others (Bartikova, Podlipna, & Skalova, 2016). Previous studies have shown that pharmaceuticals are only partially metabolized by animals (10 – 90%), so a large portion of the drugs can be found intact as parent compounds in animal waste (Kumar, Gupta, Chander, et al., 2005).

Therefore, it is important to understand the types of pharmaceuticals and typical concentrations found in manure, as land application of manure can be a pathway for contaminants to be released into the environment.

2.4.1 Antibiotics

Antibiotics are the most commonly used veterinary pharmaceutical, making up 70% of livestock pharmaceutical consumption (Song, 2014). Modern, high population density, livestock operations can be conducive to rapid spread of infections diseases. As a result, livestock in these environments commonly require aggressive infection management strategies and prophylactic antibiotic usage (Landers, Cohen, Wittum, & Larson, 2012). Annual antibiotic use on livestock is estimated to be 2.5 million kilograms in the United States. While roughly 10% of antibiotic usage is for the treatment of active infections, the other 90% is used for prophylactic care and growth promotion (EPA-NRMRL, 2004).

Since 1949, when the U.S. officially approved the use of antibiotics as a livestock feed additive (Song, 2014), antibiotics have also been used to enhance growth and feed efficiency in healthy livestock (Sarmah, Meyer, & Boxall, 2006). Antibiotics have been shown to suppress the activity and population of bacteria in livestock intestines allowing for the preservation of energy in feed that would be lost to microbial fermentation. The resulting enhancement in nutrient and energy availability is believed to promote animal growth (Song, 2014). Studies have shown the inclusion of antibiotics as a feed additive improved the liveweight gain in pigs by 5 – 6 % (Jensen, 1998).

Numerous classes of antibiotics are available for livestock use. Sarmah et al. (2006) identified ionophores, tetracyclines, penicillin, and sulfonamides as the most commonly used antibiotic classes in the U.S. In northeastern China, chlortetracycline, oxytetracycline,

sulfamethoxazole, and enrofloxacin were the antibiotics most typically found in animal feces (Y. X. Li et al., 2013). Other classes include arsenicals, polypeptides, glycolipids, elfamycins, macrolides, lincosamides, polyethers, beta-lactams, quinoxalines, and streptogramins. Table 1 lists individual antibiotics found in many of those classes.

Table 1: Common antimicrobial classes and concentrations found in manure

		Concentrations found in manure/slurry samples (mg/kg)[a]		
Antimicrobial class	Sample of drugs from Class	Swine	Poultry	Beef/Dairy
Sulfonamides	Sulfachlorpyridazine, Sulfadimethoxine, Sulfamerazine, Sulfamethazaine, Sulfathiazole, Sulfadimidine, Sulfamethazine, Sulfamethoxazole	0.01 - 29	0.004 - 91	0.015 - 791
Ionophores	Laidomycin, Lasalocid, Monesin, Narasin, Salinomycin		0.32 - 33.0	1 - 5
Tetracyclines	Tetracycline, Oxytetracycline, Chlortetracycline	0.03 - 765	0.05 - 123.3	0.13 - 872
Macrolides	Carbomycin, Erythromycin, Oleandomycin, Tylosin	0.05 - 7.9	0.2 - 0.4	0.2 - 116
Lincosamides	Lincomycin, Pirlimycin	0.07 - 0.24		
Polypeptides	Bacitracin, Polymixin B		0.01-1.76	
Flouroquinolones	Danofloxacin, Enrofloxacin	0.13 - 0.75	0.01 - 8.3	1.72 - 46.7

a. Data compiled from Song and Guo (2014), Arikan et al. (2009), Cessna et al. (2011), Hou et al. (2015), Li et al. (2013), Martinez-Carballo et al. (2007), Ravindran and Mnkeni (2017), Xie et al. (2016), Zhang et al. (2015), and Zhao et al. (2010)

In the last 20 years, there has been increased interest in the concentrations of antibiotics found in animal manure. Studies have reported that 25 to 90% of administered veterinary antibiotics were excreted in an antimicrobial active form in urine and manure (Punamiya, Sarkar, Rakshit, Elzinga, & Datta, 2016). Concentrations found in manure and slurry samples have ranged from single digit µg/kg to over 800 mg/kg (Table 1).

2.4.2 Hormones

Livestock naturally generate, metabolize, and excrete hormones depending on the animal's gender and reproductive state (EPA-NRMRL, 2004). In addition, hormones are used extensively in veterinary medicine to treat sick animals, to treat reproductive

disorders, to improve feed efficiency, and as growth promoters (Bartikova et al., 2016). The USDA has approved the use of estrogens (17β-estradiol, zeranol), androgens (testosterone, trenbolone acetate), and progesterones (progesterone, melegestrol acetate) for veterinary use in cattle as an ear implant or in feed (EPA-NRMRL, 2004).

Livestock urine and manure contain a combination of naturally generated hormones and metabolized veterinary hormones. An estimated 2300 tons of steroid hormones are excreted each year by livestock (Lange et al., 2002). Table 2 summarizes by hormone class the concentration ranges that have been found by manure type. Numerous studies have detected hormone concentrations in the μg/kg to the mg/kg range.

Table 2: Common hormone classes and concentrations found in manure

		Concentrations found in manure/slurry samples (mg/kg)[a]		
Hormone class	Sample of drugs from Class	Swine	Poultry	Beef/Dairy
Estrogens	estrone, 17α-estradiol, 17β-estradiol	0.024 - 0.593	0.002 - 0.004	0.014 - 1.46
Androgens	dihydroestosterone, androstenedione, testosterone	0.072 - 0.497	0.133 - 0.670	0.002 - 0.100
Progesterones	pregnenolone, progesterone	0.003 - 1.271	0.180 - 0.391	0.011 - 0.030

a. Data compiled from Bartelt-Hunt et al. (2013), Derby et al. (2011), Hansen et al. (2011), Bin Ho et al. (2014), Hutchins et al. (2007), Khan et al. (2008), Rodriguez-Navas et al. (2013), Shore et al. (1993), Zheng et al. (2008)

Estrogens are an importance class of hormones because they can be found in high concentrations in manures and because they are considered to be highly potent hormone-active compounds that can affect aquatic life at low levels (Goeppert, Dror, & Berkowitz, 2014). Although 17β-estradiol is administered to animals as an implant, it quickly metabolizes to estrone and estradiol. Geoppert et al. (2014) found 17β-estradiol was able to transform to estrone both abiotically or through microbial activity, and that once transformed, estrone was more persistent than 17β-estradiol.

2.4.3 Other Pharmaceuticals

Numerous other classes of pharmaceuticals are administered to livestock for therapeutic, and not growth promotion, purposes. Kools et al. (2008) extrapolated the annual use of therapeutic drugs for E.U. countries in 2004 based on pharmaceutical sales data. Of the 6051 tons of veterinary medicines used in 2004, 194 tons were antiparasitics, 221 tons were active substances used to treat the alimentary tract and metabolism related disorders, 120 tons were central nervous system active substances, 60 tons were substances used for blood and blood forming organs, and 52 tons were for the muscles and skeleton related disorders. Other drug classes include non-steroidal anti-inflammatory drugs (NSAID), tranquilizers, enteric bloat preparations, antacids, and diuretics (Bartikova et al., 2016). While numerous studies are published for concentrations of antibiotics and hormones found in manure, the other therapeutic drugs have been studied to a lesser degree. However, one study by Weiss et al. (2008) found concentrations of the antiparasitic drug flubendazole in liquid swine manure ranging from 26 – 56 μg/L.

2.4.4 Emerging Contaminants From Reclaimed Municipal Wastewater

Water shortages in dry states such as Colorado are creating a growing need for sustainable agricultural practices such as the use of reclaimed water from municipal wastewater treatment plants. The reclaimed water can be used as a water source for AD or for crop irrigation; however, this can introduce additional contaminants not removed by wastewater treatment plants. Classes of contaminants typically found in reclaimed water include pharmaceuticals such as non-steroidal anti-inflammatory drugs, blood lipid regulators, antidepressants, and antibiotics. In addition, compounds found in personal care products or cleaning supplies containing disinfectants have also been detected (Caracciolo,

Topp, & Grenni, 2015). Kosma et al. (2014) tested wastewater from eight wastewater treatment plants and found concentrations of lipid regulators, antiepileptics, steroids, and disinfectants in the ng/L range, while antibiotics and anti-inflammatory drugs were found in the low µg/L range. Salicylic acid had the highest measured concentrations (89.1 µg /L) of ECs found in wastewater influent (Kosma et al., 2014).

2.5 Environmental Impact of Land Applied Livestock Waste

The method of spreading manure, compost, anaerobic digestate, or wastewater onto fields is a cost-effective method of disposing of waste. However, the emerging contaminants found in the waste products can impact the environment by interacting with soil microbial communities, by leaching into ground and surface water, and by interacting with aquatic life. The worldwide use of veterinary pharmaceuticals in confined animal operations has resulted in an estimated discharge of 3,000 to 27,000 tons of drug chemicals into the environment per year (Song, 2014). In addition, there is a potential risk to human health due to the spread of antibiotic resistance in microbes or from prolonged exposure to low concentrations of ECs in food crops and drinking water.

2.5.1 Impacts on Soil

There has been a growing concern about the risk of ECs leaching from soils that have been fertilized with EC contaminated livestock waste; therefore, many research projects have focused on determining concentrations of ECs that can be found in soils. Awad et al. (2014) found concentrations of tetracyclines ranging from 0.01 µg/kg to 46.34 µg/kg in soil, and sulfonamides ranging from 0.04 µg/kg to 0.61 µg/kg. In a review article, Wang et al. (2016) found examples of concentrations at higher levels in various soils around China (9.1 µg/kg to 2860 µg/kg) with oxytetracycline having the highest measured

concentrations. Other studies have looked at soils fertilized with municipal wastewater or sludge. Antibiotics such as ciprofloxacin, norfloxacin, and sulfadiazine were found at concentrations up to 450 μg/kg soil and NSAIDs such as diclofenac and ibuprofen were at concentrations up to 5 μg/kg (Verlicchi & Zambello, 2015). In addition, persistence of ECs in soils is greatly dependent on soil and compound properties.

ECs found in soil can pose risks other than leaching into the environment. Many studies have been done to determine EC concentrations that impact soil microbial communities and the health of crops grown in contaminated soils. Soil microbes play a vital role in the health of soil ecosystems. Microbes are important degraders of organic matter such as plant residue, and they are essential for nutrient recycling (Carvalho, Basto, Almeida, & Brix, 2014). Microorganisms are also involved in ecosystem self-purification processes, with biodegradation considered to be the more important process for contaminant removal. However, removal of contaminants is only possible if the toxicity of ECs does not inhibit microbial activity (Caracciolo et al., 2015). Studies on soil microbial diversity have shown that concentrations of sulfonamide antibiotics as low as 1 μg/kg to 900 μg /kg can impact biomass and microbial community structure in soils (Caracciolo et al., 2015). Other studies have focused on the impacts antibiotics have on plant growth, although it is not known if negative impacts to plants are due to inhibition of microbial activity or from direct damage to the plants themselves. A review article by Carvalho et al. (2014) summarized toxicity levels of antibiotics for a variety of crop plants. While many antibiotics were only toxic at levels much higher than concentrations typically found in soil, others where toxic well within normal ranges. Sulfamethoxazole and chlortetracycline were toxic to alfalfa at concentrations of > 10 mg/kg and > 5 mg/kg respectively, while

chlortetracycline had toxic effects at levels as low as 7.2 µg/kg. Toxicity was also plant dependent, as sulfamethoxazole and chlortetracycline were found to have toxic effects on carrots starting at 2.5 µg/kg and 65 µg/kg.

2.5.2 Plant Uptake

Several studies have shown that antibiotics have the ability to accumulate in crops that are grown in EC contaminated soils. Ahmed et al. (2015) tested tetracyclines and sulfonamides and showed high levels of the antibiotics in the roots, leaves, and other nonedible parts of the plant to be as high as 94.6% of the total addition (5 mg/kg) to the soil. Wang et al. (2016) found sulfamethoxazole and doxycycline accumulation in both radishes and pak choi in the range of 7.3 – 221.5 µg/kg. Another study of plants grown in chlortetracycline and tylosin-contaminated swine manure (0.58–1.58 mg/kg soil) found that while chlortetracycline accumulated in plant tissues (2–17 µg/kg fresh weight) tylosin did not, indicating uptake is dependent on EC chemical properties (Kumar, Gupta, Baidoo, Chander, & Rosen, 2005).

The extent of contaminant uptake is governed by the relationship between EC concentrations in soil, EC chemical properties, soil properties and plant type. Studies have shown that concentrations in plant tissues tended to increase as concentrations in land applied animal manures increased (H. B. Zhang et al., 2015). In addition, if ECs act as weak acids or bases, the soil pH will impact the charge on the compound and determine if root uptake will occur at a moderate rate due to electrical attraction or more quickly due to hydrophobic sorption (X. Q. Wu, Ernst, Conkle, & Gan, 2013). Hydrophobicity (usually expressed as log K_{ow}) is believed to be the most important property in determining plant uptake and the distribution of ECs throughout the plant (J. M. Wang et al., 2016).

Hydrophobic contaminants tend to accumulate in plant roots while hydrophilic compounds are more easily translocated through a plant by water movement due to plant transpiration (X. Q. Wu et al., 2013). Tuber plants also are believed to have an increased risk in EC accumulation from diffusion through the tuber skin. One study found sulfamethazine accumulation in a potato with concentrations decreasing when moving from the peel to the center of the potato (Carvalho et al., 2014).

Overall, the estimated dietary intake by humans consuming plants grown in EC contaminated soils has been found to be negligible. Wu et al. (2013) studied the concentrations of over 20 ECs found in lettuce and spinach and estimated the annual human consumption of contaminants to be less than 70 μg for all ECs tested. However, additional research needs to be done to determine health risks associated with long-term consumption of these drugs at low levels.

2.5.3 Transport to Ground and Surface Water and Impact to Aquatic Life

Leakage from livestock waste storage and runoff from manure-fertilized fields are two sources of ground and surface water contamination. The transport of ECs through the ground is dependent on EC chemical properties and their interaction with clay minerals and organic matter in soil (Song, 2014). The strength of EC and soil interaction is also influenced by temperature, moisture, and the soil solution chemistry (Kumar, Gupta, Chander, et al., 2005). Two parameters used to predict sorption include the distribution coefficient K_d (the ratio of the quantity of compound sorbed per unit mass of soil to the amount of the compound remaining in solution at equilibrium) and K_{oc} (K_d normalized to the organic fraction of the soil). Compounds with increased aromaticity and electropolarity tend to have higher K_d and K_{oc} values, which usually indicates greater sorption (Song,

2014). K_d values for common classes of ECs include 420 – 1030 L/kg for tetracyclines, 7.5 – 600 L/kg for sulfonamides, and 3 – 255 L/kg for estrogens (Roberts, Higgins, & McCray, 2014; Song, 2014). Other soil properties such as soil pH impact the charge of the ECs and how well they sorb to soils (Bradford, Segal, Zheng, Wang, & Hutchins, 2008). Some ECs such as tetracycline antibiotics also tend to form complexes with divalent cations (Mg^{2+}, Fe^{2+}, Zn^{2+} and Ca^{2+}) found in soils. These complexes tend to block sorption and can increase compound mobility to rates faster than would be predicted by the K_d value (Solliec et al., 2016).

Although studies have confirmed that ECs have been detected in streams and aquifers near animal production operations (Bradford et al., 2008), persistent contaminants have also been measured at low levels in surface and ground water across the US. Kim and Carlson (2007) measured tetracyclines (4.5 to 32.8 μg/kg) and sulfonamides (1.2 to 3.4 μg/kg) in sediment samples collected from the Cache la Poudre River in northern Colorado at sites near farms and wastewater treatment plants. Recent USGS surveys found widespread dissemination of antibiotics in surface and ground waters throughout the U.S. Antibiotics were detected in 48% of samples taken from 139 streams across 20 states (Bradford et al., 2008). Another survey detected antibiotics in 81% of the tested groundwater from 47 sites across 18 states, with sulfamethoxazole the most frequently detected compound (Richardson, 2009).

Hormones and NSAIDs have also been detected in surface waters and have been shown to negatively impact aquatic plants, invertebrates and vertebrates. In a review paper, Mohapatra et al. (2016) summarized biological effects due to NSAIDs ranging from growth reduction, gill damage, and embryo mortality among different fish species, to

problems with reproduction and larval development in crustaceans and mussels. Hormones have also been known to physiologically affect fish and aquatic vertebrate species (Goeppert et al., 2014). Exposure to estrogens at concentrations as low as 25 ng/L of estrone and 1ng/l of 17β-estradiol (EPA-NRMRL, 2004) have been implicated in fish fertility changes, increases in the number intersex fix, and increases in the female to male ratio in fish (N. A. Zhou, Lutovsky, Andaker, Gough, & Ferguson, 2013).

2.5.4 Antibiotic Resistance

One of the greatest risks to human and livestock health from the release of antibiotics into the environment is the possible increase in antibiotic resistant microbes. Low-level concentrations of antibiotics can kill or inhibit the growth of non-resistant microbes, which allows for resistant microbes to flourish and become dominant (Song, 2014). This can happen within the gut of antibiotic-administered livestock, in animal waste storage, in soils fertilized with manure, or in ground and surface waters (Storteboom et al., 2007). Additionally, antibiotic resistant genes (ARGs) can be transferred (usually as plasmids) from microorganism to microorganism, by bacteriophages, or they can be released into the environment after cell lysis. Microbes that encounter these plasmids in the environment can take up the plasmids through their cell membranes via transformation (EPA-NRMRL, 2004). Recent studies have shown ARG persistence in stored manures and on fields spread with manure (Joy et al., 2014; Peng, Wang, Zhou, & Lin, 2015). Mounting evidence suggests that the release of antibiotic residues, ARGs, and resistant bacteria into the environment increases the risk of multi-resistant animal pathogens, reducing the effectiveness of antibiotics (Lin et al., 2017).

2.6 Degradation Studies for Emerging Contaminant

Most veterinary pharmaceuticals have been found to be biodegradable (Dolliver, Gupta, & Noll, 2008), therefore much research has been focused on determining the effectiveness of biological treatment methods such as AD and composting at reducing or removing ECs from animal waste.

2.6.1 EC degradation during anaerobic digestion

A wide range of EC removals have been reported in literature for AD systems. Table 3 summarizes degradation rates found in literature for ECs during livestock or municipal waste AD. Estrogenic hormones have generally been found to be persistent under anaerobic conditions (Zheng, Li, Yates, & Bradford, 2012). Noguera-Oviedo et al. (2016) reported that while total hormone concentrations (estrone, 17α-estradiol, 17β-estradiol, estriol) had no significant removal in a full-scale dairy farm AD, the relative abundance of the hormones changed. Estrone increased from 20% relative abundance of total estrogens in raw manure to 80% after digestion. Tetracycline and sulfonamide antibiotics removal percentages have been reported over a wide range from no removal to 99% removal (Table 3). Non-steroidal anti-inflammatory removals ranged from 5% to 93% (Carballa, Omil, Ternes, & Lema, 2007; Samaras, Stasinakis, Thomaidis, Mamais, & Lekkas, 2014). Variations in AD operating conditions and microbial communities are most likely responsible for the wide range of EC removals.

