ABSTRACT

Title of Thesis:	FATE OF ANTIMICROBIALS AND NUTRIENTS IN DAIRY MANURE MANAGEMENT SYSTEMS
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Anaerobic digestion (AD) and composting manure management strategies were explored at the field scale to monitor antimicrobial degradation, nutrient transformations, and optimize mitigation of these pollutants in manure fertilizer to decrease their entry to waterways. Removal of antimicrobials and antimicrobial resistance genes (ARGs) were explored at the bench scale, where AD degraded >85% of antimicrobials. At the field-scale, antimicrobials were not consistently removed, persisting in concentrations up to 34,000 ng/g DW in the AD effluent. The tetM genes were reduced during bench-scale AD suggesting that AD could be an effective treatment for removing tetracycline ARGs from manure. The 100% reduction of sulfadimethoxine antimicrobials during AD did not correspond with Sul1 reduction, illustrating differences in antimicrobial versus gene reductions during manure treatment. Antimicrobials did not degrade significantly during field scale composting, likely due to a shortened composting period (33-days). The field-scale results illuminate limitations of tracking antimicrobials in complex treatment systems.

FATE AND TRANSPORT OF NUTRIENTS AND ANTIMICROBIALS IN DAIRY MANURE MANAGEMENT SYSTEMS

by

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List of Abbreviations

Anaerobic Digestion (AD)

Antimicrobial Resistant Genes (ARGs)

Biochemical Methane Potential (BMP)

Collaborating Farm (CF)

Concentrated Animal Feeding Operation (CAFO)

High Intensity Management (HIM)

Hydraulic Retention Time (HRT)

Low Intensity Management (LIM)

Solid Liquid Separation (SLS)

Sulfadimethoxine (SDM)

Sulfamethazine (SMZ)

Tetracycline (TC)

Total Ammonia Nitrogen (TAN)

Total Kjeldahl Nitrogen (TKN)

Total Maximum Daily Load (TMDL)

Total Phosphorus (TP)

Total Solids (TS)

Veterinary Feed Directive (VFD)

Volatile Fatty Acids (VFAs)

Volatile Solids (VS)

1.0 Introduction

1.1 Role of agriculture in pollution of the Chesapeake Bay watershed

The Chesapeake Bay is the largest of over 100 estuaries in the United States. Its watershed extends over six states and is home to over 18 million people and 3600 species of plants and animals (Chesapeake Bay Program, 2017). The Chesapeake Bay watershed is utilized as a recreational area by many and holds vital economic significance. The value added by seafood to the economy from Maryland Chesapeake Bay fisheries was estimated to be $600 million (Maryland State Archives, 2017). The economic productivity of the Chesapeake Bay tributaries depends on the health of the Bay and its fisheries.

Excess nutrient loads are causing eutrophication and hypoxic zones in the Chesapeake Bay and its tributaries, limiting fishery productivity. The Chesapeake Bay Total Maximum Daily Load (TMDL) was established by the Environmental Protection Agency in 2010 to reduce pollution to the Bay by setting pollution limits of 185.9 million lbs of nitrogen, 12.5 million lbs of phosphorus, and 6.45 billion lbs of sediment per year, that are to be met by 2025. A midpoint goal in 2017 was set, where 60% of overall pollution reductions for the 2025 TMDL were to be achieved. Pollution loads tabulated from 2017 showed that while targets were met for phosphorus reductions, the goal for nitrogen reduction was missed by 15 million lbs (EPA, 2018a).

Most of the pollution reductions to date are currently attributed to updates in wastewater treatment plants, however, more reduction is needed in the agriculture and urban sectors to meet the TMDL due to the significant contributions in these sectors to overall pollution to the Bay. The agricultural industry is the largest contributor to nitrogen, phosphorous, and sediment loads into the Chesapeake Bay (Chesapeake Progress, 2017), so there is a significant need for improvement in farm management practices to better control nutrient pollution to the Bay.

Agriculture is not only introducing nutrients into the Bay, but it is also transporting antimicrobial residues in treated and untreated manure fertilizer runoff. Antimicrobial use in animal husbandry is more pervasive and less strictly regulated than in humans. The Union of Concerned Scientists found that a yearly average of 24.6 million pounds of antimicrobials were used in animal husbandry for non-therapeutic purposes. Non-therapeutic use in humans is less than one sixth of that amount (Mellon et al., 2001). Farmers use antimicrobials for active treatment, however, antimicrobials are widely used for preventative treatment as well. Landers et al. (2012) reported that while approximately 16% of cows receive active intramammary treatment, nearly all cows receive preventative treatment (penicillins, cephalosporins, and beta-lactams) following lactation to prevent future occurrences of mastitis. Up to 90% of certain antimicrobials can be excreted unchanged in manure, making manure fertilizer application a transport mechanism for these antimicrobials to enter terrestrial and aquatic environments. Antimicrobials are now being detected in the Chesapeake Bay and its tributaries. A 2005 study detected oxytetracycline in two Chesapeake Bay tributaries (Simon, 2005), and a 2017 study testing for 43 different antimicrobials in 3 different drug classes at 14 sites along the Eastern Shore of the Chesapeake Bay, detected veterinary antimicrobials in concentrations up to 94 ng/L (He et al., 2019).

The impact of different manure treatments on antimicrobial degradation and their relationship to nutrients and other manure characteristics are not well known. Methods of manure management and treatment and subsequent use as fertilizer are highly varied. Understanding fate and transport of nutrients and antimicrobials during manure management can be used to better inform on-farm management of these pollutant inputs in manure fertilizers and subsequently to the Bay.

1.2 Manure management techniques

There are many different methods and approaches to manure management. Scrape or flush systems can be used to transport manure from stall to storage pits. The manure transport type can significantly alter the dilution of manure. Once manure is collected, several processes can be utilized to store or treat it. Manure management systems can utilize different configurations of solid-liquid manure separation, separated solids composting, anaerobic digestion (AD), and lagoon storage. Additionally, some manure systems co-digest livestock manure with other components, such as food waste.

Composting is a controlled method of aerobic decomposition utilized to break down organic matter into a stable soil amendment. Composting is often implemented on the manure solids that are separated out of liquid manure in dairy operations with solid liquid separation systems. Nutrients stabilize during the composting process, which slows their release to the soil after land application (Larney et al., 2006).

AD, a technology that is utilized in multiple industries ranging from large-scale wastewater treatment facilities to small-scale dairy manure management systems, is becoming more widely used in the dairy industry for processing manure. AD is a microbial process that degrades organic material in the absence of oxygen. The products of AD include biogas (methane, carbon dioxide, and other trace gases) and the liquid effluent from the digester (digestate). The implementation of AD on livestock farms has increased in the United States from 160 projects in November 2011 to 253 operational projects as of April 2018. Out of the 253 operational projects, 203 are located on dairy farms (EPA, 2018). The adoption of AD has beneficial impacts for nutrient management, as studies have shown that AD digestate increased N uptake and crop yields when used as a field fertilizer, compared to untreated manure (Möller et al., 2008).

Several options are available to farmers for long-term storage of manure. Liquid storage lagoons are open to the elements and reduce odors and break down organic matter. Slurry manure is thicker than liquid manure and contains approximately 5-15% solids. Fabricated manure storage tanks, generally made from concrete, can be used to store slurry.

Dairy farmers utilize the nutrient-rich lagoon storage water, compost, or digestate for field-application as a crop fertilizer, therefore, nutrients and antimicrobials present in the fertilizer can then enter waterways through fertilizer runoff. Most farms are required to have nutrient management plans to control the release of nutrients into the environment and mitigate their environmental impact, however, the impact of antimicrobial use and its relationship to manure nutrients during manure management is not well understood.

1.3 Antimicrobial use in animal husbandry

Historically, antimicrobials have been used in the livestock industry for a multitude of reasons, including treating disease, managing infection, and promoting livestock growth (Chee-Sanford et al., 2009; Watanabe et al., 2010). Several antimicrobials are simultaneously administered to a farm's dairy herd to treat the variety of illnesses that occur on farm. Different amounts of antimicrobials are used throughout the year depending on the number of sick cows and the illnesses present. Treatments can also be seasonally dependent. Mastitis, one of the most commonly treated illnesses on dairy farms, is a bacterial infection that occurs in the teat canals of a lactating cow, which causes udder inflammation.

Clinical mastitis treatment was reported in 14% of lactating cows, 8% of cows in early/late drying off periods, and 5% of heifers during a survey of antimicrobial administration at 113 farms in Pennsylvania. Cepaphirin was the preferred antimicrobial treatment for mastitis on 49% of farms. Twenty-four different antimicrobials were administered including drug classes of beta-lactams, spectinomycin, florfenicol, and tetracyclines (Sawant et al., 2005).

Ceftiofur was the most commonly administered antimicrobial in a survey of 99 dairy farms in Michigan, Minnesota, New York, and Wisconsin. On average, 79.8% of herds were treated with antimicrobials for clinical mastitis (Zwald et al., 2004). A survey of 20 Wisconsin farms said that farmers reported penicillin as the most common dry-cow therapy, and cephapirin as the most common treatment for clinical mastitis (Pol et al., 2007).

In December 2016, the US Federal Drug Administration established the Veterinary Feed Directive (VFD), which put policies in place to more strictly regulate the therapeutic use of medically relevant antimicrobials in water and food-producing animals by requiring licensed veterinarian authorization for the use of these antimicrobials (FDA, 2018). Studies monitoring antimicrobial administration on US dairies have not been reported since the implementation of the VFD.

1.4 Antimicrobial persistence in manure

The literature has shown that antimicrobials do not break down completely in the digestive tract of the cow and antimicrobial parent compounds and metabolites may pass through the cow's digestive system into their manure. Up to 90% of the antimicrobials that are administered orally to livestock can be excreted by the animal unchanged (Kumar et al., 2005). The varied antimicrobial administration and excretion can create fluctuating spikes of antimicrobial residuals in the manure management system and the digestate/compost or lagoon waste applied to the soil.

Several studies suggest that residual antimicrobials in livestock waste that is utilized as an agricultural fertilizer may cause rural surface waters to become a pool for veterinary antimicrobials and antimicrobial resistant bacteria (Kay et al., 2004; Zhang et al., 2014). Other studies have demonstrated antimicrobial uptake in plants that are fertilized with antimicrobial laden manure (Kumar et al., 2005; Tasho et al., 2016).

Antimicrobial persistence in raw or treated manure fertilizers presents environmental and human health concerns. Several low doses of common agricultural antimicrobials have been shown to be toxic to soil organisms and plants (Kumar et al., 2005). Additionally, some antimicrobials, such as penicillin, can elicit an allergenic response in some humans during exposure (Kemper et al., 2008). Several studies have examined the impact of antimicrobials on compost manure nutrients (Ho et al., 2013; Selvam et al., 2012), indicating that antimicrobials presence and use could have significant implications for nutrient management on farms.

Consistent use of antimicrobials in livestock and agricultural systems has also raised concern for the spread of antimicrobial resistant genes (ARGs), and the implications that the spread of ARGs poses towards human health. Antimicrobial resistance in bacteria develops when those bacteria are exposed to an antimicrobial and develop a resistance against that antimicrobial agent. Those bacteria then have the means to transfer that resistance to their daughter organisms and potentially other bacteria through genetic exchange using plasmids (Khachatourians, 1998).

There are structural similarities between some veterinary antimicrobials (VAs) and antimicrobials used on humans. Bacteria that has developed a resistance to one or more VAs could foster resistance in a structurally similar human antimicrobial. Multi-drug resistant bacteria limit treatment options for infections. The World Health Organization (WHO) released a list of antimicrobial-resistant priority pathogens in February 2017 to encourage research and development of new antimicrobials to fight these 12 families of bacteria (WHO, 2017). Development of new antimicrobials is a short-term solution to increasing abundance and spread of antimicrobial resistance, if current infrastructure promotes antimicrobial-resistance development.

1.5 Study objectives

Understanding how antimicrobials and nutrients perpetuate through manure management systems can aid in developing best management practices on farms to mitigate antimicrobial resistance development and nutrient pollution at the source. Anaerobic digestion or composting could potentially be used to control the spread of ARG in environmental systems. This study examines the fate of antimicrobials and nutrient concentrations in various manure management systems to help better inform farmers on best management practices for nutrient application and antimicrobial resistance mitigation and to achieve the overall goal of reducing these two pollutants of concern in the environment.

Chapter two explores the fate of tetracyclines, sulfonamides, and nutrients at the field scale before and after anaerobic digestion of dairy manure. This research illuminates the impact of anaerobic digestion on antimicrobial degradation and nutrient transformation at the field scale using a variety of farm management and digester operating conditions.

Chapter three examines the fate of antimicrobials and nutrients during field scale composting. The objective of this study was to monitor nutrient transformations and antimicrobial degradation under real farm management practices and environmental conditions to better understand trends at the field scale. Understanding fate at the field scale is key to applying relevant and effective farm management techniques to mitigate nutrient runoff and reduce antimicrobial spread to the watershed.

Chapter four examines degradation of antimicrobials during bench scale mesophilic anaerobic digestion of dairy manure. The goal of this study was to monitor degradation patterns of antimicrobials under a more controlled environment and provide a comparison to trends of degradation monitored at the field scale.

This research can be used to further the understanding of nutrient transformations and antimicrobial degradation under various manure management conditions. The research seeks to compare trends seen in antimicrobial degradation in the field scale to the bench scale and give perspective on varying conditions when scaling up results found at the bench scale to field scale environments.

2.0 Antimicrobial and nutrient field scale monitoring during anaerobic digestion of dairy manure

2.1 Introduction

Concentrated animal feeding operations (CAFOs) are considered point sources of nitrogen, phosphorus, and sediment pollution by the United State (US) Environmental Protection Agency, and manure and wastewater from CAFOs are regulated for pollutant discharge to US waters. CAFOs use a variety of manure management systems to handle their manure, and farmers utilize nutrient-rich manure lagoon storage water, compost, or anaerobic digestate for field-application as a crop fertilizer. The implementation of anaerobic digestion (AD) on livestock farms in the US has increased from 160 projects in November 2011 to 253 operational projects as of April 2018. Out of the 253 operational projects, 203 are located on dairy farms (EPA, 2018).

AD systems provide many benefits to farmers, including renewable energy through generation of biogas and nutrient rich fertilizer from the AD effluent digestate, giving farmers incentive to adopt this technology. Digestion systems in the dairy industry vary widely on farms depending on farm management and capacity, and therefore, understanding nutrient trends during digestion and under different management and operating conditions is critical to provide comprehensive information for farm nutrient management. Comparisons of nutrient dynamics and antimicrobial degradation in different on-farm digestion systems have yet to be explored.

In addition to introducing nutrient pollutants to waterways through agricultural runoff, manure fertilizers can also be a source of antimicrobial introduction to the environment. The potential of AD technology to mitigate the spread of antimicrobials at the field scale is still relatively unknown, as digester systems on farms are variable in terms of design, scale, capacity, and operating parameters. While some bench scale experimentation has been performed to understand tetracycline (TC) degradation during dairy manure digestion, field scale studies are lacking, and the fate of sulfonamide degradation during AD is not well explored at any scale.

Arikan et al. (2008) examined the fate of chlortetracycline (CTC) during bench scale mesophilic AD of beef cow manure, where the cows were dosed with 22 mg/kg/day of CTC for 5 days. They reported 75% reductions of CTC after the 33-day digestion period. Degradation has also been observed during bench scale thermophilic digestion of dairy cow manure. Beneragama et al. (2013) recorded 70-80% reduction in oxytetracycline (OTC) when spiked at concentrations of 30-90 mg/L, while maintaining digestion process stability. Mitchell et al. (2013) is one of the only dairy manure AD studies that examines the fate of sulfonamides during a 40-day mesophilic bench scale digestion incubation period. Sulfamethazine did not degrade significantly when added in concentrations up to 280 mg/L to the digester.

Results from the literature at the bench scale suggest that AD has the potential to significantly reduce the concentrations of antimicrobials, thereby lowering the concentration present in the effluent that is applied to agricultural fields and exposed to plants and waterways. However, scaling up findings confirmed at the bench scale may not be an accurate assumption. Few on-farm monitoring studies exist that monitor tetracyclines in AD systems. Younquist et al. (2016) provided a comprehensive review of the existing literature on antimicrobial degradation during AD and did not cite a single study that examined antimicrobial degradation during AD at the farm-scale.

Field digestion studies examining antimicrobial degradation during AD are not well explored. However, Wallace et al. (2018) examines the fate of several antimicrobials during advanced AD with pasteurization and food waste co-digestion at a 2200-cow dairy. Sulfonamides were reported to be absent in most manure samples, while concentrations of TCs increased in solid fractions of manure post-digestion, differing largely from typical degradation patterns seen at the bench scale.

On-farm digesters are complex and diverse systems that are exposed to a variety of changing environmental factors, and therefore, understanding how results observed in bench scale reactors compare to on-farm practice is critical to providing management strategies that optimize AD performance and nutrient management while mitigating antimicrobial persistence in AD digestate fertilizers. Studies at the bench scale often observe concentrations of antimicrobials that far exceed concentrations actually observed at the field scale, so this study will determine the range of concentrations seen on existing operations using different manure management techniques for further study.

The objectives of this study are to: 1) quantify the impact of AD on tetracycline and sulfonamide mitigation in AD effluent at the field scale; 2) monitor volatile solids and volatile fatty acid changes during digestion, and 3) monitor nutrient (total ammonia nitrogen, total Kjeldahl nitrogen, total phosphorus) fate before and after digestion in multiple field scale management scenarios. The overall goals of this research are to better understand anaerobic digestion at the field scale under a variety of farm management conditions, to illuminate antimicrobial and nutrient fates before and after digestion, and to enhance on-farm management of pollutants into the surrounding waterways.

2.2 Methods

2.2.1 Farm descriptions

Six collaborating farms (CFs) in the Northeast US participated in this study, three of which are located within the Chesapeake Bay watershed (CF1, CF2, and CF3), and three in the Great Lakes watershed (CF4, CF5, and CF6). Farm information and digester descriptions and operating parameters from each CF with AD systems that were quantified are shown in Table 1.

Briefly, CF1 milked approximately 400 cows and used manure lagoon effluent to flush manure and soiled bedding from the barn. Dairy milking parlor wastewater was also emptied to the manure blend pit as well, further diluting the manure. Manure from the blend pit was screened through a solid liquid separation (SLS) system prior to entering the 2,570 m^3 ambient temperature digester, where it was co-digested with multiple food waste sources during a 15-day hydraulic retention time (HRT).

CF2 milked approximately 2,400 cows, and manure was scraped from barns into the manure blend pits. The healthy cow blend pit corresponds with ‘Influent A’ and the sick cow blend pit corresponds with “Influent B’. There was some dilution of raw manure with recycled liquid manure, before it was pumped into the 3,780 m^3 heated plug-flow digestion system with a 16-day HRT.

CF3 milked around 630 cows, and manure was scraped from barns into the blend pit, where solid food waste was added before the manure flowed into the 1,820 m^3 digestion system that had a 13-day HRT. In March 2017, this farm switched from solid to liquid food waste additions.

CF4 milked approximately 4,300 cows. Manure and soiled bedding were scraped to the blend pit and then, in addition to milking parlor waste, diverted to the 14,200 m^3 below-grade plug flow digestion system, and digested for 25 days

CF5 milked approximately 2050 cows. Scraped manure and soiled bedding were stored in a blend pit before pumping, along with milking center waste water, into the 7,200 m^3 below-grade modified plug flow AD system for a 33-day digestion period.

Lastly, CF6 milked approximately 1820 cows. Scraped manure and soiled bedding flowed by gravity to the blend pit and went through pasteurization before entering the 8,300 m^3 above-grade continuously mixed digester. Manure was co-digested with off-farm organics, including pre-consumer food waste and some septic waste for 28 days.

Table 2.1: Digester operating parameters for each Collaborating Farm (CF).

	Farm Management				Digester Information			
CF	Cows	Bedding	Flush/Scrape	Pre-treatment	Type	Capacity (m^3)	HRT[1] (Days)	Co-digestion Material
1	400	Sand	Flush (Recycled AD effluent)	SLS[2]	Ambient Temperature Covered Lagoon	2,570	15	Pre-consumer liquid food waste
2	2,700	RMS[3]	Scrape	N/A	Heated Below-Grade Modified Plug-Flow	3,780	16	N/A
3	690	RMS	Scrape	N/A	Heated Below-Grade	1,820	13	Food waste[4]
4	4,300	RMS	Scrape	N/A	Heated Below-Grade Modified Plug-Flow	14,200	25	N/A
5	2,050	RMS	Scrape	N/A	Heated Below-Grade Modified Plug-Flow	7,200	33	Bunker Silo Leachate
6	1,820	RMS; Wood Shaving	Scrape	Pasteurization	Heated Above-Grade	8,300	28	Pre-consumer food waste; Septic waste

[1]HRT=Hydraulic Retention Time; [2]SLS=Solid Liquid Separation; [3]RMS=Recycled Manure Solids; [4]CF3 switched from solid (spoiled produce) to liquid food waste additions in March 2017.

It should be noted that the data presented in this study are part of a larger monitoring study of 11 farms, where manure was sampled between each step of the manure management systems. Oliver et al. (2018) contains detailed descriptions of each farm manure management system referenced in this study. It should be noted that the farms labeled as CF1-3 in this study correspond with CF8, CF10, and CF11, respectively, in Oliver et al. (2018), while CF 4, 5 and 6 in this study are labeled similarly in Oliver et al. (2018). Oliver et al. (2018) calculated the hydraulic retention times of each AD system by estimating daily volumes of inputs in the digester (manure, milking center waste water, and imported organics) and multiplying that by the treatment volume of the AD vessel.

2.2.2 Field Sampling

Samples from six dairy farms in the Northeastern US with an AD system were collected approximately every six weeks from September 2016 to August 2017 from the influent and the effluent of the digester. Liquid manure was collected using sterile one-gallon buckets. If the manure storage pits had an industrial mixer installed, that mixer was turned on for a period of five minutes before collection from the pit. When manure was collected from a continuously flowing pipe, composite samples were taken over a period of ten minutes and mixed together. A YSI Pro-Plus meter was used to determine the temperature of liquid manure samples. The pH was verified before and after acidification using an Oakton portable pH meter.

After collection from a manure pit or continuously flowing pipe, liquid samples were homogenized in 5-gallon bucket using a drill operated mixer before being transferred to labeled 150-mL bottles, 200-mL bottles, 50-mL corning centrifuge tubes, and 50-mL light sensitive centrifuge tubes for analysis. Samples were transferred to the laboratory in a cooler with ice. All containers, excluding the 50-mL light sensitive centrifuge tubes, were stored in a 4°C refrigerator until analysis. The 50-mL light sensitive centrifuge tubes were frozen at -20 °C until lyophilization.

2.2.3 Solids, nutrients, and volatile fatty acid analysis

A 150-mL bottle was filled with liquid manure and used for total solids (TS) and volatile solids (VS) analysis. A 200-mL bottle was filled with liquid manure that was acidified in the field to 1.5-2 pH with 5.25 N sulfuric acid for volatile fatty acid (acetic, butyric, propionic, and valeric), and nutrient analysis (total phosphorus (TP), total Kjeldahl nitrogen (TKN), and total ammonia nitrogen (TAN)). Solids (TS and VS) were analyzed in triplicate using the Standard Methods for the Examination of Water and Wastewater (APHA, 2005).

For TKN and TP analyses, samples were Kjeldahl digested and analyzed on a Lachat autoanalyzer using Method 13-107-06-2-D (rev 2012) and QuikChem Method 13-115-01-1-B (rev 2006), respectively. Acidified samples were filtered through a 0.45-micrometer filter before Lachat NH_3-N analysis, under QuikChem Method 10-107-06-2-0.

For VFAs, samples were filtered through a 0.22- micrometer filter, then analyzed on the gas chromatograph (Agilent Technologies, Inc.; Shanghai China; model 7890 A) with a flame ionization detector (FID) operated at 300°C and 7693 autosampler (Agilent Technologies model 7693), with a DB-FFAP capillary column (Agilent J&W; USA), and He as the carrier gas at 1.80 ml/min. The injection temperature was held at 250°C and the oven operated at 100°C for 2 min and subsequently ramped at 10°C/min for a total run time of 10 min. VFAs concentrations are presented based on chemical oxygen demand (COD).

2.2.4 Antimicrobial Analysis

Samples for antimicrobial analysis were collected in separate 50-mL light sensitive polypropylene Corning centrifuge tubes, pre-washed with 2% 15.9 M nitric acid. The samples were frozen at -20°C before lyophilization, then shipped to the University at Buffalo (Buffalo, NY, USA) for analysis.

Antimicrobial solid-liquid extraction and analysis were performed via liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Agilent 6410 triple quadrupole, Santa Clara, CA). Mass of antimicrobials results are presented per nanograms of dry weight (DW) of manure. The following analytes were tested on all pre- and post-digestion samples: tetracycline (TC), 4-epitetracycline (ETC), oxytetracycline (OTC), anhydrotetracycline (ATC), chlorotetracycline (CTC), 4-epichlorotetracycline (ECTC), anhydrochlorotetracyline (ACTC), and sulfamethazine (SMZ) and sulfadimethoxine (SDM).

2.2.5 Antimicrobial administration data

Antimicrobial administration data were collected, when available, from farms over the course of the study. Data was available daily from CF1 and on a monthly basis from CF3. CF2 was only able to share their monthly antimicrobial purchasing data. Based off the farmer’s description of their antimicrobial administration at CF2, values of administration shared for this farm were presented under the assumption that they used the entirety of their purchase during that month. Administration data was collected for CF4-6 by another university collaborating in this study and will not be shared as a part of this thesis.