Most EC removal studies were conducted at temperatures typical in mesophilic (35°C – 40°C) or thermophilic (55°C – 60°C) AD reactors. Oxytetracycline consistently had moderate removals (38% to 75%) from swine and cattle manure under mesophilic conditions (Akyol, Ince, Cetecioglu, Alkan, & Ince, 2016; Alvarez, Otero, Lema, & Omil, 2010;

O. A. Arikan et al., 2006). Varel et al. (2012) reported that chlortetracycline had little removal (7%) at 22°C, but high removal in the mesophilic and thermophilic range (80% to 90%). Arikan et al. (2008) reported similar removal of chlortetracycline (75%) under mesophilic conditions. Few of the above studies included abiotic controls so it is not possible to determine if degradation was due to microbial degradation or temperature related abiotic degradation. However, Withey et al. (2016) ran water-only controls and found similar chlortetracycline removal between the water-only reactors and the lab-scale

Table 3: Fate of ECs during AD of manure or wastewater

Drug	Test Condition	Duration (days)	Temp (°C)	Initial Concentraiton (mg/kg)	Removal %	Reference
Antibiotics						
Sufonamides						
Sulfadiazine	lab scale batch reactor, swine manure	14	35	6.0	90%	Chu et al., 2017
Sulfathiazole				6.5	86%	
sulfamethazine				6.3	75%	
sulfadiazine	lab scale batch reactor, pig manure	40	15, 52	~ 0.6	none, none	Feng et al., 2017
sulfamethizole				~ 0.7	26%, none	
sulfamethoxazole				~ 0.5	98, 98 %	
sulfamethoxazole	lab scale sequencing batch reactor, wastewater	90	35	25	37%	Aydin et al., 2014
sulfamethazine	lab scale batch reactor, cattle manure	40	37	0.28–280	none	Mitchell et al., 2013
Macrolides						
clarithromycin	lab scale batch reactor, pig manure	40	15, 52	~ 0.6	33, 36%	Feng et al., 2017
erytrhomycin					20, 99%	
Tetracylines						
	steady state reactor, cattle manure					Akyol et al., 2016
oxytetracycline	one-stage, lab scale	60	37	3.2	38%	
oxytetracycline	two-stage, lab scale				48%	
oxytetracycline	lab scale batch reactor, calve manure	64	35	9.8	59%	Arikan et al., 2006
chlortetracycline	lab scale batch reactor, calve manure	33	35	9.9	75%	Arikan, 2008
oxytetracycline	lab scale batch reactor, pig manure	21	35	10, 50, 100	58, 53, 68%	Alvarez et al., 2010
chlortetracycline				10, 50, 100	91, 91, 90%	
chlortetracycline	lab scale batch reactor, swine manure	21	22, 38, 55	8.3	7, 80, 99%	Varel et al., 2012
Hormones						
estrone	full scale AD system, dairy manure	22	37	0.007	negligible	Noguera-Oviedo, 2016
megestrol	lab scale batch reactor, cattle manure	28	55	100	80%	Withey et al., 2016
17α-estradiol	lab scale batch reactor, dairy lagoon	52	35	5	15%	Zheng et al., 2012
17β-estradiol	water			5	22%	
estrone				5	33%	
NSAID						
naproxen	lab scale continuous fed digesters, wastewater, 30 day SRT	5 months	37, 55	0.01	82, 93%	Carballa et al., 2007
ibuprofen					30, 40%	
diclofenac					5, 30%	
naproxen	lab scale continuous fed digesters, wastewater, 20 day SRT	133	37, 55	2.83	79, 76%	Samaras et al., 2014
ibuprofen				2.83	93, 90%	
diclofenac				1.97	93, 93%	

AD reactors when operated at 55°C, but only minimal removals with 25°C water-only reactors. This likely indicates that abiotic thermal degradation is in part responsible for chlortetracycline removal during AD. EC removal trends relating to temperature are less clear for other ECs such as sulfonamide antibiotics. Feng et al. (2017) reported no removal of sulfadiazine over 40 days of anaerobically digesting swine manure at both 15°C and 52°C. Mitchell et al. (2013) also had no removal of sulfamethazine from cattle manure at 37°C. In contrast, Chu et al. (2017a) reported 90% removal of sulfadiazine and 75% removal of sulfamethazine from swine manure after 14 days of AD at 35°C. It is possible that degradation rates between these two studies differed due to variations in microbial community composition, which was not examined as part of either research study.

Fewer studies were found that investigated the impact of other operational conditions (such as pH and moisture content) on EC removal in AD systems. Although Chu et al. (2017b) did not directly study the impact of pH and moisture content on chlortetracycline removal, the authors conducted a single factor linear regression and determined that chlortetracycline removal and moisture content had good correlation (with higher chlortetracycline removal at higher moisture content). Chu et al. (2017b) found no correlation between pH levels and chlortetracycline removals. Another study by Akyol et al. (2016) compared oxytetracycline removal between one and two-phase AD systems. Lab scale steady state reactors were used to digest oxytetracycline spiked cattle manure. The single-phase system had a solid retention time (SRT) of 20 days, while the acidogenic and methanogenic digesters of the two-phase system had 5 and 15 day SRTs, respectively. Although oxytetracycline removal was higher in the two-phase system (48%) versus the one-phase system (38%), the results did not specify whether the reduction in

oxytetracycline was due to degradation, mineralization, or sorption. Therefore, additional research is needed to understand the mechanisms that influence EC degradation under anaerobic conditions.

2.6.2 EC degradation during composting

Degradation rates are typically high for common ECs during composting. Wu et al. (2011), found the removal of CTC and OTC to vary between 74% and 94% in swine manure. Dolliver et al. (2008) and Arikan et al. (2007) both found half-lives for tetracycline antibiotics to be between 1 to 3 days when composting manure. Naproxen had a half-life of 14 hours in a bench scale study with activated sludge (Suzuki, Kosugi, Hosaka, Nishimura, & Nakae, 2014). Table 4 lists removal efficiencies found in literature for composting and other aerobic systems.

Table 4: Fate of ECs during aerobic composting of manure or wastewater

Drug	Test Condition	Duration (days)	Temp (°C)	Initial Concentraiton (mg/kg)	Removal %	Reference
Antibiotics						
Sufonamides						
Sulfamethoxazole	lab-scale manure/straw	35	20 - 55	22.8	> 99 %	Liu et al., 2015
Sulfamethazine				24.0	> 99 %	
Sulfamethoxazole				22.8	68%	
Sulfamethazine				24.0	60%	
Sulfamethoxazole	batch reactors, activated sludge	2	22	0.1	23 - 39%	Li et al., 2010
Sulfamethoxazole	SMX degrading bacterial suspensions		28	100	80%	Hu et al., 2017
			43	100	40%	
Ionophores						
monensin	lab scale batch reactors, dairy manure	84	22, 65	6.8	95%, 85%	Arikan et al., 2016
lasalocid				10.3	35%, 40%	
salinomycin				10.0	>90%, >90%	
Tetracylines						
Chlortetracycline	swine manure + mushroom residue, windrow	52	55		74%, 8.25 days	Wu et al., 2011
Oxytetracycline					92%, 1.14 days	
tetracycline					70%, 10.02 days	
Oxytetracycline	managed compost pile	35		123.3	80%	Ravindra et al, 2017
Chlortetracycline		40	40 to 60	1.5	>99%	Dolliver et al., 2008
Chlortetracycline	laboratory composters, calve manure	30	25, 55	113	49%, 99%	Arikan et al., 2007
Hormones						
Estrone	compost pile, beef manure	40	30 to 60	0.15	74%	Bartelt-Hunt et al., 2013
Estrone	compost pile, swine manure	36	10 to 50	0.03	75 to 82%	Derby et al., 2011
Estrone	composting of municipal sludge	92	35	0.54	>99%	Butkovskyi et al., 2016
NSAID						
Ibuprofen	composting of municipal sludge	92	35	0.48	>99% by day 92	Butkovskyi et al., 2016

Temperature is believed to be one of the most important factors impacting degradation of ECs during composting (K. R. Kim et al., 2012) which has temperatures that range from air temperature up to 70°C in piles that are turned or aerated. Chai et al. (2016) compared the removal of tetracycline antibiotics between a compost pile that reached 50°C and one that was incubated at 25°C. The pile with the elevated temperature had near complete removal of tetracyclines within 14 days while the 25°C control only had 73% removal after 49 days. Sulfamethoxazole extraction efficiency is also influenced by temperature, except it experiences higher removal at lower temperatures. Hu et al. (2017), tested sulfamethoxazole removal over a range of temperatures and found that optimal removal occurred at 28°C. Similarly, Zheng et al. (2013) tested removals of estradiol between 15°C and 45°C and found that the shortest half-lives occurred at 35°C.

To a lesser extent, pH has been studied in aerobic treatment systems. Tadkaew et al. (2010) found higher removal of sulfamethoxazole between pH 5 and pH 7, however, removal rates dropped to 70% at pH 9. Hu et al. (2017) tested removal rates between pH 2.5 and pH 6.5 and found sulfamethoxazole removal decreased with increasing pH. Sorption is a mechanism that is closely related to pH. Depending on the EC chemical properties, the compounds can have a charge at different pH levels. Negatively charged particles tend to be repelled by negatively charged sorption sights and are therefore more likely to stay in solution. Many studies have found sulfamethoxazole to have little sorption at pH levels common in compost or in aerobic reactors (Feng et al., 2017; Larcher & Yargeau, 2012; B. Li & Zhang, 2010; X. Zhang et al., 2014).

2.7 Microbial Identification Studies

As described in the previous section, microbial degradation is believed to be an important contributor to EC removal from livestock waste. In the last decade, several studies have begun to identify EC degrading microbes from compost, soil, anaerobic digesters and wastewater treatment plants. Chang et al. (2014) isolated tetracycline resistant microbes from swine manure and then cultivated them individually in a media with oxytetracycline, chlortetracycline, and tetracycline as the sole carbon sources. Five microbes were able to successfully degrade the tetracyclines (*Inquilinus* sp., *Xanthobacter flavus*, *Runella* sp., *Afipia genosp*, and *Starkeya* sp.). Ma et al. (2016) isolated two bacteria from activated sludge (*Sphingomonas* sp. and *Sphingobium* sp.) that were able to degrade estrone and 17ß-estradiol as the sole carbon and energy source (2 mg/L) at 30 °C. *Mycobacterium* and *Pseudomonas* were also identified as hormone degraders, specifically for estrone and testosterone (Isabelle, Villemur, Juteau, & Lepine, 2011; Y. Y. Yang, Borch, Young, Goodridge, & Davis, 2010). Some microbes were found to partially degrade sulfamethoxazole to degradation products (Larcher & Yargeau, 2012), while one study was able to confirm the complete mineralization of radioactively labeled sulfamethoxazole by measuring radioactivity in captured CO_2 (Bouju, Ricken, Beffa, Corvini, & Kolvenbach, 2012). *Bacillus* has been found to degrade numerous ECs including SMX, OTC, progesterone, estrone, and naproxen (Ravindran et al., 2017; Zhou et al., 2013).

Research has also focused on identifying conditions that are optimal for microbes that degrade ECs. Some microbes have been found to degrade ECs only when a co-substrate such as glucose is present (Larcher & Yargeau, 2012). Zhou et al. (2013) studied the ability of a wide range of substrates (proteins, sugars, or alcohols) to support the

growth of isolated EC degrading cultures. More recently, Hu et al. (2017) isolated *Enterobacter cloacae* from swine manure and measured SMX degradation under various pH and temperature conditions. The optimal temperature for *Enterobacter cloacae* was 28 °C (degradation decreased at higher and lower temperatures), while lower pH levels had the best degradation (tested range was pH 2.5 to pH 6.5). Additional research is still needed in this area to understand how the degradation results of isolated microbes will translate to results in more complex microbial communities and more complex growth matrices.

2.8 Analytical Chemistry Methods

Liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) is the preferred method for measuring ECs in animal waste and compost because of its ability to quantify, and identify compounds (L. J. Zhou et al., 2012). Triple–quadrupole and quadrupole-time-of-flight are the most widely used tandem mass spectrometers (Yu & Wu, 2012). The high sensitivity of an LC-MS/MS system allows for limits of detection (LODs) that are low enough to measure ECs in manure and in environmental samples. Table 5 lists LC-MS/MS LODs for common ECs found in recent studies.

Table 5: LC-MS/MS limits of detection (LODs) from recent studies

Compound	Matrix	LOD (µg/kg)	Study
Sulfamethoxazole	Manure	1.6 – 3.6	Lin et al. (2017)
	Manure/compost	0.35	Zhang et al. (2015)
Sulfadiazine	Compost	4.3	Shi et al. (2016)
Oxytetracycline	Compost	3.8	Shi et al. (2016)
	Manure/compost	0.44	Zhang et al. (2015)
Chlortetracycline	Manure	4.66	Wang et al. (2015)
	Manure/compost	2.29	Zhang et al. (2015)
Estrone	Lagoon water	40	Zheng et al. (2013)
Naproxen	Sludge	2.95	Martin et al. (2015)

Samples in complex media like manure or compost typically require extraction and cleanup before analysis with LC-MS can be performed (Song, 2014). Methods and extraction solutions vary greatly in the literature, however most methods follow similar steps which include: combining samples with an extraction solution, extracting analytes through vigorous shaking or sonication, and separation of sample solids and extraction solution through centrifugation. Extractants can further be concentrated and purified by using a combination of evaporation, filtering or solid phase extraction (SPE).

Extraction solutions consisting of weakly acidic buffers and organic solvents are recommended to recover residual antibiotics from manure samples (Song, 2014). Methanol and acetonitrile are commonly used as the organic solvent for a wide range of pharmaceuticals including antibiotics, hormones, and NSAIDs (Kumirska, Migowska, Caban, Lukaszewicz, & Stepnowski, 2015; C. X. Wu, Spongberg, & Witter, 2008; Yuan, Wang, Yates, & Peterson, 2010). Most extraction solutions include acids such as acetic acid (Liu, Yang, Sun, Zhao, & Liu, 2018; C. X. Wu et al., 2008; Zhao et al., 2010), formic acid (C. W. Yang, Hsiao, & Chang, 2016), or McIlvaine buffer (citrate-phosphate buffer)(Chang et al., 2014; Ravindran & Mnkeni, 2017; Shi et al., 2016). However, some studies have extracted pharmaceuticals in pure methanol when extracting ionophore antibiotics from dairy manure (O. A. Arikan, Mulbry, & Rice, 2016). Tetracyclines in particular are sensitive to the pH level during extraction. Yuan et al. (2010) found that almost no oxytetracycline was extracted from manure when acids were excluded from the extraction solvent, but when the pH was decreased to 4.23, oxytetracycline extraction recovery reached 39%. Yuan et al. (2010) speculated that the lower pH helped oxytetracycline to protonated and become less adsorptive to sample media. Yuan et al. (2010) also studied the effect of methanol to water

ratio on oxytetracycline extraction and found the efficiency was least (30%) with a ratio of 20/80 and highest (over 50%) with a ratio of 80:20. Ethylenediaminetetraacetic acid (EDTA) is commonly added to extractions solutions, especially when tetracyclines are extracted. EDTA forms complexes with metals that would otherwise interact with tetracyclines, and reduce extraction recoveries (Tso, Dutta, Inamdar, & Aga, 2011). Wu et al., (2008) did a study comparing seven different combinations of methanol, acetonitrile, phosphate buffer, acetic acid, and EDTA and found that a methanol:acetic acid:EDTA(2:1:1) extraction solution had the greatest extraction recovery of antibiotics from municipal biosolids.

Extraction methods also vary in mixing technique, the number of extractions, and sample cleanup. Extraction is typically done using sonication, mechanical shaking, or some combination of the two. Yuan et al. (2010) found that 30 minutes was the optimal shaking time when extracting oxytetracycline from manure. Other extraction methods such as accelerated solvent extraction, and microwave-assisted extractions have been used to improve extraction efficiency (Song, 2014; H. Z. Wu et al., 2015). Multiple extractions of the same sample are common in the literature, however studies have also been done with only single extractions (Butkovskyi, Ni, Leal, Rijnaarts, & Zeeman, 2016; C. W. Yang et al., 2016; Zheng et al., 2013). Extractants can be concentrated for easier detection by evaporating under nitrogen and can be cleaned through filtration. More commonly, samples are concentrated and purified using SPE, which uses the affinity of the dissolved analytes for a solid to isolate the analyte from background compounds. Therefore, proper SPE selection is dependent on analyte and media chemical properties (Yu & Wu, 2012). However, SPE

can be expensive and time consuming, especially if a large number of samples will be analyzed.

2.9 Summary

The livestock industry in dry states such as Colorado has a need for the advancement and application of low water and sustainable agricultural practices to help with management of livestock waste. The shift to larger and more concentrated feeding operations exacerbates these problems as high volume waste production exceeds the land application capacity of surrounding croplands. Current technologies such as composting and AD help to reduce waste volume, and remove pathogens, while providing costs benefits such as producing a high quality marketable fertilizer or energy production with AD. MSAD is a more advanced AD technology that improves methane production and can be used as a low-water technology when leachate is recirculated. Additionally, the MSAD system is capable of being converted to an in-vessel composting system after the AD phase is complete allowing for more stabilized waste prior to land application. AD and compost have also been shown to reduce or remove many ECs found in manure such as antibiotics, hormones, and other veterinary pharmaceuticals. By removing ECs prior to land application, contamination of soil, groundwater and surface waters can be reduced and impacts to the environment and aquatic species can be minimized. In the last few decades, advances in analytical chemistry methods have allowed researchers to measure low concentrations of ECs in water or complex media types. In addition, recent studies have started to focus on identifying phylogenic profiles of AD and compost microbial communities and to identify microbes capable of degrading ECs. However, few have identified the AD operational conditions or compost phases under which EC degrading

compounds thrive. Also, most EC degradation research to date has been for either compost or AD, therefore, more research on EC degrading microbes and optimal system conditions is required for combined systems such as MSAD with a final composting phase.

3: EMERGING CONTAMINANT DEGRADATION UNDER VARIOUS OPERATING CONDITIONS

3.1 Introduction

The shift in the U.S. livestock industry from small farms to large-scale concentrated animal feeding operations (CAFOs) has given rise to two challenges: how to cost effectively dispose of large quantities of generated waste, and how to minimize the environmental impact of the growing use of veterinary pharmaceuticals. A common method of livestock waste disposal is through application to cropland as a fertilizer. However, this method is becoming less cost-effective due to higher transportation costs as the scale of waste generated by CAFOs overwhelms the capacity of adjacent land (EPA-NRMRL, 2004). In addition, the land application of manure creates a pathway for veterinary pharmaceuticals and other emerging contaminants (ECs) to be released into the environment through runoff into surface waters or by leaching into groundwater (Richardson, 2009; X. Zhang et al., 2014). Annual antibiotic use on livestock is estimated to be 2.5 million kilograms in the United States (EPA-NRMRL, 2004) and as much as 90% of the administered antibiotics (as the parent compound or their metabolites) are excreted in animal waste (Larcher & Yargeau, 2012). Recent studies have found these ECs can negatively impact soil microorganisms (Caracciolo et al., 2015), and can be taken up into crop roots or plant tissues (Ahmed et al., 2015; J. M. Wang et al., 2016). The negative impacts on terrestrial and aquatic ecosystems have also been widely reported (Goeppert et al., 2014; Mohapatra et al., 2016), and antibiotics in the environment have been linked to increases in quantities of antibiotic resistant bacteria (Joy et al., 2014; Peng et al., 2015). In addition the potential

negative impact on human health from routinely consuming these contaminants at very low levels in food and in water are unknown at present. Since land application of manure is a valuable means of waste disposal and soil enrichment, it is essential that cost effective technologies are developed and optimized to reduce livestock waste and the concentrations of ECs prior to land application.

Manure management technologies such as composting and anaerobic digestion (AD) have many benefits including: waste volume reduction (as high as 30 to 50%), odor reduction, killing of pathogens (L. Chen et al., 2011), and cost benefits from energy production with AD systems (Bernet & Beline, 2009). When livestock manure has been co-digested with food waste, methane production has more than doubled (C. S. Zhang et al., 2013) providing even more cost benefits. However, traditional AD has a disadvantage in dry states such as Colorado because it is a water intensive technology. A more advanced AD technology known as multi-stage AD (MSAD) combined with leachate recirculation allows for reduced water use plus it has other benefits such as shorter digestion time and greater methane production (El-Mashad, van Loon, Zeeman, Bot, & Lettinga, 2006). MSAD isolates the AD phases into three separate reactors so conditions can be optimized for different biological processes (hydrolysis, acidogenesis/acetogenesis, and methanogenesis), with the end products of each phase moving through the system in the recirculating leachate. Recent testing on the MSAD system demonstrated it was possible to convert the hydrolysis reactor to an in-vessel composting process by adding forced aeration after the AD phase was completed (Sandefur, 2017). This method (called a combined MSAD/AC system) was able to combine the benefits of in-vessel composting, such as faster composting rates and smaller land requirements, with the benefits of MSAD.

Over the last few decades many research projects have studied the impact of composting and AD on EC removal from animal waste. Research has shown that AD can leave residual amounts of up to 90% for tetracycline antibiotics (Akyol et al., 2016; Varel et al., 2012), 100% for sulfonamide antibiotics (Feng et al., 2017), and 100% for hormonal estrogens (Noguera-Oviedo & Aga, 2016). However, composting (an aerobic biological process) has been shown to remove estrone (between 75 and 100%) and other contaminants not removed during AD (Bartelt-Hunt et al., 2013; Butkovskyi et al., 2016). Therefore, another advantage to the combined AD/AC system is the ability to remove contaminants that are persistent during AD.

The objectives of this project were to determine: 1) which operating conditions lead to the greatest biotic and abiotic degradation rates of five ECs, and 2) identify microbial communities that originate from AD digestate that have the ability to degrade ECs under aerobic conditions. To achieve these objectives, inocula were obtained from a combined MSAD/AC system as it was transitioning from the anaerobic phase to the aerobic phase. In advance of the microbial degradation studies, microbial communities were pre-acclimated to a synthetic manure/food digestate feed in growth media under test conditions that were designed to mimic the temperature, pH, and carbon composition of different composting phases. Concentrations of ECs spiked into bench scale-reactors were measured over 29 days using an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) method developed as part of this work. Initial microbial community structures were analyzed for all reactor conditions based on next-generation sequencing of 16S rRNA genes.

3.2 Methods and Materials

3.2.1 Experimental design

This experiment was designed to measure the impact on EC degradation by altering various AD/AC operational parameters including: temperature, pH, and carbon source composition. The temperatures selected for this experiment were 35 °C, 45 °C, and 55 °C, which are common temperatures achieved during the four phases of thermophilic composting. As shown in Figure 2, the first phase includes the rapid increase in compost temperature (between 25 °C and 55 °C) as microbial activity releases heat. Next, the thermophilic stage can last a few days to weeks and is typically maintained at temperatures greater than 50 °C. The third phase includes a slow cooling of the compost pile until it stabilizes at 35 °C for the final curing phase (L. Chen et al., 2011). The pH of composting piles typically starts at pH 7 and then can increase as high as pH 9 due to the release of ammonia from microbial activity before dropping down again between pH 7 and 8 during curing (O. A. Arikan et al., 2009). Therefore, pH values of 7, 8, and 9 were selected for testing.

The last operational condition studied was carbon source composition. It is not uncommon for livestock manure to be co-digested with food waste as a method to increase methane production (C. S. Zhang et al., 2013). Therefore, a synthetic growth media was developed to approximately mimic the carbon and protein composition of digestate in a low-water AD/AC system with a mix of manure, bedding or other bulking agents, and food waste (Appendix A). Three different media recipes were developed (Table 6) to roughly represent concentrations of protein, carbohydrates (easily biodegradable carbon), and cellulose (more recalcitrant carbon) that could be found in digestate with differing solid

retention times (SRTs), ranging from shorter than normal to the long end of normal. Media names indicate the protein (p), carbohydrate (c), and cellulose (ce) concentrations (g/L) for each media recipe. The default setting used for the reactors was 45 °C, pH 8, with the p10:c7:ce23 (moderate level of easily degradable carbon) media. As one operational condition was tested, the other parameters were held constant at default levels. A summary of the test conditions can be found in Table 7.

Table 6: Protein and carbohydrates for carbon source composition test media

Media Name	Levels Easily Degragable Carbon	SRT Length	Protein	Carbohydrates	Cellulose
			---------- g/L (% change from) ----------		
p16:c13:ce23	High	Short	15.9	12.7	23
p10:c7:ce23	Moderate	~ 3 weeks	10.4 (-35%)	7 (-45%	23
p5:c1:ce18	Low	Long	4.7 (-70%)	1.3(-90%)	18 (-22%)

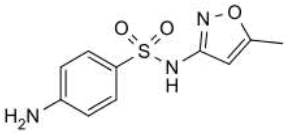
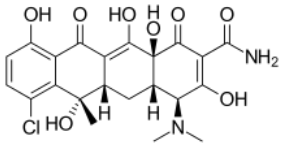
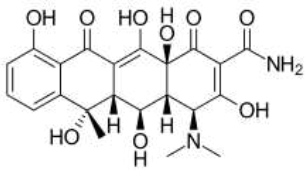
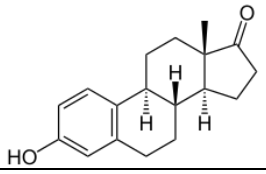
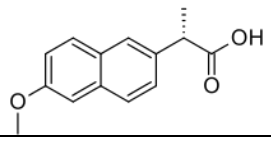
Table 7: Operational parameter settings for each test

Experiment	Temperature (°C)	pH	Media
Temperature	35, 45, 55	8	p10:c7:ce23
pH	45	7, 8, 9	p10:c7:ce23
Carbon content composition	45	8	p16:c13:ce23, p10:c7:ce23, p5:c1:ce18

Five ECs were selected for this study including three antibiotics, one hormone, and one non-steroidal anti-inflammatory drug (NSAID). Tetracycline antibiotics (oxytetracycline, chlortetracycline) and a sulfonamide antibiotic (sulfamethoxazole) were selected because they are commonly used worldwide as veterinary antibiotics (Sarmah et al., 2006). Estrone (E1) is an estrogen hormone that is naturally produced in livestock. E1 is also a metabolite of 17β-estradiol, which is frequently administered as an ear implant in cattle. Naproxen (NPX), an NSAID, and sulfamethoxazole (SMX) can be found in treated wastewater and can be introduced to livestock waste if municipal wastewater is used as a

water source for the low-water multi-stage AD/AC system described above. The five compounds were also selected as they have varying levels of removal during AD (Table 3) and would therefore be likely to be present during the AC phase of the MSAD/AC system. EC chemical structures and relevant chemical properties are summarized in Table 8.

Table 8: Emerging contaminant chemical properties

Drug	Chemical Structure	Log K_{ow}	pKa	Water solubility (mg/L)
Antibiotic				
Sulfamethoxazole		0.9	1.8, 5.7	610
Chlortetracycline		-0.62	3.3, 7.6, 9.3	630
Oxytetracycline		-1.22	3.2, 7.5, 8.9	1000
Hormone				
Estrone		2.95	10.4	30
NSAID				
Naproxen		3.2 – 3.3	4.15	15.9

Data compiled from Kasprzyk-Hordern et al. (2008), Roberts et al. (2014), and Zhou et al. (2012)

Starting drug concentrations were 200 μg/L for the temperature test, and 600 μg/L during the pH and carbon content composition tests. Degradation of oxytetracycline (OTC) and (chlortetracycline) CTC was rapid during the temperature test and variances based on temperature were difficult to determine. Therefore, a higher concentration was used for the later tests to facilitate observing clearer trends. Starting EC concentrations where selected within ranges commonly found in manure or slurry water. NPX levels are typically in the low ng/L levels; however higher concentrations were selected in order to measure degradation over two orders of magnitude above the detection limit. In addition, NPX concentrations could accumulate from wastewater that is re-circulated in an MSAD/AC system. Typical removal percentages as well as concentrations found in livestock waste or wastewater are listed in Table 3 and Table 4.

3.2.2 Chemicals and Standards

The five emerging contaminants analyzed in this study including oxytetracycline hydrochloride (OTC, CAS 2058-46-0), chlortetracycline hydrochloride (CTC, CAS 64-72-2), estrone (E1, CAS 53-16-7), sulfamethoxazole (SMX, CAS 723-46-6), and naproxen (NAP, CAS 22204-53-1) as well as the internal standard demeclocycline hydrochloride (CAS 64-73-3) were purchased from Sigma Aldrich (Milwaukee, WI). Analytical stable isotope-labeled compounds used as internal standards including naproxen-d_3 (α-methyl-d_3), estrone-2,4,16,16-d_4, and sulfamethoxazole-d_4 (benzene-d_4) were purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec). All stock solutions of the EC drugs were prepared by dissolving the drugs in LC-MS grade methanol and stored at -20 °C in the dark.

3.2.3 Bioacclimation of cattle manure digestate derived microbes

A combination of cattle manure and wood chip (added as a bulking agent) digestate was collected from the leachate bed reactors of a demonstration scale MSAD/AC at the end of a three-week AD phase as the system was being transitioned to aerobic operations. A schematic of the MSAD/AC system can be seen in Section 2.3, Figure 3, and the experiment is described in full in Sandefur (2017). Briefly, the leachate bed reactors consisted of six acrylic plastic columns (2'8" long and 8" diameter) with airtight caps on both ends. During the AD phase of operations, leachate was passed through the leachate bed reactor columns through tubing attached to the top caps, and allowed to drain from tubing attached to the bottom caps. For this experiment, cattle manure and wood chip digestate samples were collected from the top, middle and bottom of six columns and were combined into a glass storage container that was stored in the dark at -4 °C.

In an attempt to minimize the complexities of microbial community dynamics, the inoculum cultures were pre-acclimated to growth media and test conditions for at least 6 weeks prior to each experiment. One semi-continuous batch reactors was set up in 2-L flasks for each of the test conditions (a combined total of nine reactors for the three tests). Each reactor contained a combined 700 ml of sterilized growth media containing a synthetic manure/food digestate feed (see Appendix A), inoculum, and stock solutions of the 5 EC drugs. Inoculum for the three reactors was prepared by transferring 15 g of the mixed manure-woodchip digestate to a falcon tube along with growth media (up to 45 ml). The combined digestate and growth media was vortexed for 2 minutes and then 15 ml was added to each of the pre-acclimation reactors. All reactors were wrapped in foil to keep in dark conditions, kept at a constant temperature, and continuously mixed at 120 rpm to

maintain aerobic conditions. Every three to four weeks half of the volume of the reactors was replaced with fresh growth media that was spiked with EC drugs at starting level concentrations. This method ensured the EC drug concentrations would not accumulate to higher than starting level concentrations.

3.2.4 Reactor setup and operation

Acclimated cultures were then used to inoculate biodegradation experiment reactors as shown in Figure 4 (illustrated using the temperature test conditions as an example). Triplicate batch reactors were set up in 250-mL flasks containing 100 mL of the growth media described above for each of the operational levels tested. All glassware was pre-rinsed in 1 M HCl for 8 hours before use. EC drugs were spiked into reactors at starting concentrations of 200 µg/L for the temperature test and 600 µg/L for the pH and carbon source composition tests. Methanol from the drug stock made up less than 0.5% of the total reactor volume to minimize impact to reactor microbes. In order to measure abiotic losses, triplicate killed controls were prepared by autoclaving reactors at 121°C for 30 min before spiking with EC drugs.

Starting biomass was normalized among reactors based on DNA concentrations for inocula. Other typical methods for measuring biomass concentration, such as optical density and protein concentration were not possible due to the high concentrations of cellulose particles and protein from the growth media. DNA samples (1.2 ml) were collected from each of the pre-acclimation reactors and then centrifuged for 5 minutes (10,000 x g). The supernatant was removed, and DNA was extracted from the remaining pellets using the Qiagen Dneasy PowerSoil Kit (Hilden, Germany) according to

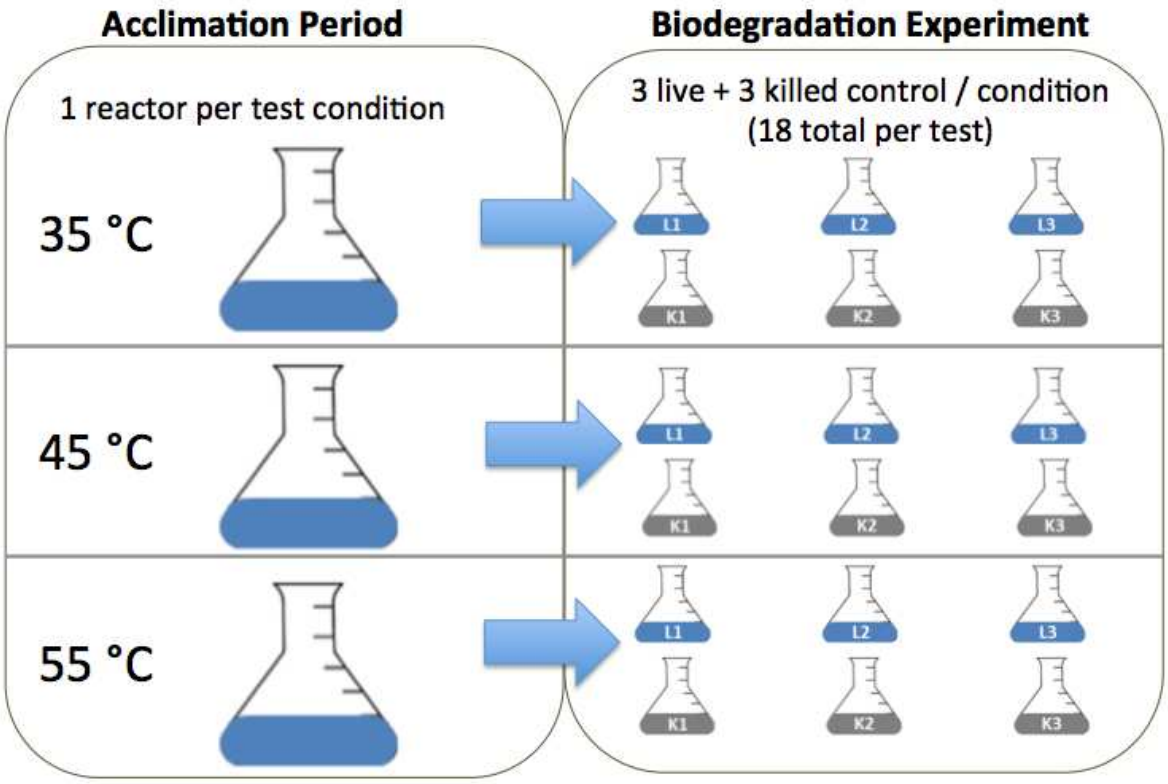


Figure 4: Acclimation and biodegradation reactor setup for the temperature test.

manufacturer's instructions. DNA concentrations were then measured by optical density at 260nm (BioTek Synergy 2 Multi-Detection Multiplate Reader). DNA concentrations were then used to determine the volume of bioacclimation inoculum needed to transfer an equivalent biomass (~ 50 ng DNA/ml) to each reactor. Before adding to test reactors, the inoculum was rinsed with new growth media to minimize carryover of EC drugs. The wash step involved centrifuging the inoculum (10 min, 4,000 x g), removing the supernatant, and then re-suspending the biomass pellet in fresh growth media. This process was repeated twice.

Reactors were covered in tin foil and operated for 29 days at constant temperature with constant mixing (120 rpm) in incubator shakers (Figure 5). Reactor pH levels were maintained at ± 0.5 pH for temperature and carbon source composition tests, and ± 0.4 pH

for the pH tests (with the exception of the 7 pH live reactors reaching ~7.65 pH on day 9 of the experiment). Samples for measuring EC concentrations were collected at 10 time points for the temperature test, and at 12 time points for the pH and carbon content composition tests. Additional samples were collected from all live reactors on days 9, 17, and 29 for DNA extraction in order to estimate cell growth based on DNA concentration. The DNA was extracted using the Qiagen Dneasy PowerSoil Kit (Hilden, Germany) as described above.

Figure 5: Photos of reactors in incubator shakers. All reactors were covered in foil, kept at constant temperature, and constantly shaken at 120 rpm.

3.2.5 EC drug extraction and analysis

EC drug concentrations were measured by using a liquid-liquid extraction followed by ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis using methods developed as part of this work (see Appendix B). Samples (250 µL)

were collected at 10 time points for the temperature tests and at 12 time points for the pH and carbon content composition tests. At time of collection, 250 µL of MeOH was added to the sample to slow estrone degradation (Zheng et al., 2013). The sample pH was then dropped to approximately 3 by adding 50 µL of glacial acetic acid to stabilize tetracycline drugs (Soeborg, Ingerslev, & Halling-Sorensen, 2004). All samples were stored at -80 °C until samples could all be extracted at the end of the experiment. The solid and liquid phases of samples were extracted simultaneously by adding 700 µL of 6:1 MeOH: 0.1 M EDTA mixture spiked with internal standard drugs. After horizontally vortexing samples for 30 min, samples were incubated at -80 °C for 12 hours. Samples were then centrifuged (10 min, 12,000 x g) and supernatant collected and stored at -80 °C. The extractable concentrations of EC drugs were measured by UPLC-MS/MS within 24 hours of extraction.