2.2.6 Statistical Analysis

Analysis of variance was used for multiple comparison of group means for tetracycline analysis. If significance was detected in the ANOVA, then a Tukey-Kramer Honest Significant Difference (HSD) test was performed to determine if significant differences existed between any of the individual treatments. All analyses were conducted using an alpha level of 0.05. All statistics were performed in Microsoft Excel using the Analysis Toolpak and the StatFi Excel add-on.

2.3 Results and Discussion

2.3.1 Antimicrobial administration and antimicrobial persistence during anaerobic digestion (AD)

TC concentrations in the AD effluent were not significantly reduced compared to the AD influent at any of the farms. Tetracyclines were administered irregularly at CF2 and CF3 during the study period, ranging from 0 to 6,480,000 mg/month (Figure 2.1), and administration patterns were not consistently related to trends in TC detection in the AD influent and effluent. Sulfonamide antimicrobials were not reportedly administered on CF1-3 during the study period, which is likely why sulfonamide concentrations in the influent and effluent on farms were negligible.

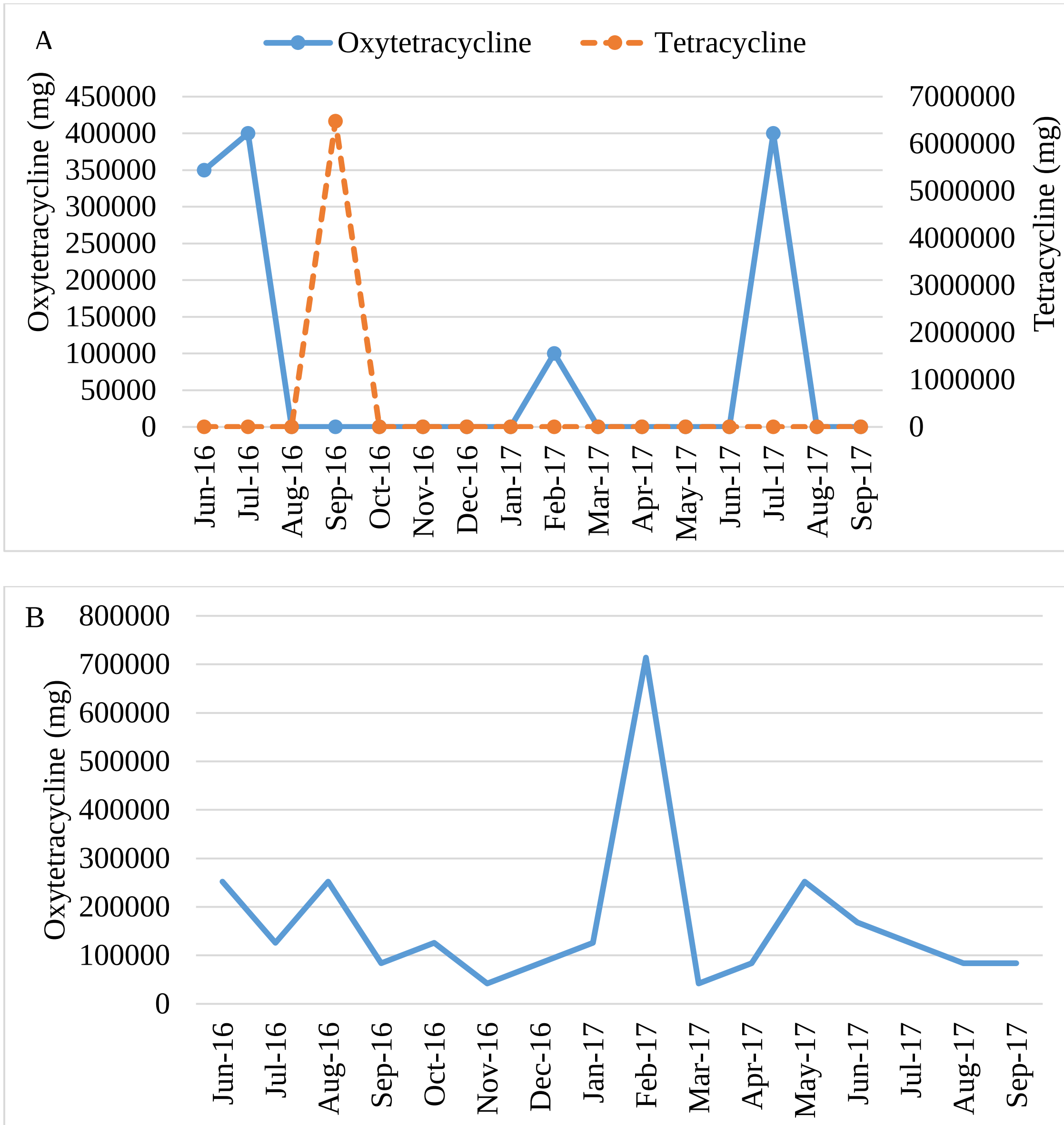

Figure 2.1: Tetracycline and oxytetracycline administration at CF2 (A) and Oxytetracycline administration at CF3 (B) from June 2016 to September 2017.

SDM and SMZ were not detected at CF1, CF3, CF4, or CF6 during any sampling events, and only detected at CF2 during the September 2016 sampling at concentrations <37 ng/g DW. Sulfonamides were not detected during most sampling events at CF5, however, there was a peak of 1140 ng/g DW in AD effluent during the June 2017 sampling event (Figure 2.2).

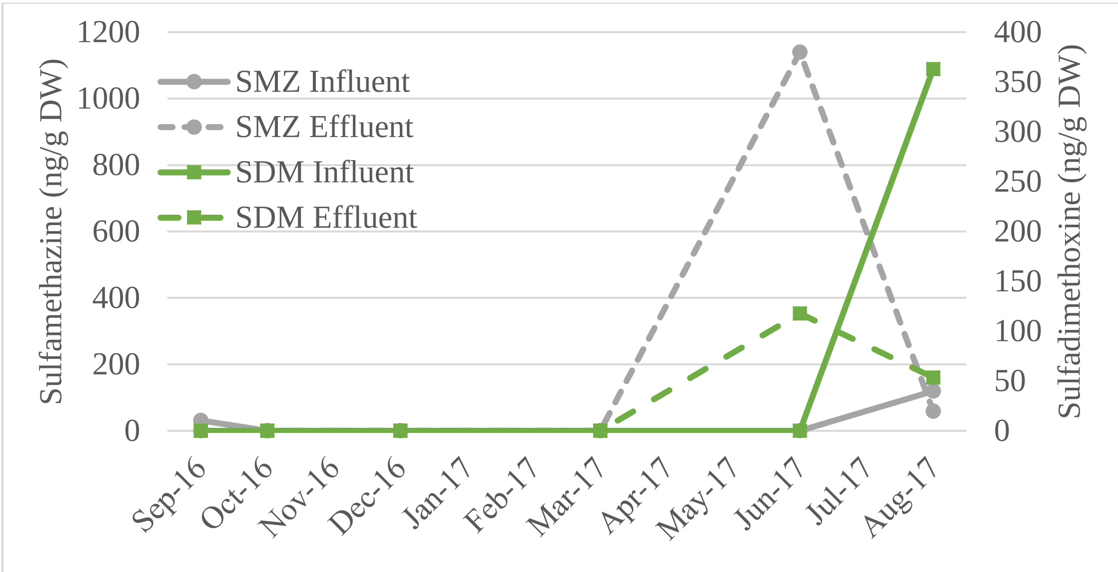

Figure 2.2: Sulfamethazine (SMZ) and sulfadimethoxine (SDM) concentrations at CF5 between September 2016 and August 2017.

Tetracyclines in the AD influent and effluent ranged from 0 to 6,000 ng/g dry weight (DW) of manure at CF1, CF3, and CF4-6. CF1 and CF6 had the lowest peak TC concentrations in the AD effluent at 1700 and 1400 ng/g DW, respectively. CF2 had much larger peak tetracycline concentrations at 34,000 ng/g DW (Figure 2.3), compared to other farms. While effluent concentrations exceeded influent concentrations during certain sampling periods at all farms, all farms (except CF5) also had occurrences of non-detectable concentrations of antimicrobials in the AD effluent as well (Figure 2.3). TC concentrations did not significantly decrease during digestion at any of the farms (*p*-

values ranging from 0.159 to 0.988), which does not follow trends in the literature of antimicrobial degradation found at the bench scale.

The hydraulic retention times (HRT) of AD systems refers to how long manure is in the digestion system, before exiting as AD effluent. HRTs are a strongly influential factor when considering the relationship between influent and effluent parameters in an AD system. Based on available farm data HRTs were calculated for the farms with AD systems in Oliver et al. (2018). The average HRT for CF1-6 are 15, 16, 13, 25, 33, and 28 days, respectively (Table 1). Given that samples were collected approximately every 6 weeks on each farm, it is difficult to make comparisons between influent and effluent concentrations in AD systems with HRTs of < 4 weeks. Even though the comparisons that can be made between influent and effluent concentrations are limited, it is important to note that peak concentrations between 1,400 and 34,000 ng/g DW (Figure 2.3) were observed in the digester effluent, which is an indication of the limitation of degradation seen in AD systems during certain sampling conditions. Conversely, non-detectable concentrations were observed in the AD effluent during other sampling periods, which likely could corresponds to lower antimicrobial administration but could also indicate increased treatment.

CF2 had a large spike of tetracycline administered (7 million mg) during September 2016 (Figure 2.1) that corresponded with a spike in tetracycline concentration detected (34,000 ng/g DW) in the AD effluent during the September 2016 sampling event. This detection could be linked to the short hydraulic retention time of the system (~16 days), indicating that the antimicrobials were moving through the system quickly. Interestingly, trends of antimicrobial administration at CF3 did not correspond to spikes of TCs in the

AD influent and effluent. Both CF2 and CF3 had similar HRTs, however, the administration at CF3 was much lower (700,000 mg) than at CF2 which could explain why corresponding trends in the manure antimicrobials were not observed.

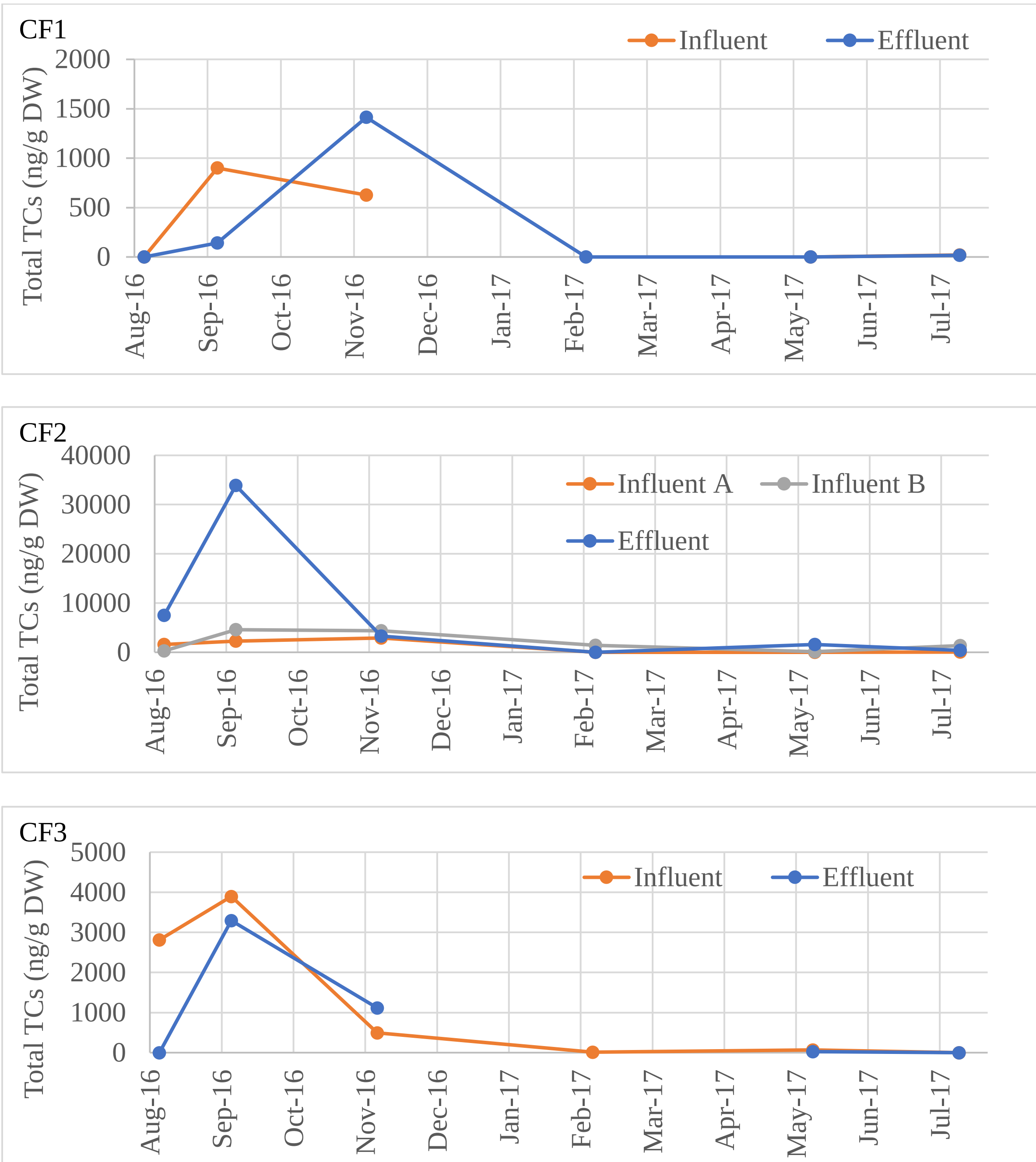

Figure 2.3: Total tetracyclines (TCs) concentrations, as ng/g dry weight (DW), in the anaerobic digester (AD) influent and effluent at each collaborating farm (CF). CF2 has two manure influents in to the digester; influent A from the healthy cow barn, and influent B from the sick cow barn.