UPLC-MS/MS was performed on a Waters Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer. Chromatographic separations were carried out on a Waters iKey HSS T3 microfluidic device (150 µm x 50 mm, 1.8 µm). Mobile phases were methanol (B) and water with 0.1% formic acid (A). The analytical gradient was as follows: time = 0 min, 0.1% B; time = 0.5 min, 0.1% B; time = 5.5 min, 95% B; time = 6.5 min, 95% B; time = 6.51 min, 0.1% B; time 10.0 min, 0.1% B. Flow rate was 12 µL/min and injection volume was 2 µL. Samples were held at 4° C in the autosampler, and the column was operated at 45° C.

The MS was operated in selected reaction monitoring (SRM) mode, where a parent ion is selected by the first quadrupole, fragmented in the collision cell, then a fragment ion selected for by the third quadrupole. Product ions, collision energies, and cone voltages were optimized for each analyte by direct injection of individual synthetic standards.

Transition ions are listed in Appendix B. Inter-channel delay was set to 3 ms. The MS was operated in positive ionization modes with the capillary voltage set to 3.6kV. Source temperature was 150° C and desolvation temperature was 120° C. Desolvation gas flow was 800 L/hr, cone gas flow was 150 L/hr, and collision gas flow was 0.2 mL/min. Nebuliser pressure was set to 7 Bar. Argon was used as the collision gas, otherwise nitrogen was used. Analyte limits of detection (LODs) and limits of quantification (LOQs) were determined considering a signal/noise ratio of 3 for LOD and 10 for LOQ (see Table 9). Linear calibration ranged from 0.5 to 1,000 ng/mL. Calibration curves were always essentially linear, with correlation coefficients greater than 0.99.

Chromatograph peak quantification was performed using SkyLine (MacCoss Lab Software). The chromatograph for CTC had three distinct peaks indicating the parent compound and two of its transformation products were captured. For this experiment, the combined concentrations of CTC and its transformation products were reported to be conservative with respect to removal. An additional breakdown of CTC peaks and the corresponding transformation products can be found in Appendix D.

Table 9: EC LODs, LOQs, and Extraction Efficiencies

	LOD ng/ml	LOQ ng/ml	Extraction Efficiency %
SMX	2.4	7.9	43
Naproxen	4.9	16.3	56
E1	5.6	18.5	73
OTC	5.6	13.4	36
CTC / eCTC / other	9.6	32.0	85

3.2.6 Calculations and statistical analysis

To compare the concentration data between the live reactors, statistical analysis was performed with SAS Studio 3.7.1 (Basic Edition) for Windows. A one-way ANOVA with the GLM procedure was conducted to determine if there was a statistical difference in concentrations between operational conditions (e.g. the three temperatures of the temperature test) at each time point. If the ANOVA analysis suggested there was a significant difference (P-value < 0.05) in concentrations between the three operating conditions, a Tukey-Kramer HSD analysis was conducted to identify which combination of operating conditions had a significant difference. In addition, comparisons between the live and killed control reactors were done using the T.DIST.RT function in Microsoft Excel to determine if results were statistically significant between biotic and abiotic reactions. Tables summarizing the results from all statistical analysis are located in Appendix C. When comparing results, only conditions where at least three time points in a row had a P-value less than 0.05 were considered to be significantly different.

Data presented in figures were corrected for evaporation using the following calculations. During the course of each set of experiments all volumes added to (for pH correction) and removed from (for sample collection) reactors were recorded and the final volume of each reactor was measured. The evaporation rate (E_{rate}) for each reactor was then calculated using the following formula:

$$E_{rate} = \frac{V_{n,final} - V_f}{N_{days}}$$

where $V_{n,final}$ is the expected final volume if no evaporation occurred (i.e. start volume + all volume added for pH adjustments – all volume removed for samples), V_f is the actual final

reactor volume, and N_{days} is the number of days of the experiment. A simplifying assumption is made that the evaporation rate remains constant throughout the experiment. An average evaporation rate was then calculated for each temperature (35°C. 45°C, and 55°C). Correction factors were then calculated (for each reactor) to multiply by the measured concentrations. For each sampling time, two volumes were calculated. One was the volume expected (V_N) if no evaporation had occurred up to that point in time (i.e. the starting concentration plus all the volume added and removed up to that point). The other volume (V_E) was the expected volume if evaporation had occurred (i.e. $V_E = V_N$ – the estimated evaporation rate * the number of days in the experiment up to that point). Using those volumes, a correction factor was generated and applied to the measured concentration (based on simple dilution calculations)

$$V_N C_N = V_E C_E \quad \rightarrow \quad C_N = C_E \frac{V_E}{V_N}$$

where C_N is the expected concentration with no evaporation, C_E is the actual measured concentration when evaporation occurred, and V_E/V_N is the correction factor.

3.2.7 Analysis of microbial community at beginning of degradation tests

As reported above, DNA was extracted from bioacclimation inocula on day 0 to normalize biomass among live reactors. Those extractions were stored at -80 °C until they were sent to Research and Testing Laboratories (Lubbock, TX) for gene sequencing. 16S rRNA gene amplicon sequencing was performed with Illumina MiSeq by using primers 28F (5' –GAGTTTGATCNTGGCTCAG-3') and 519R (5'- GTNTTACNGCGGCKGCTG-3'). The filtered and trimmed sequences were classified using the RDP classifier against the SILVA 123 database.

3.3 Results

Table 10 summarizes the maximum removal percentages for each of the five EC drug and for each of set of operating condition experiments. Any values with a greater than sign indicate concentrations dropped below the UPLC-MS/MS detection limits. Also included is the number of days required achieve maximum removal.

Table 10: Maximum percent removal of Ecs for each operational condition

Variable	SMX	NPX	E1	CTC	OTC
	max % removal (# days to reach max removal)				
Temperature					
35 C	≥ 99 (6)	20 (19)	≥ 97 (9)	≥ 93 (29)	≥ 97 (2)
45 C	72 (19)	20 (19)	60 (29)	≥ 91 (19)	≥ 96 (1)
55 C	39 (19)	23 (19)	54 (19)	≥ 92 (13)	≥ 97 (1)
pH					
7	≥ 99 (25)	8 (29)	52 (29)	≥ 98 (19)	≥ 99 (2)
8	77 (25)	38 (19)	44 (29)	≥ 98 (25)	≥ 99 (1)
9	68 (19)	8 (19)	78 (29)	≥ 99 (19)	≥ 99 (1)
growth media					
p16:c13:ce23	≥ 98 (29)	28 (15)	54 (29)	≥ 98 (29)	≥ 99 (2)
p10:c7:ce23	≥ 99 (29)	25 (25)	54 (29)	≥ 99 (29)	≥ 99 (2)
p5:c1:ce18	67 (25)	15 (13)	≥ 99 (19)	≥ 98 (29)	≥ 99 (2)

The fraction remaining for each EC drug was plotted over time for each test as shown in Figure 6. The concentration values used to generate Figure 6 were corrected (as described in the Methods section) to more accurately reflect EC degradation by removing the concentrating effect of drugs due to evaporation. In addition, DNA concentrations were measured from samples collected on days 0, 9, and 17 in order to estimate relative biomass concentrations in the experiment reactors (Figure 7).

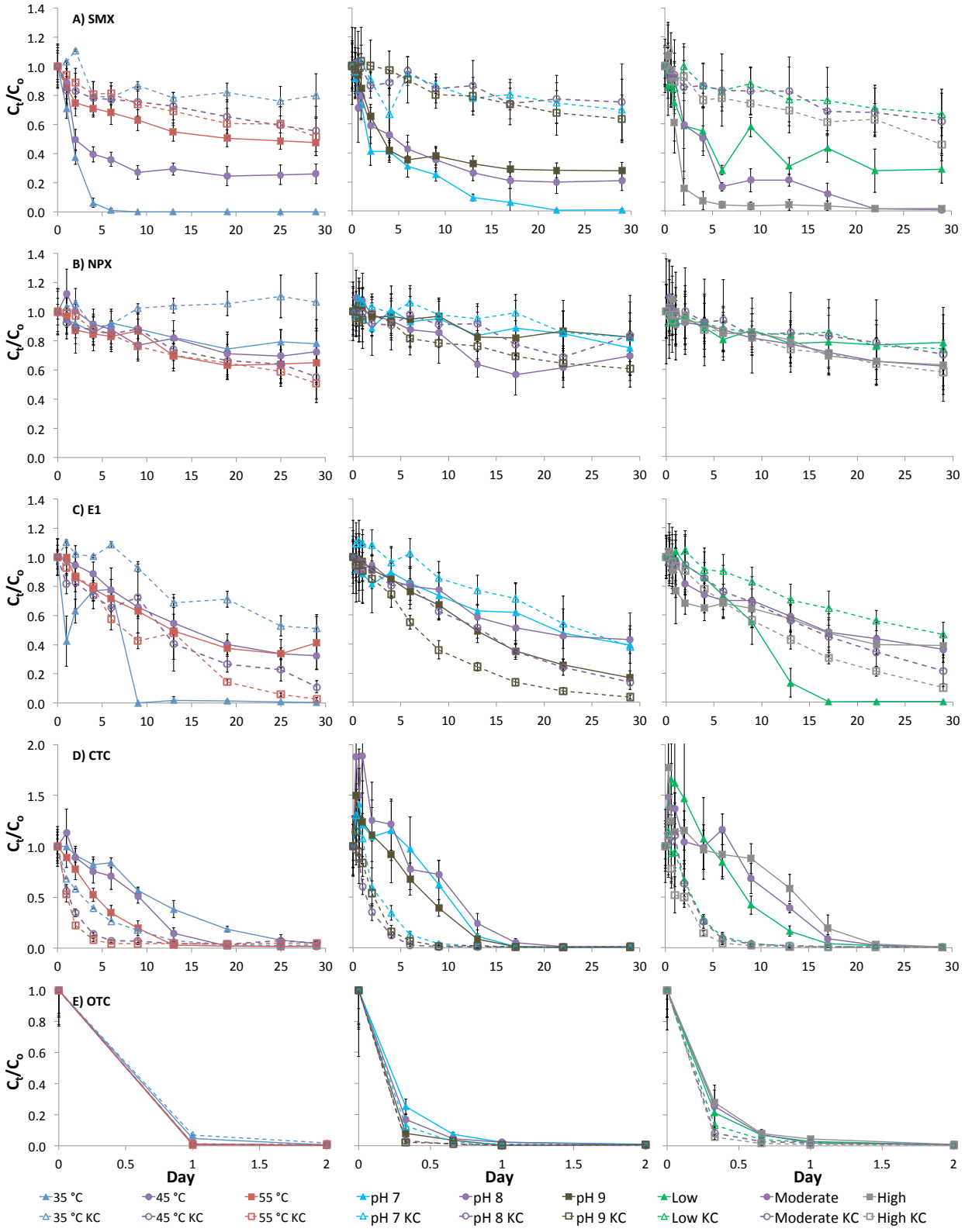


Figure 6: EC concentration change over time for temperature, pH and carbon content. The legend for the carbon composition test references levels of easily degradable carbon (low = p5:c1:ce18, moderate = p10:c7:ce23, high = p16:c13:ce23). Error bars represent the standard deviation for triplicates measures combined with evaporation rate standard deviation.

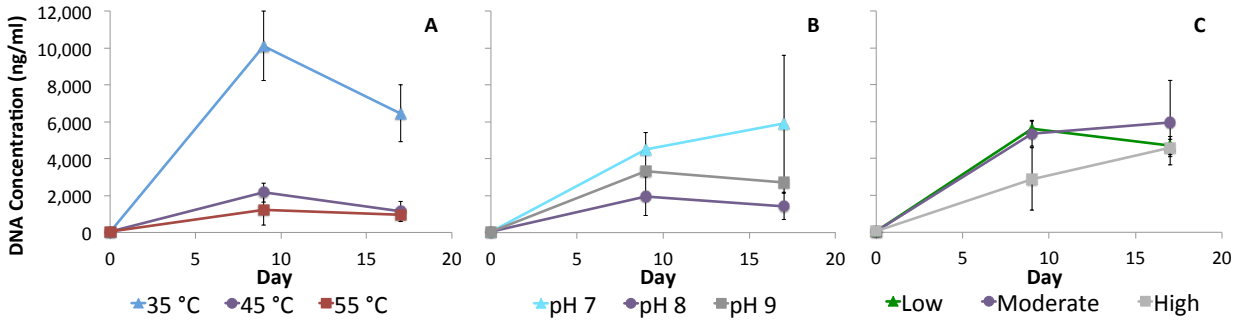


Figure 7: DNA concentrations for live reactors for all tests

Error bars represent standard deviations for triplicates measures. The legend for the carbon composition test references levels of easily degradable carbon (low = p5:c1:ce18, moderate = p10:c7:ce23, high = p16:c13:ce23)

All statistical analyses for the pH test and the carbon content composition test were conducted using the original values (not corrected for evaporation) and can be seen in Table 17 and Table 18 in Appendix C. Statistical analysis for the temperature test was done using both the original data (Table 15, Appendix C) and evaporation corrected values (Table 16, Appendix C). Only one time point had a difference in statistical significance results (the non-evaporation corrected result had no statistical significance while the evaporation corrected data did).

3.3.1 Effect of temperature on concentration of ECs

Temperature appeared to impact the extractable concentrations of ECs for several of the compounds tested. SMX, E1, and CTC all had statistically significant differences in percent EC removal ($p<0.05$ for at least 5 time points) between 35 °C and 45 °C and between 35 °C and 55 °C (Table 19, Appendix C). On the other hand, only SMX had a significant difference between 45 °C and 55 °C. For SMX and E1, lower temperatures appeared to be more conducive to removal, with both dropping to below levels of detection by day 6 and 9, respectively at 35 °C. At 45 °C and 55 °C there was only partial removal for

SMX (72% and 39% respectively) and E1 (60% and 54% respectively) after 29 days. CTC had faster removal at higher temperatures and reached levels below the detection limit by day 13 at 55 °C but not until day 29 at 35 °C. Temperature did not appear to impact the results for NPX or OTC, although NPX only had roughly 20% removal for all temperatures while OTC extractable concentrations dropped to below detection limit within 2 days for all temperatures.

The results from the killed control reactors indicate the decrease in extractable concentrations of most compounds was impacted by the presence of live microbes, i.e., was likely due to biological transformation. SMX, NPX, and E1 showed increased removal ($p < 0.05$) in live reactors compared to the killed control reactors. For SMX, increased removal occurred at 35 °C and 45 °C, and for NPX and E1 at 35 °C only. SMX had a higher percent removal at 35 °C versus 45 °C, although it is not possible to determine if this was due to microbes at 35 °C more effectively removing SMX or due to the higher level of biomass (approximately 5 times greater between 35 °C and 45 °C) leading to increased sorption. For CTC at all temperatures and E1 at 55 °C, microbial growth appeared to slow down the removal of compounds relative to killed controls. It is possible that sorption to microbes or compounds secreted by the microbes has a stabilizing effect the tetracycline drugs. No difference between live and killed control removal was observed for OTC since concentrations dropped to below detection limits for all reactors by day 2.

At 35 °C all compounds in killed control reactors decreased more slowly than in the 45 °C and 55 °C killed controls for all compounds. These differences could indicate that abiotic losses are temperature dependent and more favorable at higher temperatures. However, during the course of the temperature test two of the 35 °C killed control reactors

became contaminated with microbes and the data from those reactors became unusable. Therefore, it is possible that the differences in behavior of the 35 °C killed control reactors do not represent reproducible trends. Two of the 35 C killed control reactors showed signs of contamination by the end of the experiment, however, SMX was the only compound that behaved differently (more removal in contaminated reactors) between the contaminated and non-contaminated reactors.

3.3.2 Effect of pH on concentration of ECs

The pH levels had little impact on live reactor extractable concentrations for all ECs except SMX. At pH 7, SMX dropped to below detection limits by day 25, while pH 8 and pH 9 concentrations reached 77% and 68% respectively. Concentrations were significantly different ($p < 0.05$, Table 20, Appendix C) between pH 7 and pH 9 after day 9, and between pH 7 and pH 8 on days 13, 22, and 29. Similar to observed trends for the temperature study, CTC and OTC dropped to levels below detection limit by day 25 and day 2 respectively. NPX extractable concentrations dropped more quickly for pH 8; however, by day 29 there was no statistically significant difference in removal amounts between all pH levels. Moderate removals (44 to 78 %) were observed for E1, however results between pH levels were not statistically significant.

SMX, E1 and CTC had killed control results that indicated the decrease in extractable concentrations of most compounds was impacted by the presence of microbes. SMX had increased removal ($p < 0.05$) in live reactors compared to the killed control reactors at all pH levels, while NPX had increased removal in pH 7 reactors after day 13. Live reactor DNA concentrations at pH 7 were at least twice as high as concentrations in pH 8 and pH 9 reactors by day 17, indicating a higher concentration of biomass, which could have led to

higher or faster removal by adsorption. In killed control reactors, E1 removal appeared to be impacted by pH level, with less removal at pH 7 and greater removal at pH 9. However, microbial interaction with E1 appeared to slightly improve removal at pH 7 but decrease removal at pH 8 and pH 9. Just like during the temperature experiments, microbial growth significantly decreased CTC removal at all pH levels until day 17, but after that extractable concentrations in all reactors dropped below detection limit. No difference between live and killed control removal was determined for OTC since concentrations dropped to below detection limits for all reactors by day 2.

3.3.3 Effect of carbon content composition on concentration of Ecs

The carbon content composition appeared to significantly impact ($p < 0.05$, Table 21, Appendix C) live reactor extractable concentrations for SMX, E1, and CTC. At high levels of easily degradable carbon (p16:c13:ce23) SMX reduced rapidly to below the detection limit. SMX concentrations were significantly higher ($p < 0.05$) between p16:c13:ce23 and moderate levels of easily degradable carbon (p10:c7:ce23) until day 17, and SMX concentrations at low levels of easily degradable carbon (p5:c1:ce18) were significantly different ($p < 0.05$) from both p16:c13:ce23 and p10:c7:ce23 through the end of the experiment. There were moderate reductions in E1 concentrations for both the p16:c13:ce23 and p10:c7:ce23 reactors, while E1 levels dropped to near detection limits by day 17 in the p5:c1:ce18 reactors. OTC and CTC concentrations dropped to below detection limit for all carbon content compositions by the end of the experiment; however, CTC concentrations in p5:c1:ce18 were significantly lower ($p < 0.05$) than in p16:c13:ce23 and p10:c7:ce23 between days 6 and 17.

SMX, E1 and CTC had results that indicated the decrease in extractable concentrations of most compounds was impacted by the presence of microbes. SMX had increased removal ($p < 0.05$) in live reactors compared to the killed control reactors for all carbon content compositions. In killed control reactors, E1 removal appeared to be impacted by carbon content composition, with less removal in p5:c1:ce18 and greater removal in p16:c13:ce23. However, in live reactors E1 extractable concentrations dropped to below detection limit by day 17 in p5:c1:ce18. As with the temperature and pH experiments, microbial growth significantly ($p < 0.05$) decreased CTC removal at all nutrient levels until day 22 when extractable concentrations in all reactors dropped below detection limit. No difference between live and killed control removal was determined for NPX and OTC.

3.3.4 Effect of operating conditions on microbial community composition

The relative abundance of phylotypes for all test conditions are shown in Figure 8 at the genus level for all microbes that made up at least 1% of the microbial community. The major phylotypes in the 35 °C inoculum were *Acholeplasma* (18.9%), *Fermentimonas* (17.6%) and *Paenalcaligenes* (16.54%). In the 45 °C inoculum, *Paenalcaligenes* was also abundant (33.7%) as well *Ureibacillus* (27.1%) and *Bacillus*. At 55 °C *Bacillus* (7.95%) was also abundant as were unclassified *Bacillales* (45.6%), and *Calderihabitans* (36.8%). Additionally, unclassified *Fimicutes* was found at all three temperatures (3.0% at 35 °C, 6.14% at 45 °C, and 6.5% at 55 °C).

The major phylotypes in the pH 7 inoculum were *Vulgatibacter* (16.2%), *Bacillus* (12.5%), unclassified *Firmicutes* (9.8%), and *Gerogenia* (9.5%). At pH 8, *Bacillus* (11.8%) and unclassified *Firmicutes* (5.6%) were also present along with *Ammoniibcacillus* (52.3%)

and *Paenibacillus* (7.4%). The major phylotypes at pH 9 were *Sinibacillus* (48.7%), *Caldicoprobacter* (25.0%) and *Filobacillus* (5.0%).

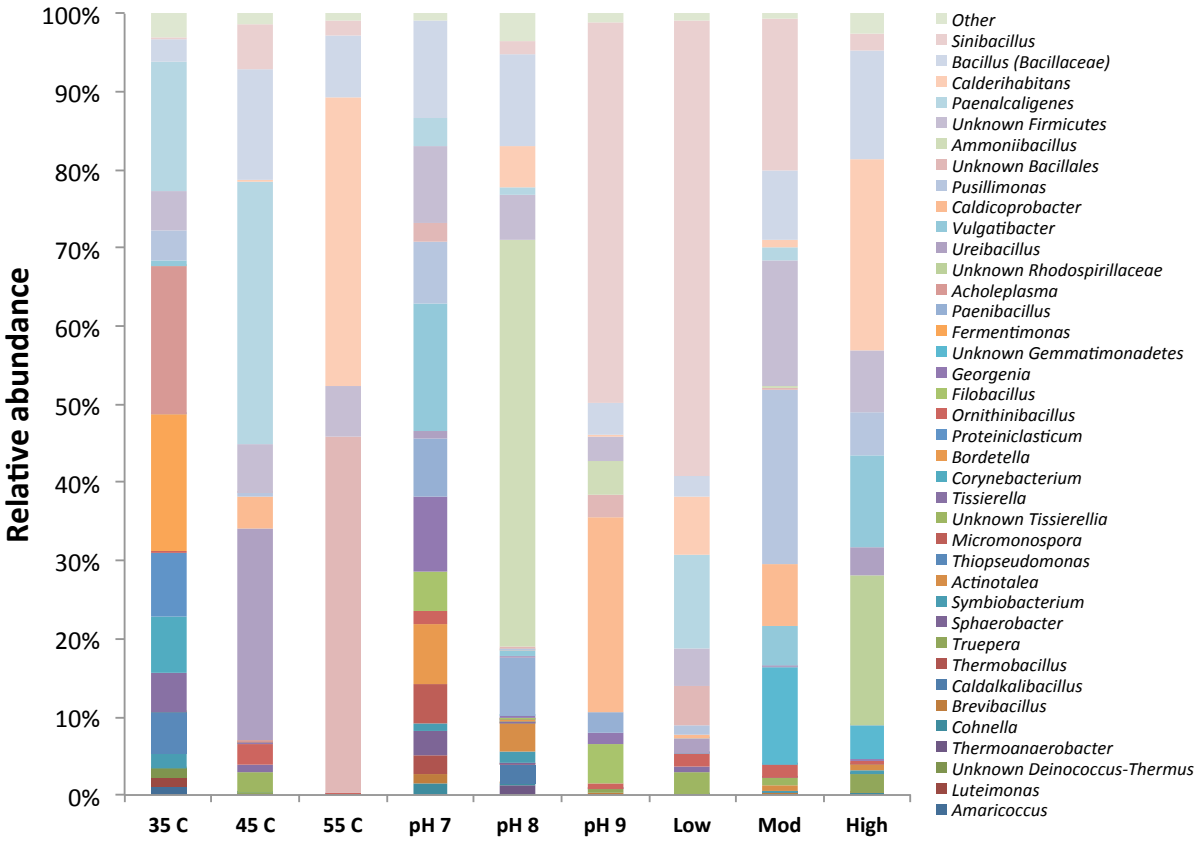


Figure 8: Microbial community structure analyzed at genus level.
Phylotypes shown for inocula taken from bioacclimation reactors before splitting into triplicate experiment reactors, therefore there is only a single result for each operating condition. All phylotypes of at least 1% abundance were included. All other phylotypes combined under "Other". The legend for the carbon composition test references levels of easily degradable carbon (Low = p5:c1:ce18, Mod = p10:c7:ce23, High = p16:c13:ce23).

The major phylotypes present in the p5:c1:ce18 reactors were *Calderihabitans* (24.5%), unclassified *Rhodospirillaceae* (19.2%), *Bacillus* (13.98%), and *Vulgatibacter* (11.8%). Phylotypes in the p10:c7:ce23 reactors included *Pusulimonas* (22.3%), *Sinibacillus* (19.37%), unclassified *Firmicutes* (16.13%), and unclassified *Gemmatimonadetes* (12.4%). *Sinibacillus* was also present in the p16:c13:ce23 reactors

and was the most abundant 58hylotypes at 58.3%. *Paenalcaligenes* (12.1%), *Calderihabitans* (7.4%), and unclassified *Bacillales* (5.1%) were also present.

3.4 Discussion

3.4.1 Degradation of sulfamethoxazole

SMX removal was significantly impacted by all the tested operational parameters. While it is not possible to rule out biosorption contributing to SMX removal, many studies with activated sludge have determined that SMX sorption is typically negligible. Goebel et al. (2007) studied sorption of different antibiotics in activated sludge and found sorption to be negligible for compounds with K_d values less than 500 L/kg (SMX had an average K_d value of 256). Other studies also determined that SMX sorption was negligible (Feng et al., 2017; Larcher & Yargeau, 2012; B. Li & Zhang, 2010; X. Zhang et al., 2014). The authors of Li et al. (2010) attributed the low sorption to the negative charge of SMX at neutral and basic pH levels, which repels the negatively charged sorption sites on biomass.

In this study lower temperatures resulted in greater SMX removals (>99, 72, and 39% at 35 °C, 45 °C, and 55 °C respectively.) Other studies found SMX removals to be a function of both temperature and manure type. Lin et al. (2017) conducted bench-scale composting studies with fresh swine and chicken manure at four temperatures (30 °C, 40 °C, 50 °C, and 60 °C) and found that chicken manure (which included bedding) had better SMX removal at 30 °C compared to higher temperatures, while swine manure had better removal at 60 °C compared to lower temperatures. SMX also had improved removal at lower pH values (>99, 77, and 68% at pH 7, 8, and 9 respectively.) Tadkaew et al. (2010) also compared removal amounts of SMX at different pH levels in a laboratory scale membrane bioreactor. The bioreactor pH was held constant for 4 days at each pH level

tested (5, 6, 7, 8, and 9) and found that SMX removal was at 90% between pH 5 and 7, but only 70% removal at pH 9. Removals of SMX from the membrane bioreactor were mostly attributed to biodegradation since SMX adsorption to the membrane was low at neutral to high pH values.

3.4.2 Degradation of tetracyclines

In this research, tetracycline removal appears to be controlled by abiotic processes. While high temperatures and low levels of easily degradable carbon contributed to faster removal of CTC, the concentrations in all test reactors dropped below detection limits within 29 days. In previous studies, CTC and OTC percent removal has varied from 74% and 92 % reduction (c_o = 3.0 mg/kg), respectively, after 50 days in pilot scale swine manure composting windrows (X. F. Wu et al., 2011) to faster removals with half-lives ranging from 1 to 3 days (c_o = 1.5 mg/kg) when composted in turkey manure windrows (Dolliver et al., 2008). Typically, OTC and CTC have similar removal rates (Chai et al., 2016); however, in this current study the extractable OTC concentrations were removed much more quickly than CTC and dropped to below detection limits within 2 days. One reason for the disparity in OTC and CTC results is that the concentrations reported herein of CTC include two of its transformation products that we were able to identify with UPLC-MS/MS. As seen in Appendix D, the CTC parent compound dissapeared as quickly as the OTC parent compound. It is possible that the majority of the OTC was quickly transformed to unmeasured transformation products, such as epi-OTC, in previous studies (Alvarez et al., 2010).

Similar to our results, many studies also observed improved removal of CTC at higher temperatures. In a study by Chai et al. (2016), a 1 m^3 composting unit filled with pig

manure and rice straw reached 65°C and maintained temperatures above 50°C for close to a week. CTC levels dropped to below detection limits within 14 days, but control samples that were incubated at 25°C only showed 73% removal after 49 days. Another study found OTC half-lives in sterilized swine manure to 84.7 hours and 19.8 hours when incubated in 10 mL glass vials at 25°C and 40°C, respectively (Ratasuk, Boonsaner, & Hawker, 2012) again suggesting an abiotic removal mechanism that is temperature dependent. An unexpected result from this current work was the improved removal of CTC in the killed controls. For all three tests killed control CTC levels dropped to near complete removal by day 13, while live reactors did not drop below detection limits until near the end of the experiment. It is possible that sorption to microbes or compounds secreted by the microbes has a stabilizing effect the tetracycline drugs.

3.4.3 Degradation of estrone

All E1 reactors had at least 44% removal from live reactors, however, it is not clear if microbial degradation had an impact on removals because all killed control reactors had substantial removals as well. There were two conditions (35°C and the reactors with low concentrations of readily degradable carbohydrates) that resulted in significantly ($p < 0.5$) more removal in live reactors (concentrations below detection limits) than killed control reactors, which indicate microbial degradation was involved. Zheng et al. (2013) studied removal of the hormone estradiol from dairy wastewater in lab scale (250 mL) reactors under controlled temperature conditions (between 15 and 45°C) and found optimal removals at 35°C as well. The removals of E1 of >99% by day 15 in this experiment were more surprising. Higher microbial activity was expected in reactors with a higher concentration of carbon and protein, however, based on DNA concentrations, microbial

growth was not significantly different (< 0.05) between media types. However, the quick drop in estrone concentration might be explained with work done by Tan et al. (2015) which also experienced a rapid decrease in estrone concentrations around the same time (day 9) while conducting degradation experiments with aged wastewater containing little carbon. The authors hypothesized that the growth of E1 degraders was hindered during the first few days of the experiment due to the recalcitrant nature of the available carbon sources in wastewater and that the E1 degraders used products produced by microbes or remnants of lysed cells as a food source. The E1 degraders then became more abundant by day 9 and E1 levels started to drop. It is unclear at this time what caused such substantial removals in killed control reactors and additional research will be required to understand that mechanism.

3.4.4 Degradation of naproxen

NPX showed moderate removal under all conditions, and with abiotic controls except at 35°C, indicating losses were due to abiotic processes. This result is in contrast to Suzuki et al. (2014) who measured a 14-hr half-live for naproxen in a bench scale-study with activated sludge; however, degradation did not occur in a sterilized control. Thus, previous studies have demonstrated the NPX is biodegradable, but the AD/AC-derived microbes were not capable of degrading NPX to a substantial degree (i.e., to levels below killed controls) or the reactor conditions failed to promote degradation. Quintana et al. (2005) attempted to degrade NPX with activated sludge-derived microbes. When NPX was provided as the only carbon source, no degradation occurred. After the addition of powdered milk as a second carbon source, naproxen was slowly degraded to 60% removal in 28 days. However, in our study, the presence of additional carbon sources still did not

promote significant removal of NPX pointing to the relevance of other variables, such as the source of microorganisms. Rather, a likely explanation for the NPX removals found in this current study is sorption. Paul et al. (2014) conducted an experiment to measure naproxen sorption in simulated domestic wastewater at different pH levels (from 3.5 to 8.5) and at different starting concentrations (ranging from 125 to 1000 ug/L) over 30 hours. Increasing pH values resulted in decreased sorption (approximately 15% sorption at pH 3.5 and 4% sorption at pH 8.5). The authors attributed the decrease in sorption to the fact that NPX is negatively charged at higher pH levels and will be repelled by negatively charged binding sites. However, sorption increased with increasing concentrations. This could partially explain the NPX losses, shown in Figure 6, that are corrected for evaporation. As evaporation occurred the NPX concentrations actually increased which could have led to increasing sorption.

3.4.5 Microbial community composition and EC degradation

Numerous EC-degrading microbes have been identified in previous studies as described in Section 2.7. Future work will be required to identify microbes capable of EC degradation in AD/AC systems and to associate them with specific EC removals. However, three microbial phylotypes previously associated with SMX and E1 removal were found in the reactors for this study. *Mycobacterium* and *Pseudomonas* were isolated from swine wastewater in E1-enriched media and were shown to degrade E1 (Isabelle et al., 2011). *Mycobacterium* was found in low concentrations in the pH 7 and pH 9 inocula, and in the p10:c7:ce23 and p5:c1:ce18 reactors. *Pseudomonas* was found in low concentrations in the 35°C and p5:c1:ce18 reactors. In this study microbes likely removed E1 in the 35°C and p5:c1:ce18 reactors; therefore, *Pseudomonas* is a candidate for the E1 degradation. In

another study, numerous strains of *Bacillus* were found to be dominant members of the microbial communities cultured from river water and sediment for a SMX bio-degradation study. While the biotic degradation of SMX was not done by isolated cultures, nine different strains of *Bacillus* were verified to have SMX resistance by plating on media mixed with SMX (Xu, Mao, Luo, & Xu, 2011). For this current study *Bacillus* microbes were also abundant in all reactors ranging from 2.6 to 14% of the microbial community. Since SMX had biotic degradation under all conditions, *Bacillus* is a candidate for SMX degradation.

It is also worth noting that while antibiotic resistant genes were not studied as part of this research, it is likely they were present in the experiment reactors as microbes were pre- acclimated to the tetracyclines and sulfamethoxazole. A recent study by Chen (2018) studied the abundance of various tetracycline, macrolide, quinolones, and sulfonamide ARGs during in-vessel compost of swine manure. The relative abundance of each ARG varied based on microbial community composition and on composting phase, but total ARG concentrations were reduced between 85 and 95% after 90 days of composting. This indicates that the composting phase of the MSAD system could have the added benefit of removing ARGs if the system is maintained aerobically for an extended period. Additional research would be required to optimize system conditions.

3.4.6 Transition to full-scale operations

The purpose of this research was to help inform operators of combined MSAD/AC systems of operational methods to improve EC removal in order to generate high-value composted material. One challenge that will need to be addressed when scaling up to an industrialized size MSAD/AC system will be the speed at which microbes will acclimate to new conditions. For this study, all cultures were acclimated to test conditions prior to the

start of the experiment. In a full-scale system, microbes will have been acclimated to ECs that were consumed by the animals producing the waste, however, they will not most likely not be acclimated to ECs introduced in reclaimed wastewater. In addition, EC degrading microbes might not have time to grow to sufficient numbers to effectively remove the ECs. Previous research found inoculating the LBR units of a MSAD system with acclimated microbes improved hydrolysis rates for AD under conditions with extreme salinity or ammonia concentrations (Wilson, Sharvelle, & De Long, 2016). Similarly, future research could determine if inoculating the LBR unit with acclimated EC degrading microbes would help to improve the EC removal rates.

Depending on the ECs identified in the animal waste being treated in the MSAD/AC system, prioritizing which compounds to remove might be necessary if optimal operating conditions are conflicting. However, results from this study show that removal for most of the selected ECs should be possible within the same system. The initial mesophilic phase with lower temperatures and higher concentrations of readily degradable carbon would be conducive to microbial degradation of SMX. The high temperatures during the thermal composting phase should result in a rapid abiotic degradation of CTC and OTC. Finally, E1 degradation would be likely during the curing phase where most of the easily degradable carbon has been spent, and E1-degrading microbes would have the chance to become established. NPX was not removed well in this study, but previous research has indicated it is biodegradable (Carballa et al., 2007; Samaras et al., 2014). Inoculum from municipal waste plants (likely the source of the reclaimed water used for recirculating leachate in the MSAD system) could potentially improve NPX removal.

3.5 Conclusion

The first objective of this research was to determine which operating conditions lead to the greatest removal of ECs during the composting phase of a MSAD/AC system. The results from this study show that temperature and carbon source composition had the greatest impact on EC removal under aerobic conditions. SMX and E1 degraded to below detection limits at 35°C (>99% and >97% removal respectively), with less removal at higher temperatures (39% and 54%, respectively, at 55°C). By contrast, CTC had increased removal at higher temperatures; however, concentrations dropped to below the detection limit at all temperatures after 29 days. Carbon content composition had a significant impact on SMX, E1, and CTC removals. In media with high levels of easily degradable carbon (p16:c13:ce23), SMX dropped to below detection limits by the end of the experiment. In contrast, E1 reached >99% removal by day 19 and CTC had more rapid removal in media with low levels of easily degradable carbon (p5:c1:ce18). SMX was the only EC to be impacted by pH, with degradation to below the detection limit (>99% by day 25) at pH 7, but only had 77% and 68% removal at pH 8 & 9 respectively.

The second objective of this study was to identify microbial communities that originate from AD digestate and that have the ability to degrade ECs under aerobic conditions. Microbial degradation appeared to be involved (since killed control reactors had significantly less ($p < 0.05$) removal than their corresponding live reactors) in the removal of SMX under all test conditions, E1, and NPX at 35°C and for E1 in media with low levels of easily degradable carbon (p5:c1:ce18). *Bacillus*, found in literature to degrade numerous ECs including SMX, OTC, E1 and NPX, was present in experimental reactors under all operating conditions. Since SMX had biotic degradation under all test conditions,

Bacillus was a likely degrader of SMX. *Pseudomonas*, found in literature to degrade E1, was only found in reactors that had microbial degradation of E1 and was likely involved it E1 removal. Further research will be needed to isolate these microbes to verify they were capable of EC degradation. CTC and OTC appeared to degrade through abiotic processes. CTC removal was actually faster in killed control reactors. Most likely CTC sorption to biomass made it less available for abiotic degradation in solution or microbes secreted compounds that stabilized CTC.