A list of monitored concentrations of tetracyclines in various samples of manure was compiled by Massé et al. (2014), who reported 110 to 10,000 ng/g DW in cow manure. Most antimicrobial concentrations observed in the present study fall within the range of values reported in the literature, except for peak concentrations at CF2 (34,000 ng/g DW in September 2016) and the non-detectable concentrations observed at CF1-4 and CF6. Most AD studies that monitor antimicrobial degradation at the bench scale or pilot scale have found significant degradation of antimicrobials during anaerobic digestion (Arikan et al., 2008; Mitchell et al., 2013, Varel et al., 2012), which was not observed at any of the farms in the present study. Degradation could potentially be limited at the field scale due to the short HRT in an AD system. Kim et al. (2005) performed a bench scale AD experiment with wastewater treatment plant sludge that examined tetracycline degradation under two different HRTs, 24 hours and 7.4 hours. TC removal was significantly lower in the treatment with the 7.4-hour HRT.

Additionally, lack of TC degradation could be related to TC sorption, as TCs are not truly degrading in the systems, but are adsorbing to the solid particles. TCs have been shown to strongly sorb to solids, both in manure amended soils (Hou et al., 2015) and activated sludge (Kim et al., 2005). Higher concentrations of antimicrobials in AD effluent (450 ng/g DW) compared to AD influent (270 ng/g DW) were also observed by Wallace et al. (2018), who reported concentration of TCs from a field-sale dairy manure digestion system. They observed that TCs in the solids portion of manure increased after digestion due to increased particulate surface area availability for TC sorption after AD particulate breakdown by microorganisms. This suggests that bench scale findings of significant antimicrobial degradation are not indicative of antimicrobial trends at the field scale.

2.3.2 Manure characteristics before and after digestion

The VS decrease during digestion ranged between 30 - 49% on all farms, indicating that the microbial breakdown of organic matter was not inhibited by the presence of antimicrobials in the manure. The VS concentrations at CF2-6 ranged from 44.3 to 161 g/L in the AD influent and from 40.4 to 82.0 g/L in the AD effluent. VS concentrations in the influent and effluent at CF1 were 5.20 and 3.70 g/L, respectively, and were the lowest out of all farms, even with the addition of organic matter from multiple food waste substrates for co-digestion (Table 2.2). CF1, as previously mentioned, utilized a significant amount of flush water to transport manure from barn stalls to manure pits, creating very dilute manure slurry with low solids and organic content.

The VS concentrations in the influent and effluent of CF3, 193 and 161 g/L, respectively, were highest out of all farms, likely due to the scrape manure system and the additions of solid food waste into the AD system (Table 2.2). CF6 also had a scrape manure system and co-digested with added organics, however, it had the second lowest value of VS in the influent and effluent at 44 and 23 g/L, respectively. This could be due to the breakdown of organics during pre-treatment pasteurization of the manure before entering the digestion system.

Table 2.2: Average concentration ± standard error of total solids (TS) and volatile solids (VS) in the anaerobic digester influents and effluents.

Farm Code	TS (g/L)			VS (g/L)		
	Influent	**Effluent**	**%diff**	**Influent**	**Effluent**	**%diff**
CF1*#	12.3 ± 4.4	6.5 ± 0.2	47.5	5.2 ± 2.4	3.7 ± 0.6	29.1
CF2	72.3 ± 2.4	54.1 ± 3.8	25.3	58.2 ± 2.1	40.4 ± 3.5	30.6
CF3*	193 ± 46	69.1 ± 4.5	64.2	161 ± 39	82 ± 28	49.0
CF4	119 ± 7.6	82.2 ± 0.9	31.1	87.1 ± 6.0	56.2 ± 1.7	35.5
CF5	79.8 ± 4.2	57.8 ± 2.9	27.5	64.7 ± 3.6	44.3 ± 2.8	31.6
CF6*+	57.4 ± 3.8	35.3 ± 2.5	38.5	44.3 ± 2.9	22.8 ± 1.7	48.7

*Farms co-digest with food-waste; #Farm uses recycled AD effluent to flush manure from barn to manure pit as opposed to manure scrape system; + Pre-treatment of manure using pasteurization before AD

VFA concentrations ranged from 1900 mg/L to 12,900 mg/L in the AD influent and from 350 mg/L to 6100 mg/L in the AD effluent (Figure 2.4) at the CFs, which is comparable to ranges found in the literature (Lee et al., 2015). VFA concentrations were significantly lower in the AD effluent than in the AD influent at CF4-6 (p-values = 0.003, 0.0005, 0.001, respectively), which indicates that digesters were functioning properly on these farms through the consumption of acetic acid by methanogens to produce biogas.

CF1, CF2, and CF3 did not show significant differences in average VFA concentrations before and after digestion (p-values = 0.354, 0.411, 0.146, respectively), likely due to more temporal variability in VFA concentrations caused by management or environmental changes.

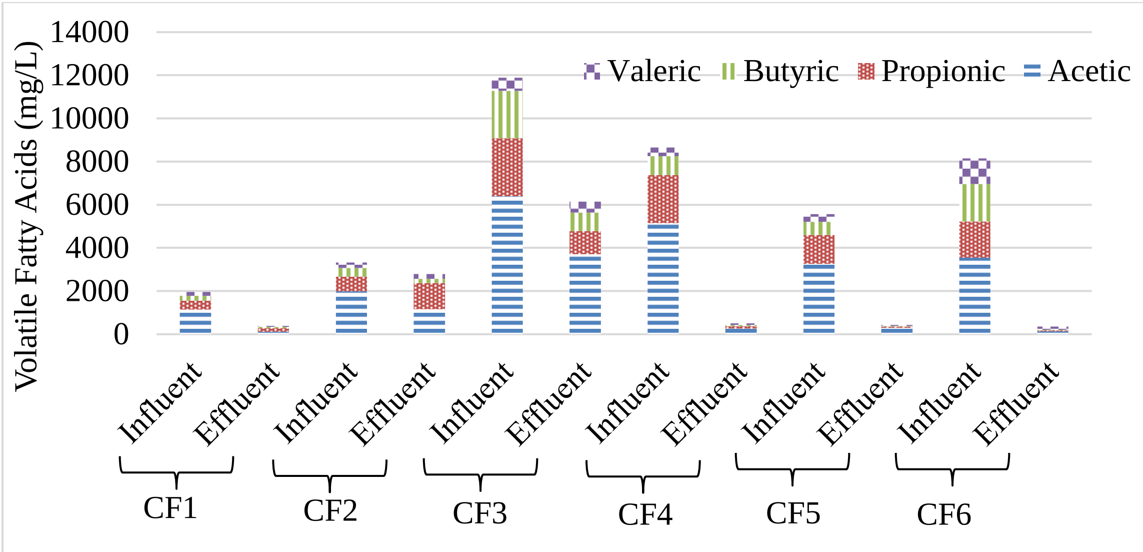

Figure 2.4: Average volatile fatty acid concentrations (acetic, propionic, butyric, and valeric) in anaerobic digestion influent and effluent over 15-month study period.

VFA concentrations at CF1 were higher in the AD effluent than in the AD influent in February 2017, indicating that there was some digestion process instability during this time, limiting methanogens from utilizing the acetic acid produced during acetogenesis and acidogenesis (Figure 2.5). This likely was caused by the decrease in digester temperature during this month, because the digester at CF1 was not heated and affected significantly by the ambient temperature. The digester at CF1 had a normal operating temperature between 23-2°C, however, in December 2016 and February 2017 the digester effluent temperature was 10.5 and 11.3°C, respectively. This process instability did not seem to affect the degradation of TC, as the effluent concentrations of TC after February 2017 were <16 ng/g DW of TC.

Variability in VFA concentrations observed at CF3 is likely due to impacts from organic food waste additions into the influent for co-digestion. Depending on the time of sampling, variability could be related to how recently food waste had been applied to the system. Concentration of total VFAs in the influent decreased over time, which could reflect the CF3's switch from solid to liquid food waste additions in March 2017.

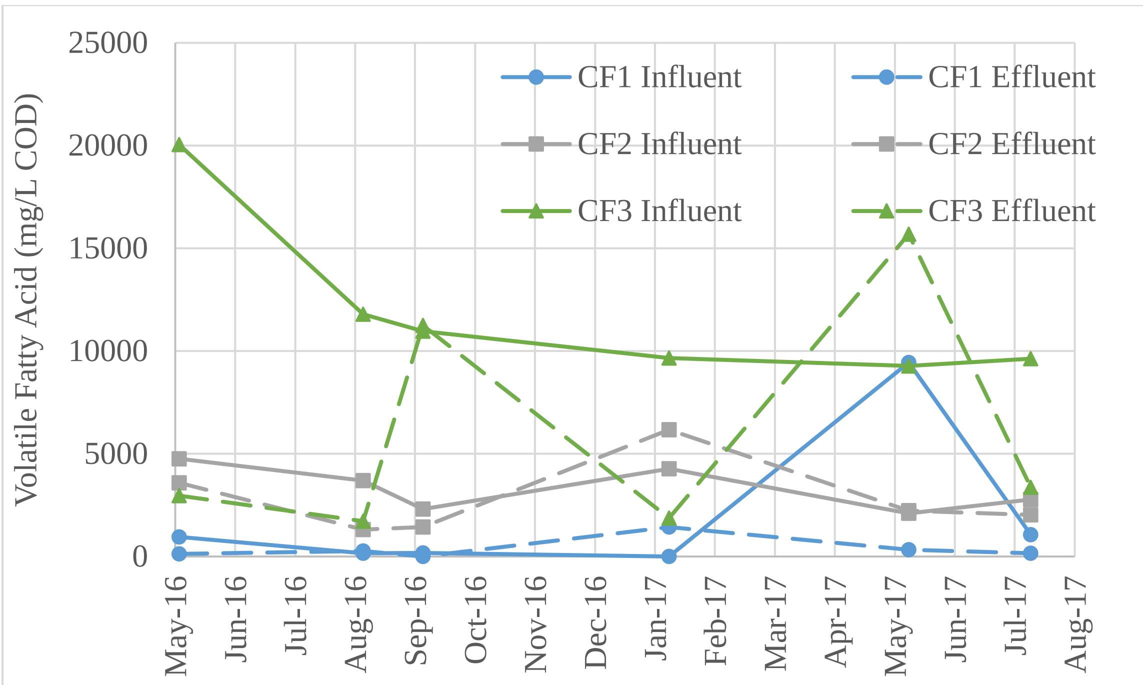

Figure 2.5: Total volatile fatty acid concentrations (acetic, propionic, butyric, and valeric), presented as mg/L of chemical oxygen demand, in AD influent and effluent at CF 1-3 over time.

2.3.3 Nutrient trends before and after anaerobic digestion (AD)

TAN, TP, and TKN were not significantly different in the AD influent compared to the AD effluent at any of the CFs, except an increase in the TAN concentrations at CF2 and CF3 post digestion due to N-mineralization. Nutrient values at CF1 were notably lower than other farms, due to the dilution of the manure with flush water.

Average TAN values consistently ranged between 888-2703 mg N/L in the AD influent and effluent at CF2, CF3, and CF4-6, which are all farms that implement scrape manure systems. The TAN values at CF1 were on average 237 mg N/L in the AD influent and 278 mg N/L in the effluent (Figure 2.6). The lower values are likely attributed to the dilution of the manure with flush water. Farms with AD systems generally have blend pit influents that hold raw manure straight from the dairy barn, however, the CF1 manure system implemented solid-liquid separation prior to AD, which further reduced the solids content of the AD influent.