It is expected that full scale MSAD/AC operations will follow similar trends as discussed above; however, additional pilot scale experiments will be necessary to determine how the time frames observed scale to larger operations as microbes acclimate to new operating conditions. The results from these experiments indicate optimal operating conditions for waste treatment technologies will be highly dependent on the contaminants that need to be prioritized for removal. Further, risk assessment studies could be conducted to set removal priorities if conflicting optimal operating conditions cannot be resolved. Based on the results of this research, MSAD/AC operators will be able to identify specific composting phases where conditions can be optimized or where inoculum can be added to enhance the removal of EC contaminants.

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APPENDIX A: NUTRIENT SOLUTION

A.1 Nutrient Solution Design

A co-digestion compost synthetic manure (CCSM) growth media was developed to mimic the protein, carbohydrate (easily degradable carbon), and cellulose (more recalcitrant carbon) composition of digestate in a low-water AD/AC system with a mix of manure, bedding or other bulking agents, and food waste. Starting concentrations were selected based on measured carbon and protein levels found in manure (G. Yang, Zhang, Zhang, Wang, & Yang, 2015) and based on estimates of food waste content. In addition, the media's carbon to nitrogen (C/N) ratio, between 7 and 8, was designed based the C/N ratio of cattle manure and maize-oat silage digestate measured by Alburquerque et al. (2012).

In a combined AD/AC system, the anaerobic digestion phase is run before the aerobic phase. Therefore, the duration of the anaerobic digestion phase will have a direct impact on the quantity and composition of carbon sources in the digestate at the start of the composting phase. Yang et al. (2015) determined the removal efficiencies for protein and carbohydrates, 35% and 45% respectively after 3 weeks of AD operation. Those removal efficiencies were applied to the synthetic growth media recipe two times in order to estimate concentrations of protein and carbohydrates that could be found in digestate with differing retention times (Table 6). In addition, cellulose levels were reduced for the long retention time recipe as it is assumed that microbes would begin to use cellulose when more readily degradable carbohydrates are no longer available. A variety of widely used and commercially available products such as beef extract, tryptose peptone, sodium

acetate, and cellulose were used to represent biomolecules you would expect to find during various stages of decomposition in an anaerobic digester.

A.2 Nutrient Solution Preparation

The preparation method for Co-Digestion Compost Synthetic Manure (CCSM) microbial growth media is listed below. CCSM stock solutions are listed in Table 11 (CCSM 3 to 8 are modified versions of media described in (Owen, Stuckey, Healy, Young, & McCarty, 1979).

1. Prepare each of the stock solutions. CCSM 4 as described in Table 11 is at 5000 times concentration. Prior to making growth media CCSM 4 needs to be diluted to 100 times concentration. This is done by adding 20 ml of the 5000x stock per liter of 100x solution.
2. Solutions 2 and 4 should be filter sterilized. All other solutions should be autoclaved for a length of time appropriate for liquid volume.
3. To prepare 1 L of growth media start by adding, under sterilized conditions:
    a. 500 ml CCSM 1
    b. 50 ml CCSM 2
    c. 15 ml CCSM 3
    d. 10 ml CCSM 4 (100x)
    e. 10 ml CCSM 5
    f. 9 ml CCSM 6
    g. 1.7 ml CCSM 7
    h. 200 ml CCSM 8
4. Add enough autoclaved water to reach a final volume of 1 L.

Table 11: CCSM concentrated stock solutions

Stock Solution	Compound	Concentration (g/L)
CCSM 1	Tryptose peptone	10
	Yeast extract	3
	Beef extract	10
	Sodium acetate	4.5
	Starch (soluble)	1.5
	L-cysteine hydrochloride	0.5
	Long chain cellulose	46
CCSM 2	Glucose	75
CCSM 3	KCl	86.7
	$MnCl_2 – 4\ H_2O$	1.33
	$CoCl_2 – 6\ H_2O$	2
	H_3BO_3	0.38
	$CuCl_2 – 2\ H_2O$	0.18
	$Na_2MoO_4 – 2\ H_2O$	0.17
	$ZnCl_2$	0.14
	$NiCl_2 – 6\ H_2O$	0.44
	$Al_2(SO_4)_3 \cdot 18\ H_2O$	2
CCSM 4	Biotin	0.1
	Folic acid	0.1
	Pyridoxine hydrochloride	0.5
	Riboflavin	0.25
	Thiamin	0.25
	Nicotinic acid	0.25
	Pantothenic acid	0.25
	B12	0.005
	ρ-aminobenzoic acid	0.25
	Thioctic acid	0.25
CCSM 5	$FeCl_2 \cdot 4H_2O$	37
CCSM 6	$CaCl_2 – 2\ H_2O$	147
CCSM 7	$MgSO_4$	120
CCSM 8	$NaH_2PO_4 \cdot H2O$	23
	Na_2HPO_4	47.3
	Tris	60.6
	NH_4Cl	26.7

The above method is to prepare the 3-week digestate growth media, which is named p10:c7:ce23. Media names indicate the protein (p), carbohydrate (c), and cellulose (ce) concentrations (g/L) for each media recipe. A more easily degradable carbon and protein

rich recipe (p16:c13:ce23) was used for the media designed to mimic digestate with a shorter solids retention time (SRT). Changes to the stock solution CCSM 1 are shown in Table 12. In addition, step 3, part b should be changed to "78 ml of CCSM 2".

Table 12: Modified CCM 1 stock solution for p16:c13:ce23 recipe

Stock Solution	Compound	Concentration (g/L)
CCSM 1	Tryptose peptone	15
	Yeast extract	8
	Beef extract	15
	Sodium acetate	8
	Starch (soluble)	3
	L-cysteine hydrochloride	1
	Long chain cellulose	46

A less easily degradable carbon and protein rich recipe (p5:c1:ce18) was used for the media designed to mimic digestate with a long SRT. Changes to the stock solution CCSM 1 are shown in Table 13. In addition, step 3, part b should be changed to "10.5 ml of CCSM 2".

Table 13: Modified CCSM 1 stock solution for p5:c1:ce18 recipe

Stock Solution	Compound	Concentration (g/L)
CCSM 1	Tryptose peptone	4.5
	Yeast extract	2
	Beef extract	4.5
	Sodium acetate	0.6
	Starch (soluble)	0.1
	L-cysteine hydrochloride	0.2
	Long chain cellulose	36

APPENDIX B: ANALYTICAL CHEMISTRY METHOD DEVELOPMENT

The objective of this method development was to find a low-cost, facile method for measuring the concentrations of five emerging contaminants (SMX, CTC, OTC, NPX, and E1) in growth media designed to mimic anaerobic digestate. Extraction methods were optimized in order to achieve detection limits less than two orders of magnitude below the degradation study starting concentration (200 µg/L – 600 µg/L). Analyte detection and quantification were performed by liquid chromatography-triple quadrupole mass spectrometry (UPLC-MS/MS).

B.1 Extraction method optimization

Acid type, incubation time, repeated extractions, and evaporation of samples (to concentrate analytes) were all tested in order to optimize extraction efficiency. Section 2.8 of the literature review contains a summary of common extraction solutions and methods used for EC detection in manure, compost, and similar media. Based on research by Wu et al. (2008), an extraction solution consisting of a weakly acidic buffer and an organic solvent was chosen. Methanol was selected as the organic solvent based on research by Zheng et al. (2013) which found that adding equal parts of methanol to samples immediately after collection decreased hormone degradation and extracted hormones sorbed to particles in anaerobic wastewater. In order to optimize extractions for OTC and CTC the extraction solution included ethylenediaminetetraacetic acid (EDTA). Without EDTA, tetracycline antibiotics chelate with metals and can be difficult to extract (Tso et al., 2011). In addition, acetic acid was added to drop samples to pH 3, which has been shown to improve tetracycline antibiotic extraction (Soeborg et al., 2004). During initial testing, samples

were evaporated under nitrogen and then extractant pellets were re-dissolved in methanol. Horizontal vortexing was selected over sonication for the extraction method because during initial testing pellets from sonicated samples were more difficult to re-dissolve in methanol that vortexed samples.

For the initial extraction method 750 µL of extraction solution (50 µL glacial acetic acid, 70 µL 0.1M EDTA, 630 µL methanol) and 250 µL samples were combined in a microcentrifuge tube. The sample was then vortexed for 30 min, precipitated at -80°C for 30 min, and then centrifuged for 10 min (12,000 x g). Supernatant (500 µL) was evaporated under N_2 and then dissolved in 90% methanol (10% water).

B.1.1 Repeated extractions

The first test was to determine if repeated extractions would improve analyte recovery. Aliquots of growth media (250 µL) were spiked with all five drugs at three concentrations (28, 52, and 100 µg/L). The first set of spiked samples was extracted once with 750 µL extraction solution. The second set was extracted three times (once with 350 µL and twice with 200 µL extraction solution) and all supernatant was combined before the evaporation step. Results are shown in Figure 9. Extracting three times had little impact on extraction efficiency; therefore the single extraction method was selected.

B.1.2 Acid selection

Acetic acid was replaced by formic acid in the extraction solution. Formic acid was selected because it was used as part of the liquid chromatograph mobile phase and therefore it was speculated that formic acid in the extraction solution might generate less background noise during UPLC-MS/MS analysis. Growth media was spiked with all five drugs at 200 µg/L. The results from this test were compared to the single extraction

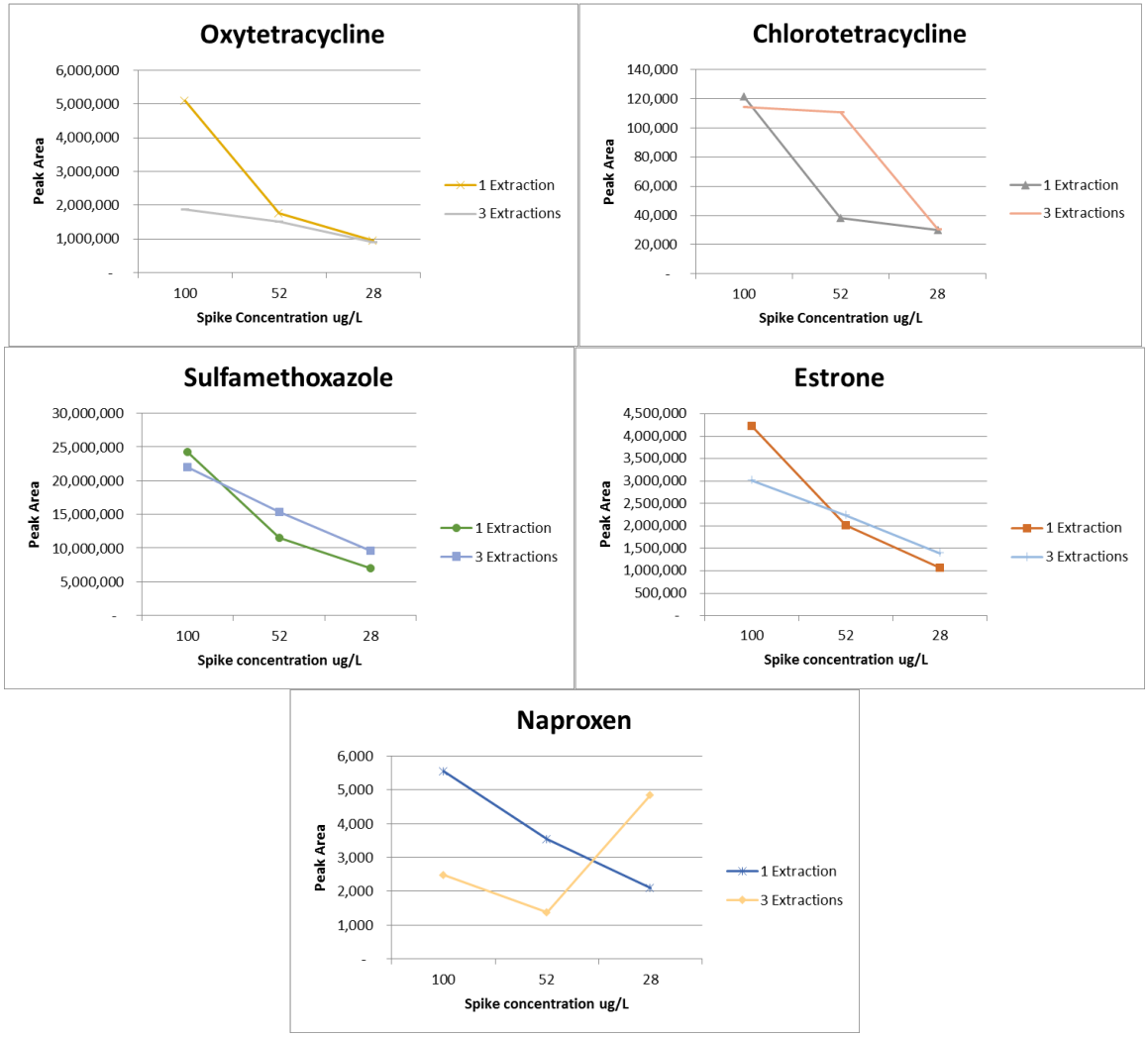

Figure 9: Comparison of extraction efficiency for single and triple extraction methods

results in the previous test. Since the acetic acid samples were spiked at 28, 52, and 100 µg/L, but not at 200 µg/L, the expected chromatograph area at 200 µg/L had to be calculated from the slope of the line generated from the three lower concentrations. Results are shown in Figure 10 and Figure 11. Acetic acid was predicted to have had better extraction efficiency for 4 of the 5 drugs and was therefore selected for the final extraction method.

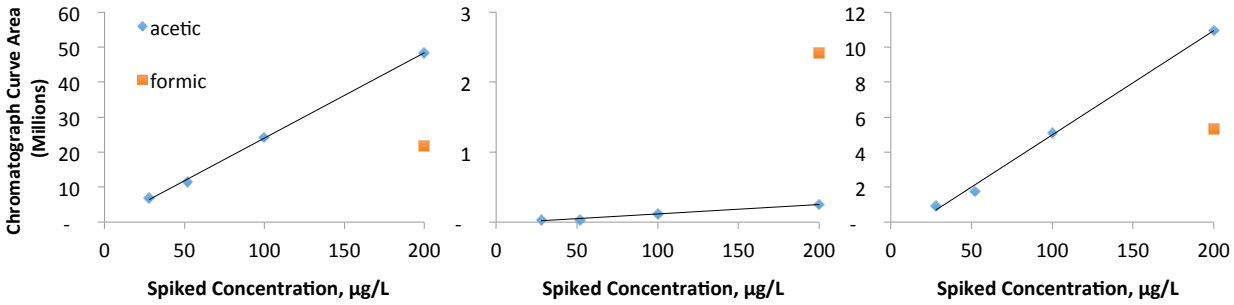

Figure 10: Peak areas comparing acetic acid and formic acid extractions for antibiotic. Peak areas for SMX (left), CTC (center), and OTC (right).

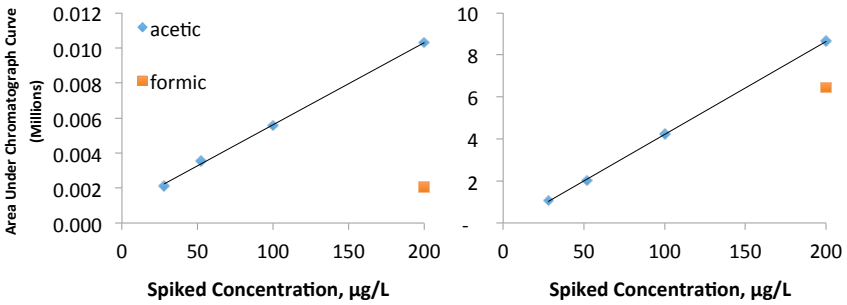

Figure 11: Peak areas comparing acetic acid and formic acid extractions for ECs Peak areas for NPX (left) and E1 (right).

B.1.3 Incubation time

Two incubation times were tested, 30 min and 12 hours. The test was to determine if increasing the incubation time would improve precipitation and increase extraction efficiency. Extraction efficiencies improved slightly for four of the drugs when incubated for 12 hours, but variability in peak are decreased for all drugs when samples where incubated for 12 hours as seen in Figure 12. A 12-hour incubation was selected for the final method.

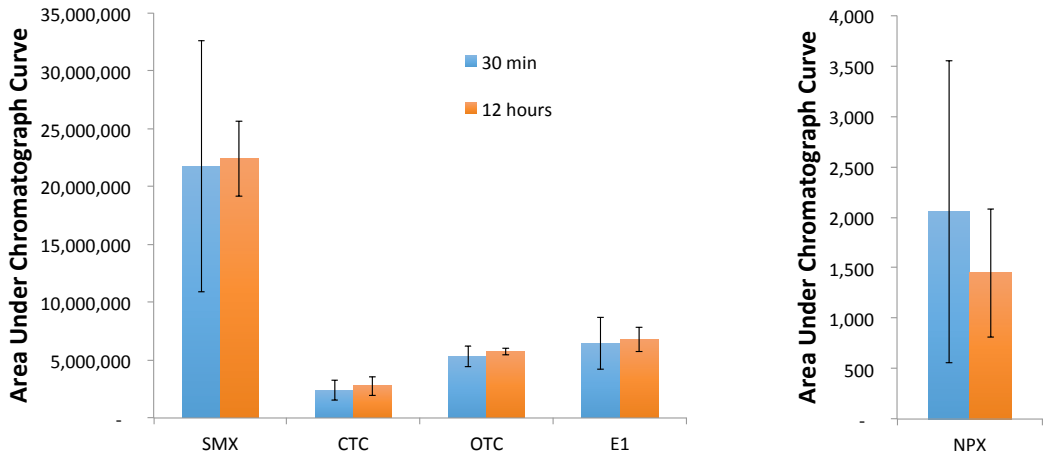

Figure 12: Comparison of 30 min and 12-hour incubations times

B.1.4 Sample evaporation

For the final test, sample extraction supernatant was directly analyzed by UPLC-MS/MS without evaporating with N_2. This produced the greatest improvement in extraction efficiency as seen in Figure 13. It is possible that analytes did not fully re-dissolve in methanol or that analytes were degraded during the evaporation process. Therefore, the evaporation step was removed from the final method. Target LODs were met after this method change.

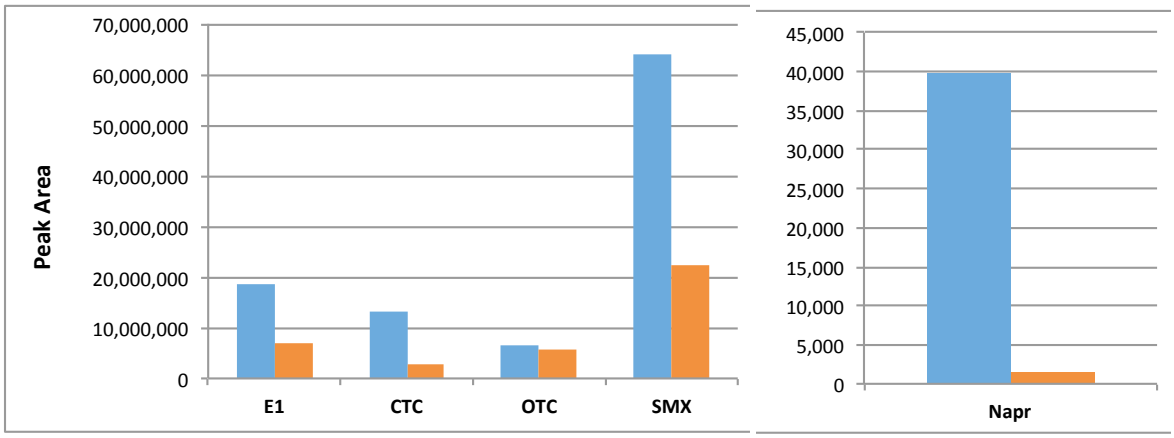

Figure 13: Comparison of extractions with and without evaporation
Peak areas for extractions without evaporation (blue) and for extractions with evaporation (orange).

B.2 Final extraction method

The finalized extraction method had an 85:15 methanol to water ratio, and a sample to extraction ratio of 1:4. Method steps were as follows:

1. At time of collection, 300 μL of MeOH and glacial acetic acid were added to the sample.
2. Samples were stored at -80 °C until each degradation test was complete so all samples from one test could be extracted at the same time.
3. The remaining extraction solution (700 μL of 6:1 MeOH: 0.1 M EDTA) and the internal standards were added to each sample.
4. Samples were extracted by horizontally vortexing for 30 min.
5. Samples were incubated at -80 °C for 12 hours.
6. Samples were then centrifuged (10 min, 12,000 x g) to settle all matrix solids into a pellet. Supernatant was collected and stored at -80 °C.
7. Samples were analyzed by UPLC-MS/MS within 24 hours of extraction. (Significant degradation was observed in samples that were stored for more than two weeks, so care was taken to analyze samples shortly after extraction.)

Ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was performed on a Waters Acquity UPLC coupled to a Waters Xevo TQ-S triple quadrupole mass spectrometer. Chromatographic separations were carried out on a Waters iKey HSS T3 microfluidic device (150 μm x 50 mm mm, 1.8 μM). UPLC-MS/MS configurations are described in the Methods and Materials section (Section 3.2.5). Product ions, collision energies, and cone voltages were optimized for each analyte by direct

injection of individual synthetic standards. Analyte retention times and transition ions are listed in Table 14. Chromatagraphs for each analyte are shown in Figure 14 through Figure 16.

Table 14: Transition table

Target Analyte	Retention Time (Min)	Cone (V)	Collision Energy (V)	Precursor Mass (m/z)	Product Mass (m/z)	pol
sulfamethoxazole	3.2	30	25	253.9	92	Positive
sulfamethoxazole	3.2	40	20	253.9	108	Positive
naproxen	4.0	30	25	230.92	170.5	Positive
naproxen	4.0	40	20	230.92	186	Positive
oxytetracycline	3.0	30	25	461	426	Positive
oxytetracycline	3.0	40	20	461	443	Positive
chlorotetracycline	3.2	30	25	479	444	Positive
chlorotetracycline	3.2	40	20	479	462	Positive
estrone-positive	4.1	35	19	271	133	Positive
estrone-positive	4.1	35	16	271	157	Positive
estrone-positive	4.1	35	19	271	159	Positive
naproxen-d3	4.0	45	25	234	173	Positive
naproxen-d3	4.0	35	15	234	188	Positive
demeclocycline-Hcl	3.1	15	15	464.8	448	Positive
demeclocycline-Hcl	3.1	15	30	464.8	289	Positive
sulfamethoxazole-d4	3.2	40	25	257.94	96	Positive
sulfamethoxazole-d4	3.2	40	25	257.94	112	Positive
estrone-d4-positive	4.1	45	10	275.98	256.9	Positive
estrone-d4-positive	4.1	20	20	275.98	135	Positive

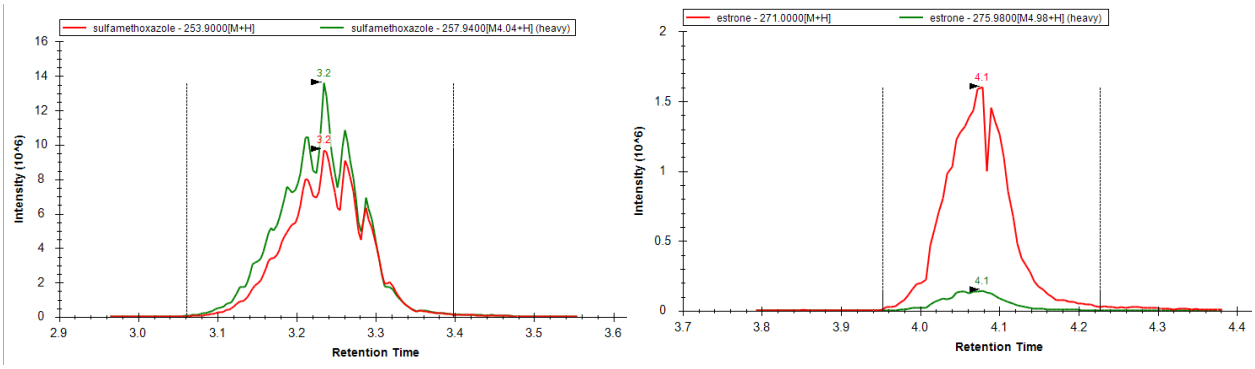

Figure 14: Chromatographs for SMX (left) and estrone (right).

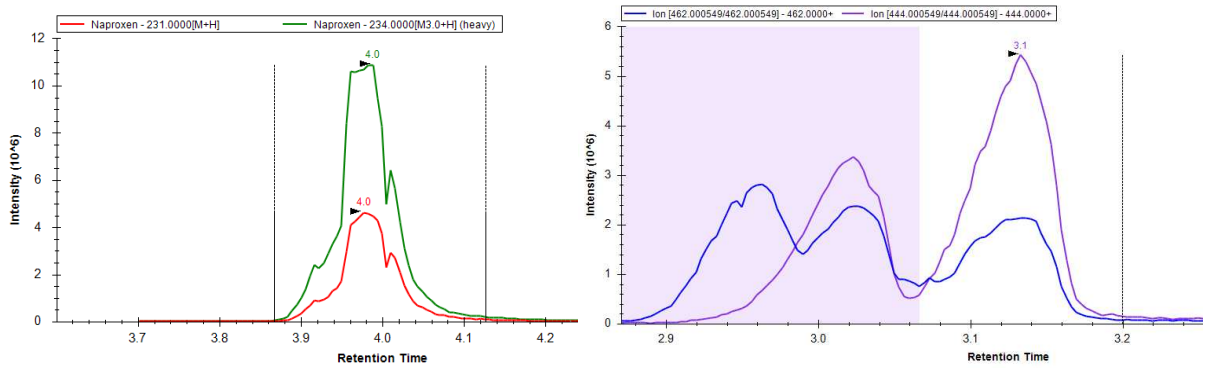

Figure 15: Chromatographs for NPX (left) and CTC (right).

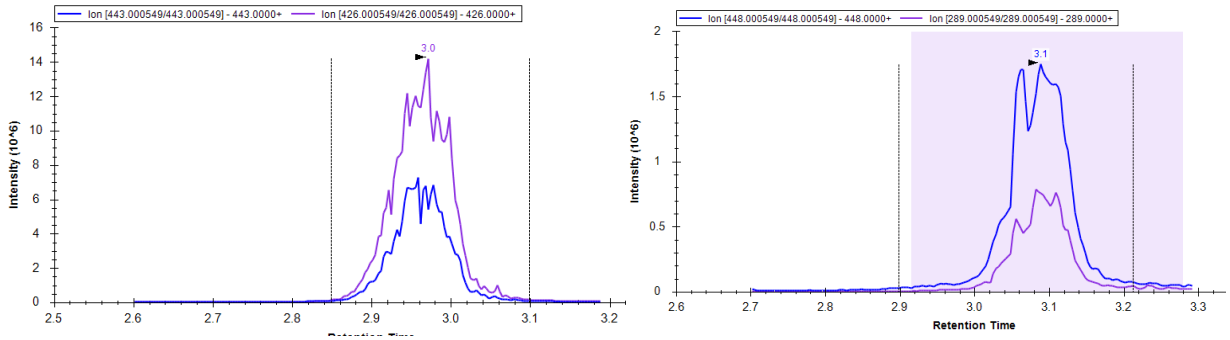

Figure 16: Chromatographs for OTC (left) and demeclocycline (right).

B.3 Method Validation

Internal standards were used to minimize between-sample quantification errors caused by slight variations in UPLC-MS/MS operations. Stable isotope-labeled internal standards were used for sulfamethoxazole (sulfamethoxazole-d3), estrone (estrone-d4), and naproxen (naproxen-d3). Demeclocycline was selected as a surrogate drug for chlortetracycline and oxytetracycline (O. Arikan et al., 2009; Shi et al., 2016). All samples were spiked with the same concentration of internal standards prior to extraction and

UPLC-MS/MS analysis so the area under each analyte's chromatograph curve could be normalized by the corresponding internal standard's chromatograph area.

Linear calibration curves were generated by spiking all 5 analytes into growth media at 11 concentrations between 0.5 to 1,000 ng/mL. Calibration curves were analyzed with UPLC-MS/MS before, after, and halfway through each batch of experiment samples. Each batch of samples consisted of roughly 70 experiment samples and took approximately 20 hours to run on the UPLC-MS/MS. Calibration curves were always essentially linear, with correlation coefficients greater than 0.99. Within-day variations of calibration curve slopes ranged between 1% for estrone and 18% for chlortetracycline (Figure 17). Average slopes of the three calibration curves were used to calculate analyte concentrations for samples run in the same batch.

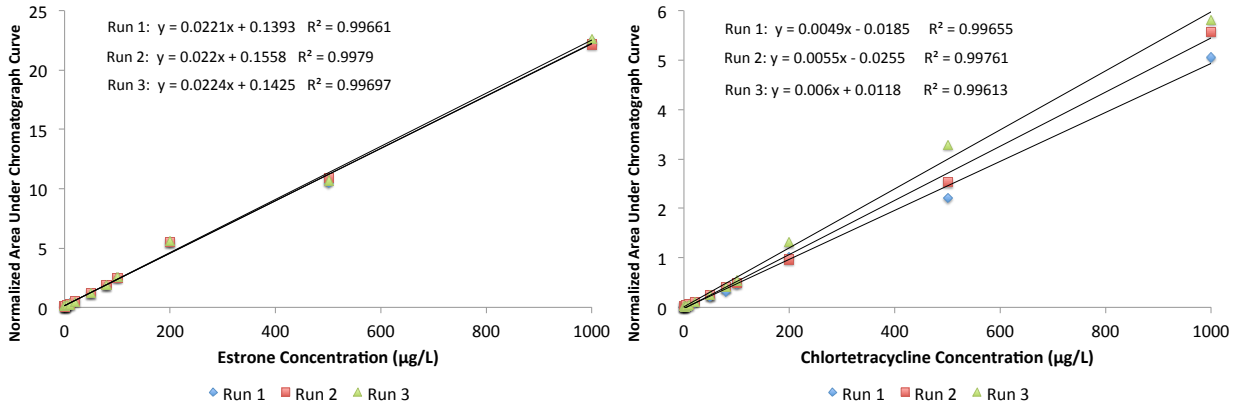

Figure 17: Example E1 and CTC calibration curves for one batch of samples.
(Run 1 = run at beginning of batch, Run 2 = run in the middle of the batch, Run 3 = run at the end of the batch)

Analyte limits-of-detection (LODs), limits-of-quantification (LOQs), and extraction efficiency were calculated and are listed in Table 9. LODs and LOQs were calculated by measuring the normalized area under the chromatograph curve for blank samples (media

with no drugs) and multiplying the resulting concentration (calculated with the calibration curve slope) by 3 for the LOD and by 10 for the LOQ. To determine extraction efficiency of all analytes, growth media was spiked with 50 μg/L of each analyte and the measured chromatograph areas were divided by the chromatograph areas measured when 50 μg/L were spiked into pure methanol.

APPENDIX C: STATISTICAL ANALYSIS TABLES

C.1 Comparison between operational conditions

Statistical analysis was performed with SAS Studio 3.71 (Basic Edition) for Windows. Multiple comparisons of the emerging contaminant (EC) concentrations between the different operating conditions were conducted by a one-way ANOVA with the GLM procedure. Results are presented in Table 15 through Table 18 (yellow highlight indicates significance level of 0.05, tan highlights indicate a significance level of 0.10). For the temperature experiment, the ANOVA analysis was conducted with both the original data (Table 15) and with the evaporation corrected data (Table 16) with little difference between the results.

While the ANOVA P-values indicate if there is enough evidence to suggest a difference between the three sample means for the operational conditions tested, it does not indicate which conditions were significantly different from each other. Therefore, a Tukey-Kramer HSD analysis was conducted on all data where the ANOVA analysis resulted in a significance level of 0.05. The results from the Tukey analysis are presented in Table 19 through Table 21, where the letter S indicates which conditions had significant differences in their sample means.

Table 15: Temperature test ANOVA analysis P-values based on original data

Day	Sulfamethoxazole	Naproxen	Estrone	Oxytetracycline	Chlorotetracyline
0	0.331	0.284	0.186	0.526	0.427
1	0.391	0.775	0.003	<.0001	0.223
2	0.001	0.946	0.021	0.218	0.008
4	<.0001	0.525	0.702	0.575	0.001
6	<.0001	0.176	0.464	0.479	0.000
9	<.0001	0.006	<.0001	0.953	0.000
13	<.0001	0.497	0.088	0.433	0.000
19	<.0001	0.593	<.0001	0.089	<.0001
25	<.0001	0.178	0.000	0.085	0.000
29	<.0001	0.277	0.009	0.857	0.003

Table 16: Temperature test ANOVA analysis P-values based on evaporation corrected data

Day	Sulfamethoxazole	Naproxen	Estrone	Oxytetracycline	Chlorotetracyline
0	0.331	0.284	0.186	0.526	0.257
1	0.405	0.784	0.003	<.0001	0.214
2	0.001	0.921	0.023	0.207	0.007
4	<.0001	0.574	0.625	0.580	0.001
6	<.0001	0.149	0.365	0.445	0.000
9	<.0001	0.006	<.0001	0.965	<.0001
13	<.0001	0.124	0.088	0.428	0.000
19	<.0001	0.280	<.0001	0.070	<.0001
25	<.0001	0.082	0.000	0.077	0.000
29	<.0001	0.283	0.009	0.799	0.002

Table 17: pH test ANOVA analysis P-values

Day	Sulfamethoxazole	Naproxen	Estrone	Oxytetracycline	Chlorotetracyline
0	0.868	0.757	0.827	0.213	0.507
0.33	0.843	0.801	0.664	0.025	0.100
0.66	0.163	0.913	0.433	0.001	0.729
1	0.230	0.903	0.766	0.030	0.426
2	0.011	0.873	0.527	0.159	0.651
4	0.356	0.253	0.253	0.228	0.747
6	0.361	0.304	0.412	0.505	0.177
9	0.030	0.012	0.415	0.148	0.247
13	0.000	0.010	0.157	0.686	0.120
17	0.009	0.016	0.057	0.962	0.250
22	0.000	0.038	0.223	0.765	0.522
29	<.0001	0.093	0.092	0.805	0.754

Table 18: Carbon content composition test ANOVA analysis P-values

Day	Sulfamethoxazole	Naproxen	Estrone	Oxytetracycline	Chlorotetracyline
0	0.670	0.427	0.410	0.040	0.184
0.33	0.298	0.388	0.494	0.635	0.762
0.66	0.697	0.665	0.423	0.391	0.378
1	0.144	0.815	0.106	0.193	0.172
2	0.091	0.468	0.121	0.928	0.659
4	0.003	0.718	0.080	0.907	0.611
6	<.0001	0.372	0.499	0.154	0.007
9	<.0001	0.312	0.180	0.530	0.004
13	0.001	0.582	0.001	0.111	0.001
17	0.001	0.239	0.000	0.101	0.136
22	0.013	0.133	0.000	0.686	0.740
29	0.001	0.042	<.0001	0.416	0.948

Table 19: Tukey analysis results for temperature test data.

	SMX			NPX			E1			OTC			CTC		
Day	35 C / 45 C	35 C/ 55 C	45 C / 55 C	35 C / 45 C	35 C/ 55 C	45 C / 55 C	35 C / 45 C	35 C/ 55 C	45 C / 55 C	35 C / 45 C	35 C/ 55 C	45 C / 55 C	35 C / 45 C	35 C/ 55 C	45 C / 55 C
0															
1							S	S		S	S				
2		S	S				S	S					S	S	
4	S	S	S										S	S	S
6	S	S	S										S	S	S
9	S	S	S	S		S	S	S					S	S	S
13	S	S	S										S	S	
19	S	S	S				S	S					S	S	
25	S	S	S				S	S					S	S	
29	S	S	S					S					S	S	

note: S indicates a significant difference in test condition results ($p < 0.05$)

Table 20: Tukey analysis results for pH test data.

	SMX			NPX			E1			OTC			CTC		
Day	pH 7 / pH 8	pH 7 / pH 9	pH 8 / pH 9	pH 7 / pH 8	pH 7 / pH 9	pH 8 / pH 9	pH 7 / pH 8	pH 7 / pH 9	pH 8 / pH 9	pH 7 / pH 8	pH 7 / pH 9	pH 8 / pH 9	pH 7 / pH 8	pH 7 / pH 9	pH 8 / pH 9
0															
0.33										S	S				
0.66										S	S				
1											S				
2		S													
4															
6															
9		S		S		S									
13	S	S	S	S		S									
17		S		S		S		S							
22	S	S				S									
29	S	S	S												

note: S indicates a significant difference in test condition results ($p < 0.05$)

Table 21: Tukey analysis results for carbon content composition test data.