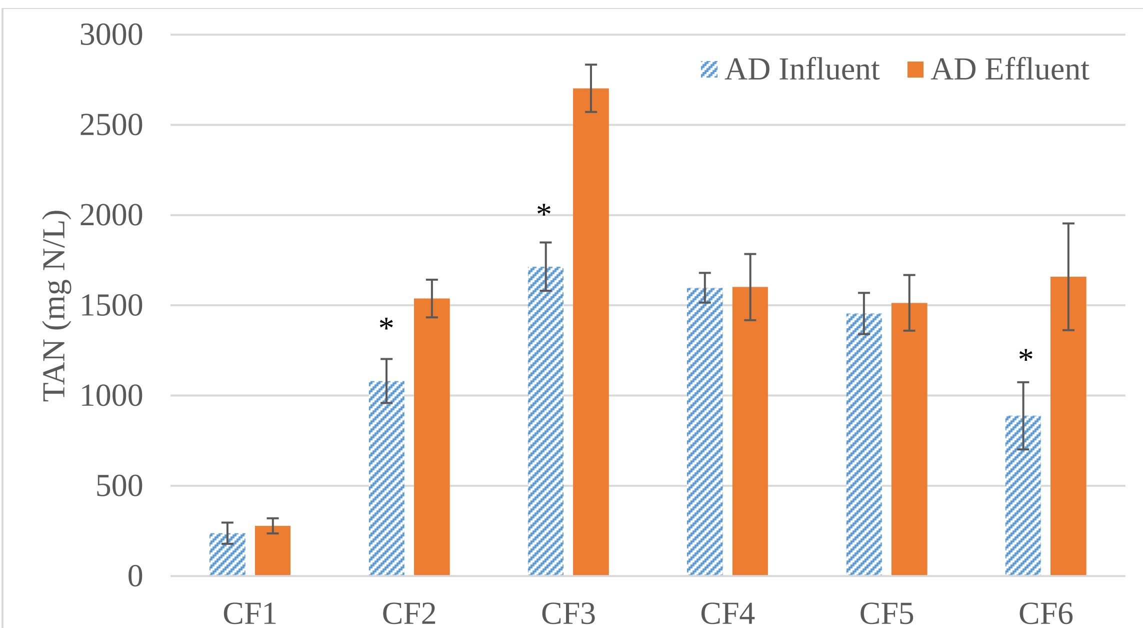

Figure 2.6: Total ammonia nitrogen (TAN) average, with error bars representing standard error in the AD influent and effluent at farms with digestion systems. * indicates significant difference between the AD influent and effluent.

Anaerobic digester effluent TAN concentrations increased compared to the AD influent at CF2, CF3, and CF6 (Figure 2.6), which is expected due to mineralization of the organic N during digestion, however, the increase was only significant at CF2 and CF3 (p-value = 0.017 and 0.0004, respectively). TAN values at CF1, CF4, CF5, and CF6 were not significantly different before and after digestion (p-values = 0.584, 0.986, 0.765, 0.052, respectively).

Rajagopal et al. (2013) performed a comprehensive review of digester inhibition from excess ammonia and reported that beneficial or non-antagonistic concentrations of TAN during digestion ranged between 50-1000 mg/L. While inhibition thresholds varied widely due to substrate, inocula, and environmental conditions, VFA accumulation and digester instability were speculated to be a result of TAN concentrations up to 1500 to 7000 mg/L.

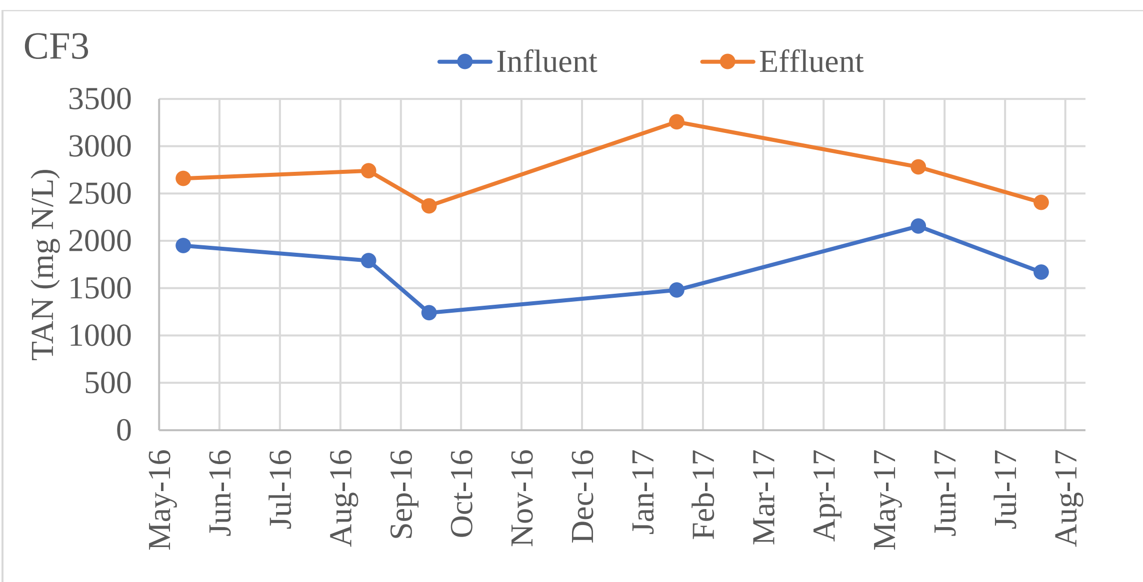

Figure 2.7: Total ammonia nitrogen concentrations (mg N/L) of anaerobic digester influent and effluent at CF3 over 15 months.

Chen et al. (2008) also reported that TAN levels can be detrimental to digester function by limiting methane (CH_4) production, due to toxicity to the methanogenic bacterial community, at levels surpassing 1700 mg/L. CF3 was the only farm to surpass this threshold, with TAN concentrations >2300 mg N/L in the AD effluent from June 2016-August 2017 (Figure 2.7). While the high TAN concentrations at this farm this did not seem to impact the utilization of VS, it could have been an influential factor in limiting VFA decrease during digestion.

Differences in digester scale, type, and HRT did not seem to impact manure TKN or TP transformations during the AD process, as concentrations of TKN or TP in the AD influent did not significantly change compared to concentrations in the AD effluent, (p-value, 0.279-0.810 and 0.050-0.802, respectively). Flush system manure management led to much lower concentrations of manure nutrients compared to scrape system farms, and farm co-digestion led to slightly higher TP values in manure.

TKN influent and effluent values ranged from 2635-3688 mg N/L at farms with scrape manure systems (CF2, CF3, and CF4-6). The flush system farm, CF1, had average influent and effluent values of 476 and 443 mg N/L respectively (Table 2.3).

TP influent and effluent values ranged from 240 mg P/L to 893 mg P/L in the manure scrape farm systems. CF1 the average values of the TP in the influent and effluent were much lower, 68 mg and 59 mg P/L, respectively (Table 2.3). Even with the pre-consumer liquid food waste added into the digestion system at CF1, TP values were not significantly different before and after digestion, likely due to the high dilution of the manure. In contrast, CF3 and CF6 had the highest influent and effluent TP concentrations

(Table 2.3), which is likely due to addition of food waste to the blend pits at these farms prior to digestion.

Güngör et al. (2008) performed an on-farm case study focusing on phosphorus availability during mesophilic digestion that included three dairy manure digesters, and TP values in the influent and effluent at these farms ranged from 400-900 mg P/L and concentrations did not significantly change after digestion, which is comparable to the findings in the present study, except for CF1. Demirer et al. (2005) observed TKN concentrations in the AD influent and effluent of a 2L batch reactor, with an HRT of 20 days, with the influent and effluent TKN values ranging between 500-3000 mg N/L, similar to the values observed in the present study.

Table 2.3: Average ± standard error of total Kjeldahl nitrogen (TKN) and total phosphorus (TP) of the anaerobic digestion influent and effluent for collaborating farms (CFs).

	TP (mg P/L)		**TKN (mg N/L)**	
Farm	**Influent**	**Effluent**	**Influent**	**Effluent**
CF1	68 ± 8	59 ± 12	476 ± 69	443 ± 60
CF2	240 ± 64	379 ± 21	2635 ± 211	2672 ± 184
CF3	893 ± 49	791 ± 89	3688 ± 272	3401 ± 291
CF4	608 ± 37	555 ± 18	3376 ± 140	3126 ± 156
CF5	419 ± 37	423 ± 9	2760 ± 163	3068 ± 130
CF6	851 ± 112	734 ± 62	2883 ± 163	2959 ± 282

2.4 Conclusions

Antimicrobial trends at the field scale did not follow the trends of degradation found in previous pilot scale and bench scale literature. Concentrations of tetracyclines in digester effluent ranged from 0 to 34,000 ng/g DW and were not significantly different from concentrations in the AD influent, indicating limitations in the degradation potential of antimicrobials during digestion, possibly related to the short HRTs of the sampled digesters (13-33 days) and high sorption potential of TCs in the AD effluent. Further research is needed on antimicrobial fate during field scale AD to better understand how the dynamics of fluctuating on-farm antimicrobial administration and digester function impact antimicrobial persistence in field-applied digestates.

Even though farms had varying digester types and operating conditions, digestion did not significantly change total nutrient concentrations. TP and TKN concentrations did not change significantly during the digestion process at all farms, however, dilution of liquid manure at CF1 resulted in lower concentrations of nutrients compared to the other farms with manure scrape systems. Additionally, farms with food waste co-digestion additions had higher influent values of TP compared to other farms, indicating that digester operating conditions can have a significant impact on nutrient content of the manure.

3.0 On farm monitoring of tetracyclines and nutrients during dairy manure composting

3.1 Introduction

Composting is a common practice used on farms to manage manure and create a nutrient-rich product that can be sold off-farm or field-applied directly as a fertilizer. The composting process can be conducted using several methods, including static piles, windrows (elongated piles), or in-vessel. Piles and windrows can be managed with varying degree of intensity, depending on the farm practice. Piles can be forced aerated or aerated through convection, watered, covered under roof, placed on a concrete pad, within a vessel, or in the open environment, and turned with differing frequency. Additionally, some farms solely compost manure solids, while others amend compost with sawdust, leaves, food waste, or other sources of organic matter (Peigné et al., 2004). Many benefits incentivize farmers to adopt composting practices, including manure volume reduction, odor reduction, and weight reduction leading to easier transport and spreading, and improvement in soil health.

In addition to providing an effective method of recycling manure solids, composting has also been shown to be an effective treatment for antimicrobial mitigation. Studies have shown composting has the potential to decrease tetracycline and its degradation products in manure, up to 70-95%. Sulfonamides, such as sulfadiazine, sulfamethazine, and sulfamethoxazole, have been shown to be reduced up to 100% during composting (Arikan et al., 2007; Arikan et al., 2009; Wu et al., 2011; Selvam et al., 2011; Liu et al., 2015). Kim et al. (2010) stated that antimicrobial degradation in composting is most likely related to abiotic factors, such as moisture content, pH, aeration, temperature, carbon to nitrogen ratio (C:N), and the nature of the composting substrate.

Previous studies have examined degradation of antimicrobials during composting with broiler and swine manure substrates (Bao et al., 2009; Dolliver et al., 2008; Kim et al., 2012; Liu et al., 2015), however, there are still limited studies that examine the fate of antimicrobials during the composting of dairy manure. Mitchell et al. (2015), one of the few studies that examined antimicrobials during dairy manure composting, found that concentrations of florfenicol, tylosin, sulfadimethoxine, and sulfamethazine decreased more than 95% after 21 days of composting at the pilot scale. Composting substrates (dairy vs. biosolids) were also compared and greater antimicrobial degradation was observed with dairy manure substrate, indicating that substrate type could impact antimicrobial degradation in composting.

Most of the literature examining the fate of antimicrobials during composting has been conducted in controlled laboratory environments (Arikan et al., 2007; Arikan et al., 2009a; Bao et al., 2009), however, this does not reflect the conditions of composting piles on-farms, which fluctuate based on dynamic environmental conditions, pile management, and ambient temperatures and weather. Dolliver et al. (2008) monitored degradation of chlortetracycline, monensin, and tylosin during composting of turkey litter in a static pile, an intensely managed pile (weekly turning and watering), and in-vessel composting, and found no significant differences in degradation between the different composting methods.

In contrast, Storteboom et al. (2008) compared low-intensity management (LIM) and high intensity management (HIM) composting of dairy manure and found significantly higher rates of tetracycline dissipation in the HIM compost both at the pilot scale and at the field scale. The fate of antimicrobial degradation during dairy manure composting at the field scale and under various management conditions is not well understood.

Ho et al. (2013) is one of few studies that correlated physio-chemical properties of the composting process, such as total nitrogen, total phosphorus, and C:N, to antimicrobial degradation using broiler manure and nine different veterinary antimicrobials (macrolides, sulfonamides, quinolones, and tetracyclines). It was suggested that the increase of total nitrogen in the initial phase of composting was due to the negative impact of antimicrobials on the nitrogen transforming microbes and an inhibition of N-mineralization. Selvam et al. (2012) monitored total Kjeldahl nitrogen (TKN) during bench scale composting of swine manure with chlortetracycline, sulfadiazine, and ciproflaxcin, and found that antimicrobials caused an inhibition of nitrogen loss during manure composting. The relationship of nutrients on antimicrobial degradation during composting is not well understood, and these relationships have yet to be explored during dairy manure composting.