	SMX			NPX			E1			OTC			CTC		
Day	Short / Med	Short / Long	Med / Long	Short / Med	Short / Long	Med / Long	Short / Med	Short / Long	Med / Long	Short / Med	Short / Long	Med / Long	Short / Med	Short / Long	Med / Long
0										S					
0.33															
0.66															
1															
2															
4	S	S													
6	S	S	S										S		S
9	S	S	S											S	S
13	S	S						S	S					S	S
17		S	S					S	S						
22		S	S					S	S						
29		S	S		S			S	S						

note: S indicates a significant difference in test condition results ($p < 0.05$)

C.2 Comparison between live and killed control reactors

The Student's t-Test was used to compare the samples collected from the live reactors and their corresponding killed control reactors. Statistical analysis was performed with the T.TEST function in Microsoft Excel for all data except the 35 °C reactors for the temperature test. Two of the three 35 °C killed control reactors became contaminated and data from those reactors was unusable. Instead, the probability that the killed control reactor value would be included in T-distribution estimated from the live reactor data was calculated (this assumes the killed control sample data would have the same standard deviation as the live sample data). This was done using the T.DIST.RT function in Microsoft Excel. Results are presented in Table 22 through Table 24 (yellow highlight indicates significance level of 0.05, tan highlights indicate a significance level of 0.10).

Table 22: Student's t-Test analysis comparing temperature experiment live and killed control reactors

Day	SMX			NAP			E1			OTC			CTC / eCTC		
	35 °C	45 °C	55 °C	35 °C	45 °C	55 °C	35 °C	45 °C	55 °C	35 °C	45 °C	55 °C	35 °C	45 °C	55 °C
1	0.010	0.520	0.348	0.095	0.085	0.687	0.008	0.074	0.398	0.051	0.392	0.098	0.016	0.023	0.004
2	0.001	0.007	0.184	0.101	0.422	0.326	0.004	0.338	0.869	0.002	0.055	0.021	0.007	0.007	0.000
4	0.000	0.000	0.221	0.226	0.551	0.767	0.014	0.124	0.766	0.008	0.384	0.045	0.004	0.000	0.005
6	0.000	0.001	0.055	0.461	0.700	0.748	0.000	0.089	0.043	0.008	0.001	0.041	0.001	0.001	0.019
9	0.000	0.462	0.173	0.023	0.566	0.478	0.000	0.312	0.040	0.022	0.514	0.051	0.001	0.000	0.056
13	0.000	0.011	0.663	0.001	0.443	0.350	0.001	0.734	0.144	0.113	0.107	0.506	0.008	0.457	0.724
19	0.000	0.002	0.185	0.013	0.940	0.498	0.000	0.050	0.006	0.033	0.469	0.030	0.003	0.046	0.041
25	0.000	0.451	0.118	0.001	0.494	0.647	0.000	0.106	0.000	0.446	0.921	0.052	0.011	0.415	0.053
29	0.000	0.491	0.148	0.001	0.345	0.906	0.000	0.042	0.063	0.142	0.485	0.016	0.044	0.403	0.074

Table 23: Student's t-Test analysis comparing pH experiment live and killed control reactors

Day	SMX			NAP			E1			OTC			CTC / eCTC		
	pH 7	pH 8	pH 9	pH 7	pH 8	pH 9	pH 7	pH 8	pH 9	pH 7	pH 8	pH 9	pH 7	pH 8	pH 9
0.33	0.649	0.473	0.855	0.969	0.293	0.594	0.274	0.453	0.610	0.053	0.026	0.001	0.913	0.003	0.336
0.66	0.063	0.253	0.723	0.190	0.313	0.146	0.203	0.501	0.501	0.053	0.057	0.052	0.533	0.056	0.093
1	0.000	0.141	0.295	0.252	0.224	0.457	0.017	0.550	0.545	0.421	0.027	0.906	0.513	0.021	0.210
2	0.000	0.031	0.045	0.057	0.439	0.731	0.017	0.693	0.260	0.669	0.516	0.755	0.086	0.014	0.018
4	0.107	0.001	0.014	0.681	0.659	0.681	0.498	0.476	0.086	0.375	0.423	0.196	0.000	0.007	0.004
6	0.000	0.001	0.024	0.066	0.376	0.173	0.048	0.605	0.039	0.573	0.187	0.687	0.004	0.036	0.028
9	0.000	0.000	0.002	0.538	0.225	0.139	0.030	0.064	0.034	0.322	0.872	0.699	0.025	0.002	0.016
13	0.000	0.021	0.000	0.008	0.058	0.453	0.023	0.438	0.015	0.704	0.807	0.491	0.060	0.071	0.033
17	0.001	0.005	0.000	0.004	0.002	0.132	0.011	0.073	0.002	0.704	0.435	0.423	0.841	0.222	0.338
22	0.000	0.001	0.014	0.033	0.034	0.106	0.180	0.004	0.000	0.908	0.423	0.759	0.308	0.639	0.897
29	0.004	0.022	0.001	0.019	0.118	0.016	0.263	0.000	0.006	0.557	0.787	0.967	0.691	0.699	0.667

Table 24: Student's t-Test analysis comparing carbon content composition experiment live and killed control reactors

Day	SMX			NAP			E1			OTC			CTC / eCTC		
	Short SRT	Med SRT	Long SRT	Short SRT	Med SRT	Long SRT	Short SRT	Med SRT	Long SRT	Short SRT	Med SRT	Long SRT	Short SRT	Med SRT	Long SRT
0.33	0.771	0.392	0.425	0.705	0.366	0.637	0.982	0.479	0.873	0.055	0.174	0.196	0.222	0.548	0.566
0.66	0.536	0.604	0.348	0.282	0.844	0.460	0.569	0.956	0.064	0.005	0.016	0.182	0.163	0.535	0.020
1	0.087	0.976	0.675	0.614	0.483	0.956	0.355	0.759	0.233	0.040	0.915	0.434	0.131	0.511	0.165
2	0.001	0.132	0.171	0.795	0.401	0.767	0.083	0.351	0.457	0.456	0.643	0.869	0.008	0.204	0.149
4	0.000	0.027	0.038	0.715	0.912	0.675	0.070	0.402	0.433	0.730	0.893	0.948	0.017	0.021	0.045
6	0.000	0.012	0.021	0.962	0.523	0.195	0.777	0.621	0.075	0.195	0.460	0.983	0.020	0.001	0.001
9	0.002	0.002	0.002	0.793	0.529	0.882	0.291	0.993	0.083	0.926	0.026	0.534	0.008	0.019	0.024
13	0.000	0.007	0.049	0.616	0.055	0.481	0.081	0.953	0.002	0.593	0.117	0.752	0.026	0.002	0.002
17	0.000	0.001	0.086	0.371	0.106	0.331	0.018	0.937	0.007	0.199	0.132	0.756	0.164	0.062	0.055
22	0.003	0.002	0.031	0.506	0.104	0.562	0.001	0.301	0.001	0.488	0.914	0.415	0.094	0.249	0.137
29	0.000	0.010	0.010	0.420	0.021	0.785	0.000	0.117	0.000	0.718	0.847	0.701	0.536	0.383	0.558

APPENDIX D: CTC Transformation Product Data

In aqueous samples the structure of chlortetracycline (CTC) allows for easy epimerization to form epi-chlortetracycline (e-CTC) as well as intra-molecular rearrangement to form isochlortetracycline (Shelver & Varel, 2012). In addition, while under acidic conditions the hydroxyl group can undergo acid degradation to form anhydrochlortetracycline (ACTC) (Diana, Vandenbosch, De Spiegeleer, Hoogmartens, & Adams, 2005). Figure 18shows six of the most common forms of CTC related compounds and the conditions under which they are formed. Numerous studies have shown it is possible to isolate the CTC related compounds using LC-MS (G. Chen, Zhao, & Dong, 2011; Diana et al., 2005; Shelver & Varel, 2012; Tylova, Olsovska, Novak, & Flieger, 2010), however retention times and the order the compounds leave the chromatograph vary based on system setup.

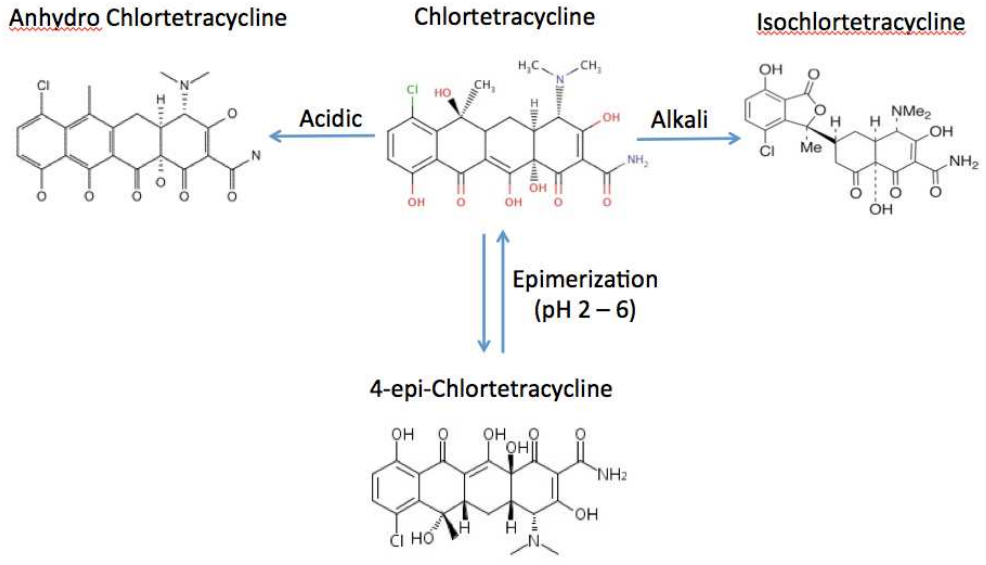

Figure 18: Chemical structures of chlortetracycline and transformation products

As with the studies mentioned above, for this study CTC was transformed into at multiple related compounds. Fragment ions at m/z 444 and 462 where used to identify CTC and its transformation products. The resulting chromatograms exhibited three distinct peaks at retention times 3.0, 3.1,and 3.2 min (Figure 19). The left figure is the chromatogram for CTC in MeOH, while the right figure is CTC in growth media. From these two figures the peak at 3.2 min is expected to be CTC. In Figure 19 the fragment ion for m/z 444 is shown in red and while m/z 462 is blue. Solliec (2016) found that the CTC and eCTC had fragment ions at m/z 444 while ICTC and its epimer EICTC did not. Although additional testing would need to be done to confirm the compounds identities, it is believed that the peak at retention time 3.1 is likely to be eCTC, while the peak at 3.0 min could be either ICTC or EICTC. Since epimerization of CTC typically occurs for pH levels between 2 and 6 it is not known if the eCTC was formed during the experiment or during the extraction process when the pH was dropped to roughly pH 3. The formation of ICTC would be expected during the course of the experiment since reactors were maintained in the range of pH 7 to 9.

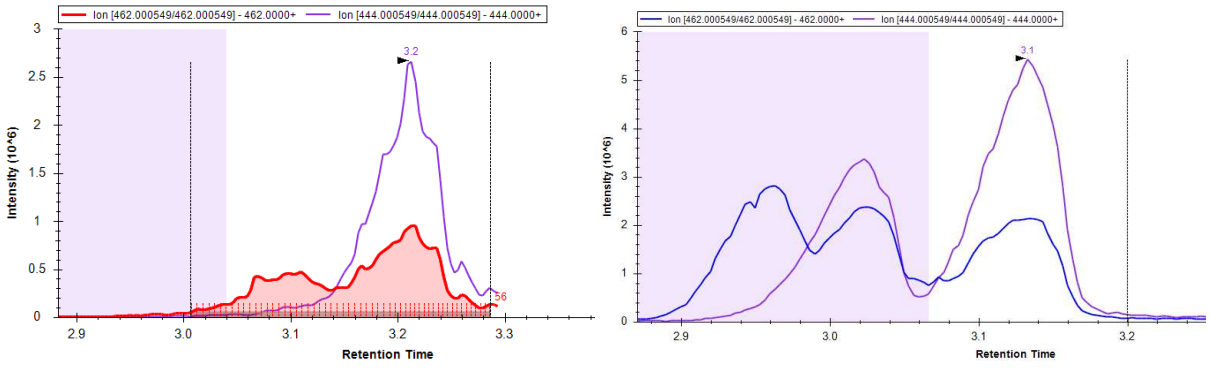

Figure 19: Chromatograms of CTC and transformation products.
Compounds in MeOH (left) and in CCSM growth media (right).

Concentrations used for the thesis experiment were calculated from the combined area under all three peaks so degradation would not be overestimated as CTC was transformed to its related compounds. Concentrations were also calculated for the three separate peaks to determine if test conditions had an impact on the rate of transformation or the subsequent degradation for the individual compounds. Figure 20 shows the resulting breakdown of concentrations based on each peak. The concentrations associated with CTC (red lines) rapidly degraded under all conditions. The concentrations associated with eCTC (green lines) initially increased and then decreased more slowly than CTC although it dropped to levels below the detection limit by the end of the experiment under all conditions. The third peak (blue lines) had the largest increase in concentration during the first two days of the experiment and then had a more gradual degradation than the other two compounds. If this compound were ICTC, the results would be similar to those in other studies. Arikan et al. (2009) found the concentration of ICTC to initially increase, and then decrease at a rate similar to combined CTC/eCTC during a composting experiment, although initial concentrations of ICTC were only a fifth of the combined CTC/eCTC. Shelver and Varel (2012) had initial ICTC concentrations that were nine times higher than CTC concentrations in manure from swine that were administered CTC. At 38 and 55 °C the CTC degraded to near detection limit over the first two weeks. At lower temperatures (22 and 38 °C), the ICTC concentrations continued to increase over the 28 days of the experiment, while at 55 °C ICTC concentrations increased for the first 5 days and then stayed level. It was not clear if there was any aeration provided during the 28 days, so this could have been under anaerobic conditions. In addition, the pH for the experiment described in Shelver and Varel (2012) ranged from 6.1 to 7.3, which was lower than the pH

for the current research. For this experiment low temperature and high concentrations of nutrients appear to have lengthened the degradation time, where high temperature and low nutrient levels appear to have shortened the degradation time. The data from the pH experiment indicate that pH had little impact on degradation time.

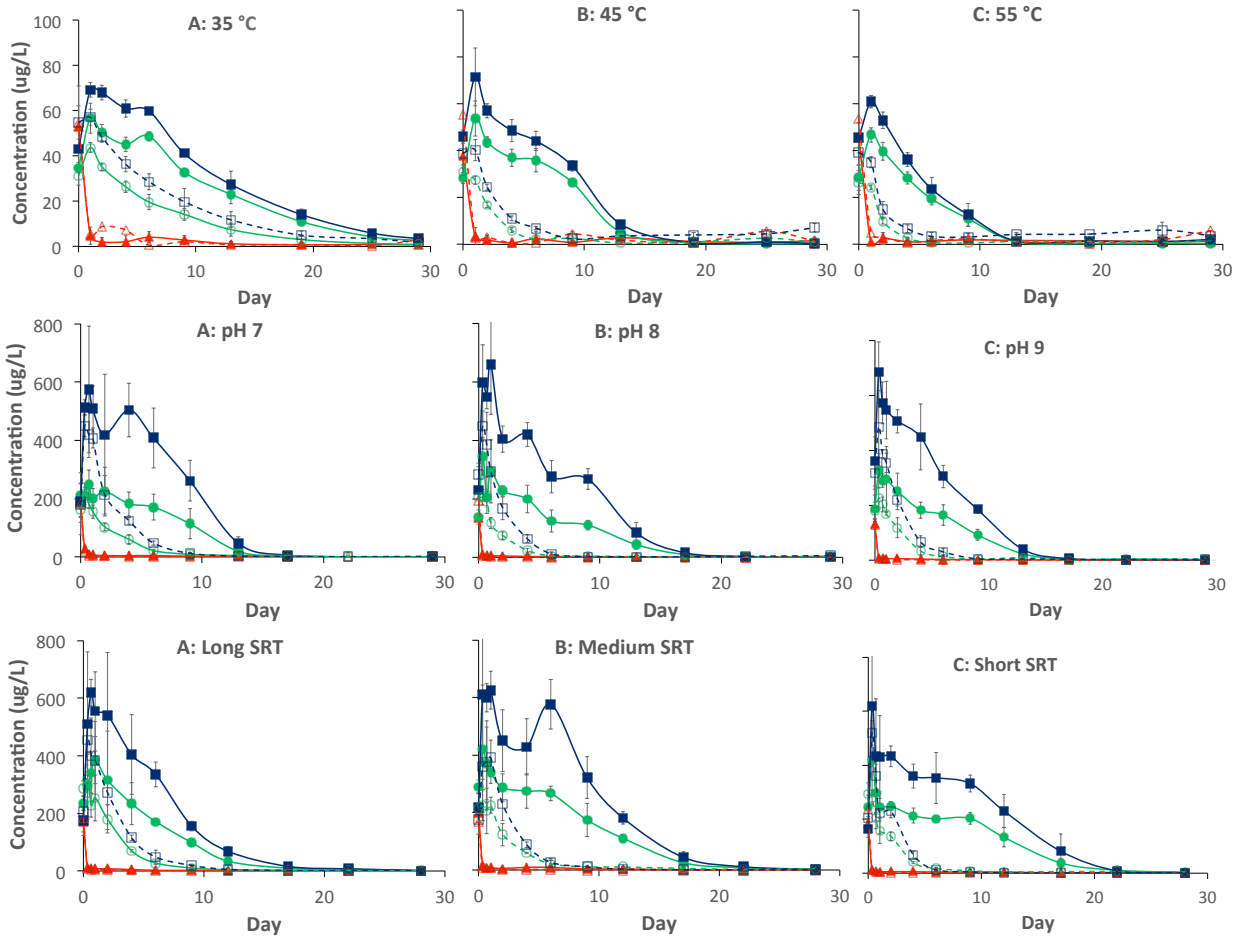

Figure 20: Relative concentrations of CTC and transformation products. Concentrations for original CTC (red triangles), transformation product believed to be epi-CTC (green circles), and transformation product believed to be isoCTC (blue squares). Solid markers and lines are for live reactors and outline markers with dotted lines are for killed control reactors.

LIST OF ABBREVIATIONS

AC	Aerobic Composting
AD	Anaerobic Digestion
AD/AC	Combined Anaerobic Digestion and Aerobic Composting System
ANOVA	Analysis of Variance
ARGs	Antibiotic Resistant Genes
C/N	Carbon to Nitrogen ratio
CCSM	Co-Digestion Compost Synthetic Manure
CTC	Chlortetracycline
E1	Estrone
EC	Emerging Contaminant
eCTC	epi-Chlortetracycline
EDTA	Ethylenediaminetetraacetic acid
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
MS	Mass Spectrometry
MSAD	Multi-Stage Anaerobic Digestion
NPX	Naproxen
NSAID	Non-Steroidal Anti-Inflammatory Drugs
OTC	Oxytetracycline
SMX	Sulfamethoxazole
SPE	Solid Phase Extraction
SRT	Solids Retention Time
UPLC-MS/MS	Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry
VA	Veterinary Antibiotic
VFA	Volatile Fatty Acids