The goals of this study are to: 1) examine the transformations of tetracycline and its degradation products during the composting process of dairy manure at the field scale, and 2) monitor nutrient and organic characteristics throughout the composting process on-farm to determine correlations between physio-chemical properties and antimicrobial degradation. It has been shown that composting characteristics are significantly impacted by management style and intensity, which vary from farm to farm. This study seeks to monitor antimicrobial degradation at the field scale under pile management conditions used on-farm to better understand how on-farm management and environmental conditions can impact the composting process. Additionally, the relationship between antimicrobial degradation and nutrient and organic characteristics were further explored. There are still relatively few studies that examine antimicrobial fate and nutrient characteristics during dairy manure composting, therefore, this study seeks to fill that gap and illuminate how a substrate may impact the physio-chemical properties of the compost and antimicrobial degradation.

3.2 Methods

3.2.1 Field Sampling

A compost windrow was created at a 1000 cow dairy farm in the Northeast US and monitored from November-December 2017 using their typical farm management practices. The pile was kept under an open-air pavilion where the farm manages composting windrows and was turned every 2-3 days. The pile was not watered during the experiment. The compost pile was constructed using separated solids packed bedding from the sick and lame cow barns. Packed bedding remained in barns for 4-6 weeks before removal for composting. No additional antimicrobials were spiked into the bedding or compost piles. Samples were collected from the pile on Days 1, 2, 3, 5, 10, 20, and 33 from the day of pile creation.

A 30-35-day composting period was typical for the farm management, which is why the pile was monitored for this length of time. This was the average length of time for this farm between pile formation and field application. Four temperature probes were distributed evenly throughout the pile to monitor the temperature of the pile over time. During each sampling event, several grab samples were taken from each side of the pile and mixed thoroughly in sterile one-gallon buckets. Three well-mixed composite samples were taken from the pile, placed in plastic sealable bags and put on ice for transfer back to the laboratory.

3.2.2 Laboratory Analysis

Samples were tested for total solids (TS), volatile solids (VS), total Kjeldahl nitrogen (TKN), total phosphorus (TP), and carbon to nitrogen ratio (C:N). The TS and VS concentrations were determined using the Standard Methods for the Examination of Water and Wastewater (APHA, 2005). The TKN and TP samples were Kjeldahl digested and analyzed on a Lachat autoanalyzer device. TKN was analyzed using QuikChem Method 13-107-06-2-D (rev 2012) and TP was analyzed using QuikChem Method 13-115-01-1-B (rev 2006). Total carbon and TN were determined using an elemental analyser (Elementar Vario Max CNS, Elementar Analysensysteme GmbH, Hanau, Germany). Moisture content (MC) of the samples were calculated using equation 3.1, where the wet weight (WW) of the sample refers to the mass of the sample taken from the field and the dry weight (DW) of the sample refers to the mass of the sample after oven drying at 105 °C.

$$\%MC = 100 \times \frac{mass_{WW} - mass_{DW}}{mass_{WW}} \qquad \text{(Eq.3.1)}$$

Samples for antimicrobial analyses were collected in 50-mL light sensitive polypropylene Corning centrifuge tubes, pre-washed with 2% 15.9 M Nitric acid. The samples were frozen and lyophilized prior to analysis. Samples were sent to the University of Buffalo, where antimicrobial solid-liquid extraction was performed, followed by analysis via liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Agilent 6410 triple quadrupole, Santa Clara, CA). The following antimicrobial compounds were analyzed; tetracycline (TC), 4-epitetracycline (ETC), oxytetracycline (OTC), anhydrotetracycline (ATC), chlorotetracycline (CTC), 4-epichlorotetracycline (ECTC), sulfamethazine (SMZ), and sulfadimethoxine (SDM). Concentrations of antimicrobials were presented in ng/g dry weight (DW) of manure.

3.2.3 Statistical Analysis

Kruskal-Wallis non-parametric ANOVA test was used to determine significance for antimicrobials, nutrients, solids, and moisture content between the different sampling events. All analyses were conducted using an alpha level of 0.05. All statistics were performed in Microsoft Excel using the Analysis Toolpak and the StatFi Excel add-on.

3.3 Results and Discussion

3.3.1 Tetracycline degradation during manure composting

Tetracycline (TC) and its metabolites (ETC, OTC, and ATC) persisted at concentrations from 13.6 to 690 ng/g DW at the end of the composting monitoring period (Day 33), while sulfamethazine (SMZ), sulfadimethoxine (SDM), and CTC, a TC degradation product, were not detected at any point during the monitoring study (Table 3.1). The TC degradation curves did not follow typical first order kinetic degradation during field scale composting, even though the temperature did reach a maximum of 60°C on Day 31, above the 55°C threshold for pathogen kill (Usepa, 1994). This is likely due to the gradual temperature increase and frequent turning (every 1-3 days) of the compost pile.

Table 3.1: Average antimicrobials, presented in ng/g dry weight in compost over a 33-day composting period. Antimicrobials include oxytetracycline (OTC), 4-epitetracycline (ETC), anhydrotetracycline (ATC), 4-epichlorotetracycline (ECTC), and tetracycline (TC).

Day	**OTC**	**ETC**	**ATC**	**ECTC**	**TC**
1	ND*	100 ± 13	7.2 ± 1.1	4.3 ± 0.5	452 ± 71
2	ND*	125 ± 6	11.0 ± 0.6	2.1 ± 0.6	569 ± 5
3	ND*	73 ± 27	7.5 ± 2.8	1.9 ± 0.5	374 ± 136
5	32.1 ± 8.7	72 ± 3	5.4 ± 0.3	0.4 ± 0.4	341 ± 27
10	27.6 ± 5.2	125 ± 29	10.4 ± 2.2	ND*	638 ± 72
20	67.1 ± 5.8	137 ± 6.1	13.1 ± 1.2	ND*	684 ± 53
33	64.2 ± 4.2	126 ± 8.1	13.6 ± 1.4	ND*	689 ± 58

*ND indicates non-detectable concentration

TC and ECTC were the only antimicrobials that showed significant differences in concentrations between sampling days (*p*-values = 0.0416, 0.0179, respectively), while, OTC, ETC, and ATC metabolites were not significantly different between sampling days (*p*-values = 0.0679, 0.0843, 0.0706, respectively). This was not expected, as most studies in the literature observe first order kinetic degradation of antimicrobials during the composting process. The TC and ETC values were found to be in the highest concentrations in all the samples, between 240-710 ng/g DW and 46.4-137 ng/g DW, respectively, whereas concentrations of OTC, ATC, and ECTC did not exceed 67.1 ng/g DW (Figure 3.1). TC, OTC, ETC, and ATC concentrations at the end of the composting period (680, 64.2, 126, and 13.6 ng/g DW, respectively) were higher than the concentrations at the start of composting (452, ND*, 100, and 7.2 ng/g DW, respectively), which likely corresponds to the loss of moisture over time, but could also highlight variability of sampling throughout the pile and throughout time.

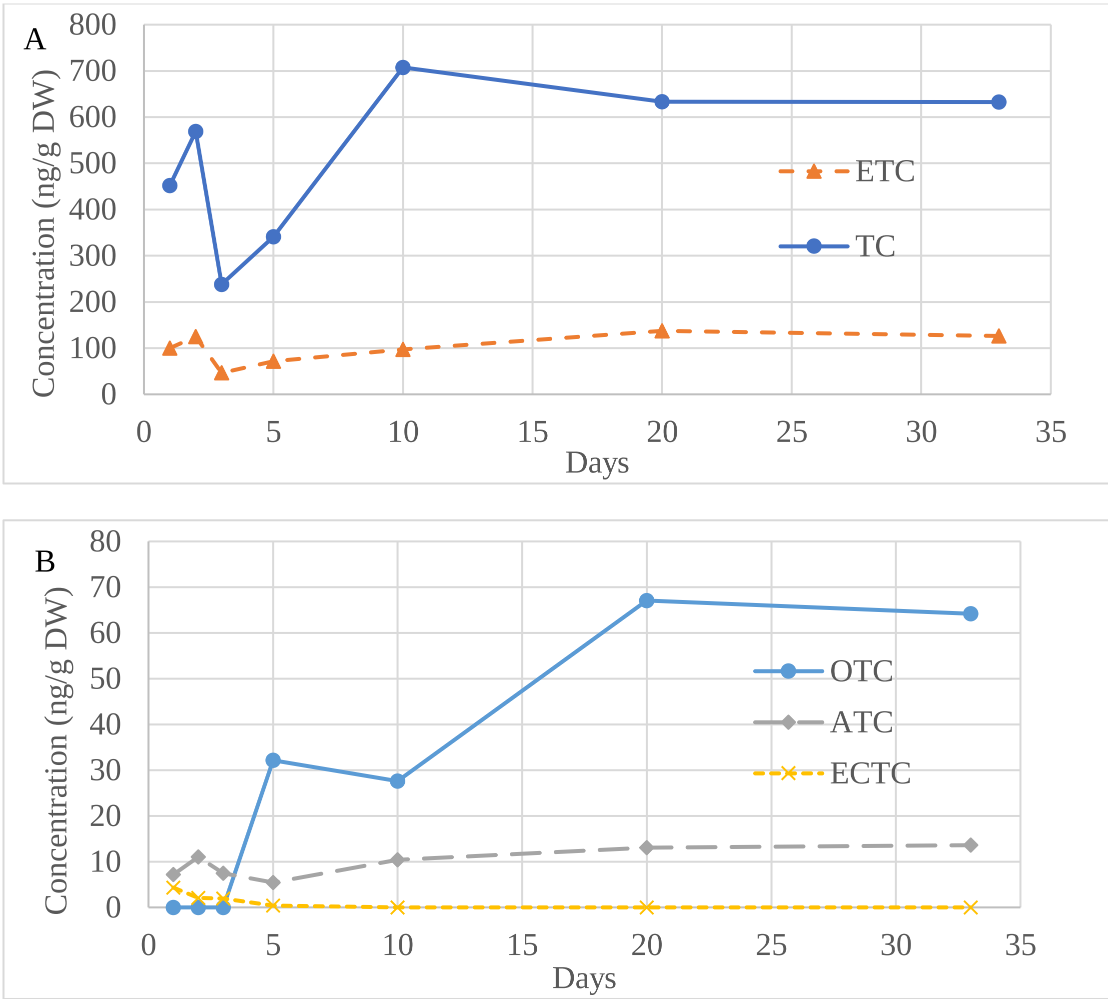

Figure 3.1: Concentrations of tetracycline (TC) and 4-epitetracycline (ETC) (A) and oxytetracycline (OTC), anhydrotetracycline (ATC), and 4-epichlortetracycline (ECTC) (B) during dairy manure composting at the field scale.

The compost pile took 31 days to reach its peak temperature (60 °C) of the monitoring period (Figure 3.2). The pile was created and monitored during the winter months (end of November-beginning of January) when ambient temperatures were lower, which could have impacted the temperature profile of the pile as cold weather increases heat loss (Rynk et al., 1994). Compost piles generally reach peak temperature between 1-

5 days (Arikan et al., 2009b; Mitchell et al., 2015), therefore, the gradual increase in temperature could indicate an inhibition of the microbial community by the tetracyclines present in the compost (Cessna et al., 2011). Additionally, solids used for compost were previously in the barns being utilized as packed bedding for the cows and remained in barns as bedding for four to six weeks. Typically, piles begin in the lower end of the mesophilic range (10-20 °C) and rise rapidly within the first four days to the thermophilic range (45-60 °C). The starting temperature of the composting pile on Day 1 was 38.9 °C, which is already at the high end of the mesophilic temperature range (10-40 °C) and approaching the thermophilic range (>40 °C) (Rynk et al., 1994). Initial phases of the composting process could already have been occurring in the barns, bringing the solids up to temperature and degrading antimicrobials, before solids were even collected for windrow composting.

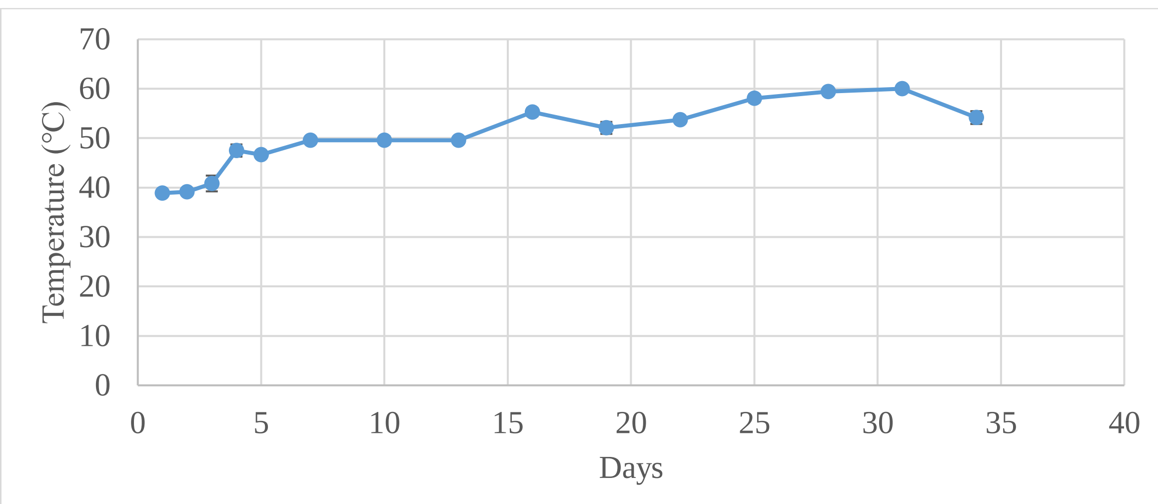

Figure 3.2: Temperature (whiskers represent standard error) of compost pile over 34 days, starting from the day of pile creation.

Volatile solids concentrations ranged from 20-32 g/kg, and similarly to the moisture content, showed significant difference between sample groups (p-value = 0.0422 and 0.0076, respectively). Even though the pile was not watered the moisture content remained between 50-56% throughout the course of the monitoring period (Figure 3.3). Moisture loss was likely limited due to low ambient temperatures during the study.

High intensity management (HIM) composting is generally considered to be a compost pile that is watered and turned, or aerated, regularly. Turning releases heat and moisture from the compost pile (Rynk et al., 1994). The pile in this present study was turned every 1-3 days but was not watered during the duration of the compost period. Although the pile was not watered and was turned frequently, moisture levels remained within optimal range for compost microbial activity (50-70%) (Peigné et al., 2004).

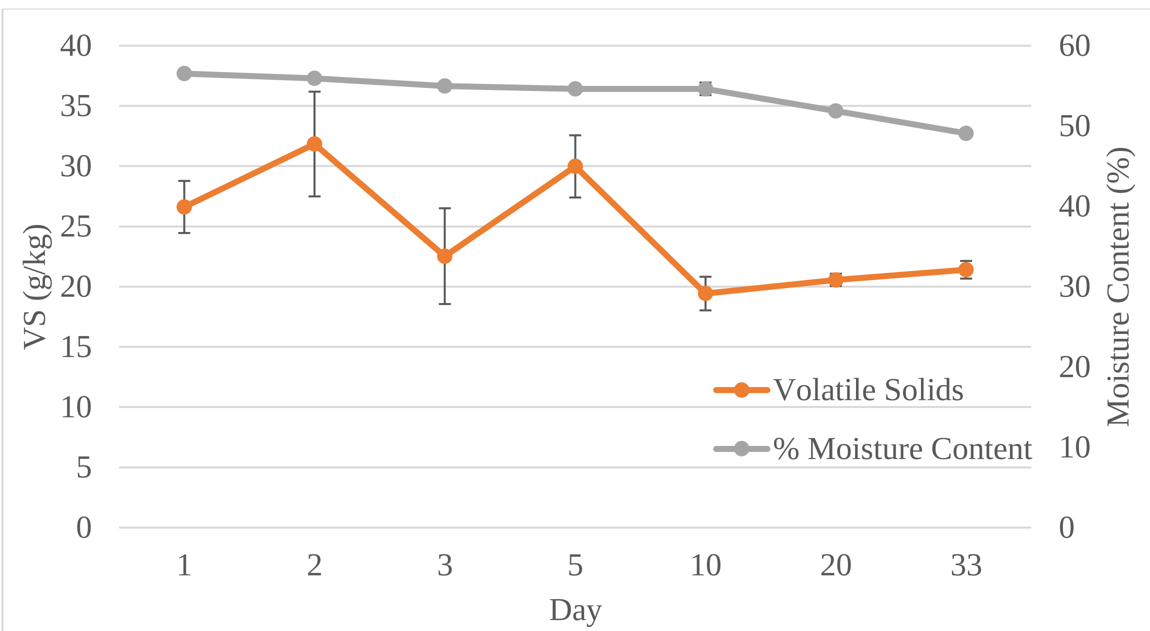

Figure 3.3: Average volatile solids (VS) concentrations (g/kg) and moisture content (%) in dairy manure compost during the 33-day monitoring period.

Starting concentrations of antimicrobials in pilot scale composting piles in the literature range from 5000 to 22,000 ng/g DW (Ho et al., 2013; Ramaswamy et al., 2010), which were at least an order of magnitude higher than the starting concentrations of TCs in the composting pile in the present study (4-450 ng/g DW). Most previous pilot scale studies have spiked large quantities of antimicrobials and observed a first order degradation of antimicrobials during the composting process (Arikan et al., 2007; Bao et al., 2009; Selvam et al., 2012), which was not observed in the present study.

The fate of degradation profiles of lower spikes of antimicrobials during composting is not well documented, however, Storteboom et al. (2008) performed a field scale experiment monitoring the degradation of TCs during 180-day composting of dairy manure, with weekly turning and consistent watering to maintain moisture levels of 31-36%. Their compost pile took about 20 days to reach peak temperature (34°C) and remained in the mesophilic stage throughout the composting process. The degradation profiles of TCs followed first-order kinetic degradation, decreasing from 300 ng/g DW to approximately 100 ng/g DW after 30 days, and finally to non-detectable concentrations after 180 days of composting.

Kim et al. (2012) performed an on-farm composting monitoring study that evaluated composting of swine manure and sawdust over an 80-day period, with daily turning and no watering. Temperature was not monitored but typical maximum farm compost temperatures had been recorded at 65 °C. They compared 80-day field scale composting to a 35-day bench scale experiment, observing that tetracyclines at the field-scale did not degrade to the same concentrations as the bench scale, even with a composting period that was twice as long. They concluded that degradation took much longer in field scale composting compared to a bench scale experiment.

Longer composting times may be needed at the field scale to achieve the same levels of degradation seen in bench scale experiments. Interestingly, the declines of TCs at the field scale did not follow a first order kinetic degradation pattern both in the present study and in the findings of Kim et al. (2012). The more gradual declines of TC could relate to the frequency of compost turning and the pile temperature.

In the literature, most compost piles reached peak temperature (50-60 °C) within 3-5 days (Wu et al., 2011; Selvam et al., 2012). Antimicrobial degradation in composting has been linked primarily to abiotic factors in the composting process (Kim et al., 2010), particularly pile temperature and aeration, indicating that pile management and physio-chemical parameters could be indicators for pile antimicrobial degradation. Environmental and management conditions impacting compost piles at the field scale are more complex than laboratory environments, which could impact the degradation profile of antimicrobials during composting. Duration of field scale composting and differences in the intensity of pile management could play a key factor in the difference in degradation profiles.

3.3.2 Nutrient transformations during composting

TKN concentrations in the compost increased steadily over time, ranging from 15.3-18.4 g/kg (Table 3.2), which corresponded to a decrease in moisture. The TKN decrease might also be attributed to antimicrobial inhibition of tetracyclines on N-mineralization. Increases in TKN concentrations did not correspond with significant changes in the C:N ratio (p-value = 0.1271), which remained consistently between 12.0-13.5 during the composting period. The starting C:N ratio was outside of the ideal composting range, likely because the manure was composted without any added carbon amendment. TP concentrations ranged from 1.2-1.8 g/kg (Table 3.2), and TKN and TP both showed a significant difference between sampling days, which corresponded to decreasing moisture and mass of the manure (p-values = 0.0087 and 0.0051, respectively).

The ideal starting range of the C:N ratio for composting is 25-35, with a reasonable range extending between 20-40. Cattle manure tends to have lower C:N ratios, with an average around 19 (Rynk et al., 1994). When C:N ratios are low (<20), then carbon is utilized without stabilizing all the nitrogen, creating a higher risk of ammonia or nitrous oxide loss to the atmosphere. Additionally, as the compost matures over time, the C:N ratio should decrease due to loss of CO_2 from the starting materials exceeding loss of nitrogen, however, if the starting ratio is <15 then the rate of carbon and nitrogen loss could be equal, resulting in little change in the C:N ratio (Rynk et al., 1994).

Increases in total nitrogen during manure composting with antimicrobials were observed in previous studies. Selvam et al. (2012) examined nutrient trends during 56-day bench scale composting of sawdust and swine manure, which was regularly moistened and aerated and spiked with chlortetracycline, sulfadiazine, and ciprofloxacin. They observed a steady increase of TKN during the composting period, starting at 1.7% and ending at 1.9%, which was attributed to inhibition of N-mineralization from antimicrobial additions. The C:N ratio declined from 29-23 during composting, corresponding to the increase in TKN.

Similarly, Ho et al. (2013) observed an increase in TN from Day 0-4 (43-45 g/kg), in a 40-day bench scale composting of broiler manure composting that was spiked with doxycycline and several other antimicrobials. Moisture content was kept consistently between 50-60% and the compost was mixed daily during the experiment. This initial increase in TN was followed by a gradual decrease to 38 g/kg until the completion of composting.

Selvem et al. (2012) and Ho et al. (2013) had similar pile management to the present study so it is unclear how pile management may have impacted compost temperature profiles, however, both studies were conducted at the bench scale, which could explain some of the differences in results when compared to the field scale results presented in this paper. Additionally, the antimicrobials present in compost could impact the temperature profile through microbial inhibition.

Table 3.2: Average and standard error of total Kjeldahl nitrogen (TKN), total phosphorus (TP), and the carbon to nitrogen ratio (C:N) of the compost over time.

Day	**TKN (g/kg)**	**TP (g/kg)**	**C:N**
1	15.3 ± 0.2	1.15 ± 0.07	13.5 ± 0.2
2	15.6 ± 0.2	1.30 ± 0.01	12.9 ± 0.1
3	15.8 ± 0.2	1.30 ± 0.03	12.9 ± 0.1
5	16.1 ± 0.1	1.36 ± 0.01	12.0 ± 0.7
10	16.5 ± 0.4	1.43 ± 0.02	12.7 ± 0.3
20	17.4 ± 0.4	1.56 ± 0.04	12.9 ± 0.0
33	18.4 ± 0.7	1.75 ± 0.11	12.0 ± 0.1

3.4 Conclusions

Antimicrobial degradation results seen at the pilot scale may not be indicative of trends at the field scale, as longer composting times may be needed at the field scale to produce similar degradation results. The length of the composting period, the time to reach peak composting temperature, and compost turning, and watering frequency could all be important management factors to consider for antimicrobial degradation at the field scale. Composting management styles may need to change throughout the year to accomplish the same goals in antimicrobial degradation, as ambient winter temperatures could impact the speed of the pile to reach peak temperature.

Antimicrobial presence in compost substrate could also impact nutrient trends, through inhibition of N-mineralization, which is pertinent information for farm nutrient management. Further research is needed at the field scale to better understand the impact that of management and environmental conditions on antimicrobial degradation and nutrient management.

4.0 Tetracycline and sulfadimethoxine degradation during anaerobic digestion of dairy manure

4.1 Introduction

The use of antimicrobials in animal husbandry for both therapeutic and non-therapeutic purposes and the extensive use of manure-based fertilizers in agriculture has raised concerns about the persistence of antimicrobials and antimicrobial resistance genes (ARGs) during manure treatment and possible impacts on terrestrial and aquatic environments (Kumar et al., 2005; Mellon et al., 2001). Concerns over antimicrobial pollutants in manure fertilizers are amplified by studies showing detection of antimicrobials in surface waters and the uptake of antimicrobials into plant tissue (Simon, 2005; Migliore et al., 2010). Economic, environmental, and energy benefits associated with AD have expanded its practice on dairy farms. Yet, compared to the number of studies investigating the fate of antimicrobials during AD of swine manure or municipal wastewater sludge (Aydin et al., 2015; Liu et al., 2018; Stone et al., 2009), few antimicrobial AD studies have focused on dairy manure, and even fewer studies have examined both the fate of antimicrobial mixtures and ARGs during AD.

Some previous studies at the bench scale, have observed significant reductions in antimicrobials in dairy manure during anaerobic digestion (AD) (Arikan et al., 2006; Mitchell et al., 2013; Turker et al., 2013). However, a study that separately analyzed the liquid and solid fractions of AD treated dairy manure suggested that while sulfonamide antimicrobials were completely removed during AD, the significant reductions in tetracyclines (TC) in the liquid fraction of manure was a result of partitioning of TC into the solid fraction of manure (Wallace et al., 2018).

Prior dairy manure AD studies have focused primarily on tetracyclines (TC) (Arikan et al., 2008; Ince et al., 2013; Turker et al., 2013). Mitchell et al. (2013) examined sulfonamide degradation in batch AD reactors and found that sulfamethazine (SMZ) did not impact biogas production at concentrations up to 280 mg/L and that SMZ did not degrade during AD. These and most other studies of dairy manure AD, to date, studied the impacts of individual antimicrobials on AD (Arikan et al., 2006; Arikan, 2008; Ince et al., 2013; Turker et al., 2013; Xin et al., 2014). In farm management, however, multiple antimicrobials of varying drug classes are present in the manure stream. The combined effect of multiple antimicrobials present in dairy manure on the AD process has only been studies in a few studies.

For example, Beneragama et al. (2013) explored the combined effects of oxytetracycline (OTC) and cefazolin (CFZ) on methane (CH_4) production. They found that while an OTC spike of 90 mg/L and a combined OTC and CFZ addition (45 mg/L each) decreased CH_4 production by 68.6% and 70.3%, respectively, the inhibitory effects of both the individual antimicrobials and the antimicrobial mixture were similar.

The concentrations of antimicrobials used by Beneragama et al. (2013) and by others in prior AD studies (1-350 mg/L) (Beneragama et al., 2013; Coban et al., 2016; Loftin et al., 2005; Mitchel et al., 2013) are not representative of concentrations observed in farm manure lagoons. Environmentally relevant AD studies are needed to evaluate the risk of manure field applications on plant uptake and surface waters.

Zhang et al. (2015) investigated the degradation of multiple ARGs during AD of municipal wastewater sludge at both mesophilic and thermophilic temperatures. The abundance of sulfonamide ARGs increased during mesophilic AD and decreased by only 3% during thermophilic AD. The abundance of tetracycline ARGs was reduced by 40-50% in both mesophilic and thermophilic AD. Ma et al. (2011) found that concentrations of tetracycline and sulfonamide ARGs decreased during mesophilic AD of wastewater sludge.

Sun et al. (2016) examined tetracycline and sulfonamide ARG changes during mesophilic AD of dairy manure and found that tetM concentrations increased and Sul1 concentrations decreased. A farm-scale study showed that copies of sulfonamide resistant genes (Sul1 and Sul2) decreased significantly during AD, while tetracycline resistant genes (tet(O) and tet(W)) remained unchanged (Wallace et al., 2018). In short, changes in ARG concentration during AD of dairy manure in the presence of multiple antimicrobials and at environmentally relevant field concentrations have rarely been studied, and the limited results available are not fully consistent.

The objectives of this study were to: 1) understand the extent of degradation of tetracycline (TC), sulfadimethoxine (SDM), and antimicrobial mixtures during AD of dairy manure; 2) examine the influence of different concentrations and mixtures of antimicrobials on the AD process at relevant concentrations; and 3) monitor ARG persistence during AD and study the relationship between antimicrobial classes and ARG abundance.

4.2 Methods

4.2.1 Experimental Design

A biochemical methane potential test (BMP) was conducted following methods described by Moody et al. (2009). Both dairy manure, used as the substrate, and AD effluent, used as the inoculum, were collected from the USDA's Beltsville Agricultural Research Center (BARC) facility in Beltsville, MD, USA. Specifically, 82 mL of dairy manure and 118 mL of inoculum were added to 300 mL batch AD reactors using an inoculum to substrate ratio (ISR) of 2:1 on a volatile solids (VS) basis and 200 ml of inoculum was used for the inoculum-only control. Triplicate manure-only reactors, triplicate inoculum-only controls, and triplicate antimicrobial treatment reactors were set up for each of five antimicrobial treatments: 1) TC at 1 mg/L (TC 1), 2) TC at 10 mg/L (TC 10), 3) sulfadimethoxine (SDM) at 1 mg/L (SDM 1), 4) SDM at 10 mg/L (SDM 10), and 5) a mixture of the two antimicrobials (TC and SDM) each at 1 mg/L (TC+SDM 1).

Antimicrobial concentrations (1 and 10 mg/L) were chosen based on results of our 11-farm monitoring study that quantified antimicrobial concentrations during all stages of the dairy manure management systems at each farm every six weeks from September 2016-September 2017 (Hurst et al., 2018). The peak concentrations in manure samples were 0.5-1 mg/L, which was chosen as the low spike, while the 10 mg/L spike was chosen to represent a worst-case scenario shock to the manure system.

The antimicrobials, in powder form, were weighed, homogenized in water, and then pipetted, during manure homogenization on a stir-plate, into the treatment bottles. Bottles were purged with 70:30 N_2:CO_2 to ensure anaerobic conditions. The AD reactors were incubated for 44 days under anaerobic conditions at 35 °C. The CH_4 production was measured daily for the first 7 days of incubation, then adjusted to every other day and then weekly as the biogas production decreased. Measurements were taken using a glass syringe. The CH_4 production from the inoculum control bottles were subtracted from presented CH_4 data.

4.2.2 Laboratory Methods

Samples were collected from each reactor before and after the digestion period, and analyzed for pH, total solids (TS), VS, volatile fatty acids (VFA), and antimicrobials (TC and SDM and their metabolites). Solids (TS and VS) were analyzed in triplicate using the Standard Methods for the Examination of Water and Wastewater (APHA, 2005). For VFAs, samples were filtered through a 0.22 μm filter, then analyzed on the gas chromatograph (Agilent Technologies, Inc.; Shanghai China; model 7890 A) with a flame ionization detector (FID) operated at 300°C and 7693 autosampler (Agilent Technologies, model 7693), with a DB-FFAP capillary column (Agilent J&W; USA), and He as the carrier gas at 1.80 ml/min. The injection temperature was held at 250°C and the oven operated at 100°C for 2 min and subsequently ramped at 10°C/min for a total run time of 10 min. VFAs were converted and presented in terms of chemical oxygen demand (COD).

4.2.3 Antimicrobial Analysis

Samples for antimicrobial analyses were collected in 50-mL light sensitive polypropylene Corning centrifuge tubes, pre-washed with 2% 15.9 M Nitric acid. The samples were frozen and lyophilized, then antimicrobial solid-liquid extraction was performed prior to analysis via liquid chromatography-tandem mass spectrometry (LC-

MS/MS) (Agilent 6410 triple quadrupole, Santa Clara, CA). Mass of antimicrobials results are presented per nanograms of dry weight (DW) of manure. Antimicrobial recovery rates were calculated using Equation 4.1. Recoveries for the sulfonamide surrogate, sulfamethazine (SMZ), ranged from 63-225%, while the recoveries for the tetracycline surrogate, demeclocycline (DMC), ranged from 9-113%, in all samples. The following analytes were tested on all pre- and post-digestion samples: TC, 4-epitetracycline (ETC), OTC, anhydrotetracycline (ATC), chlorotetracycline (CTC), 4-epichlorotetracycline (ECTC), anhydrochlorotetracyline (ACTC), and SDM.

$$\% \, Recovery = \frac{[Surrogate]_{Calculated}}{[Surrogate]_{Expected}} \times 100\% \qquad \text{(Eq. 4.1)}$$

4.2.4 Quantitative PCR

Samples for ARG analyses were collected in sterile 50-mL centrifuge tubes, frozen at -20 °C, and shipped to the University of Michigan for quantitative PCR (qPCR) analysis. Sul1, Sul2, TetM, and 16S rRNA gene copies were quantified for each sample, as indicators of sulfonamide resistance, tetracycline resistance, and microbial biomass, respectively. Sul1 and TetM genes are most commonly used for monitoring resistance in manure and/or AD systems (Kyselková et al., 2015; Wolters et al., 2016). Primers, annealing temperatures, and references for TetM, Sul1, Sul2, and 16S rRNA were based on Luo et al. (2010), Pei et al. (2006), and Fierer and Jackson (2005), respectively (Table 4.1). Widely-used primer sets were selected to enable comparison of our results to other studies. The primer sets were verified for specificity using NCBI PrimerBlast against the archaea, virus, viroid, and eukaryote nucleotide databases. qPCR reactions were carried out on an Eppendorf MasterCyler ep realplex^2 using Fast EvaGreen Fast Master Mix (Biotium, Fremont, CA, USA).

Table 4.1: Primers, sequences, and annealing temperatures for antimicrobial resistant gene analysis.

Primer	Sequence (5`-3`)	Annealing Temp (ºC)	Reference
TetM Fwd TetM Rev	CCGTTGGGAAGTGGAATGC TCCGAAAATCTGCTGGGGTA	59	Luo et al., 2010
Sul1Fwd Sul1Rev	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	62	Pei et al., 2006
Sul2Fwd Sul2Rev	TCCGGTGGAGGCCGGTATCTGG CGGGAATGCCATCTGCCTTGAG	58	Pei et al., 2006
16S Fwd 16S Rev	ACTCCTACGGGAGGCAG ATTACC GCGGCTGCTGG	54	Fierer and Jackson, 2005

The 10 µL reactions were performed following the manufacturer's recommended reaction mixture with 0.4 uM of forward and reverse primers and 0.625 mgmL^{-1} of Ultrapure BSA (Invitrogen, Carlsbad, CA, USA). Plates were centrifuged for 2 min at 500 RPM at 4ºC before thermocycling, with 1 µL DNA extracts used in each reaction. The pre-reactor samples consisted of one biological replicate and the post-reactor samples consisted of three biological replicates. qPCR reactions were run in triplicate for seven pre-AD samples (one for each antimicrobial treatment, the manure-only treatment, and inoculum-only) and in duplicate for the 21 post-AD samples. The template for the standard curve consisted of Gblock Fragments (IDT, Skokie, Illinois, USA). When the selected primer hit the CARD ARG sequences, that sequence was used as the standard curve amplicon. When the primers did not hit the exact CARD ARG sequence, a

sequence was selected from NCBI. Table 4.2 includes the sequences used for the Gblock samples.

Table 4.2: Gblock fragments (IDT, Skokie, Illinois, USA) used for standard curve for qPCR reactions.

Gene	**Accession Number**	**Target length**	**Sequence**
Sul1	gb|JF969163|+|1054-1893|sul	163	CAGTTTCTCCGGTGGAGGCCGGTATCT GGCGCCAGACGCAGCCATTGCGCAGGC GCGTAAGCTGATGGCCGAGGGGGCAGA TGTGATCGACCTCGGTCCGGCATCCAGCA ACCCCGACGCCGCGCCTGTTTCGTCCGAC ACAGAAATCGAGCGTATCGCGCCGGTGCT GGACGCGCTCAAGGCAGATGGCATTCCCG TCTCG
Sul2	NG_048113.1	191	CAGTTTCTCCGGTGGAGGCCGGTATCTGGCG CCAGACGCAGCCATTGCGCAGGCGCGTAAG CTGATGGCCGAGGGGGCAGATGTGATCGAC CTCGGTCCGGCATCCAGCAACCCCGACGCCG CGCCTGTTTCGTCCGACACAGAAATCGAGCG TATCGCGCCGGTGCTGGACGCGCTCAAGGCA GATGGCATTCCCGTCTCG
TetM	NC_004116.1:c929374-927455	196	CACCGCTTCCGTTGGGAAGTGGAATGCAG TATGAGAGCTCGGTTTCTCTTGGATACTTA AATCAATCATTTCAAAATGCAGTTATGGAA GGGATACGCTATGGTTGCGAACAAGGATTA TATGGTTGGAATGTGACGGATTGTAAAATC TGTTTTAAGTATGGCTTATACTATAGCCCTG TTAGTACCCCAGCAGATTTTCGGATGCTTG CTC
16S	CP026677.1:1353823-1354031	200	GTCCAGACTCCTACGGGAGGCAGCAGTGGGG AATATTGCACAATGGGCGCAAGCCTGATGCAG CCATGCCGCGTGTATGAAGAAGGCCTTCGGGT TGTAAAGTACTTTCAGCGAGGAGGAAGGCGTT GTGGTTAATAACCGCAGCGATTGACGTTACTCG CAGAAGAAGCACCGGCTAACTCCGTGCCAGCA GCCGCGGTAATACGGAG

4.2.5 Statistics

Analysis of variance (ANOVA) was conducted on all post-digestion reactors for pH, TS, VS, cumulative CH_4 production, and VFAs to determine if there was statistical significance between reactors, with post-hoc Fishers least significant difference (LSD) test. *P*-values < 0.05 were considered significant. All values are reported as averages ± standard error (SE). Differences between 16S rRNA gene-normalized concentrations of TetM and sul1 were compared using ANOVA. All pre-AD reactors were statistically analyzed together, under that assumption that there would be minimal ARG impact from the antimicrobial additions, since these samples were frozen immediately after antimicrobial spiking. All statistical analyses were performed in Microsoft Excel, except for the ARG analysis, which was performed in R.

4.3 Results and Discussion

4.3.1 Methane Production

SDM and TC reactors with 10 mg/L spikes (SDM 10 and TC 10) significantly decreased CH_4 production (110 ± 2 and 110 ± 1 ml CH_4/g VS, respectively) by 7.8% compared to manure-only (120 ml CH_4/g VS; *p*-values of 0.0195 and 0.0160, respectively), as shown in Table 4.3 and Figure 4.1.

The reactors had similar CH_4 production during the first four days of incubation before diverging (Figure 4.1), with no observed lag phase for any reactor at AD start-up. The TC 1 and TC+SDM 1 reactors, and manure-only reactors had similar cumulative CH_4 production of approximately 120 ml CH_4/g VS, while the SDM 1 reactor averaged 115 ± 3 ml CH_4/g VS (Table 4.3; Figure 4.1).

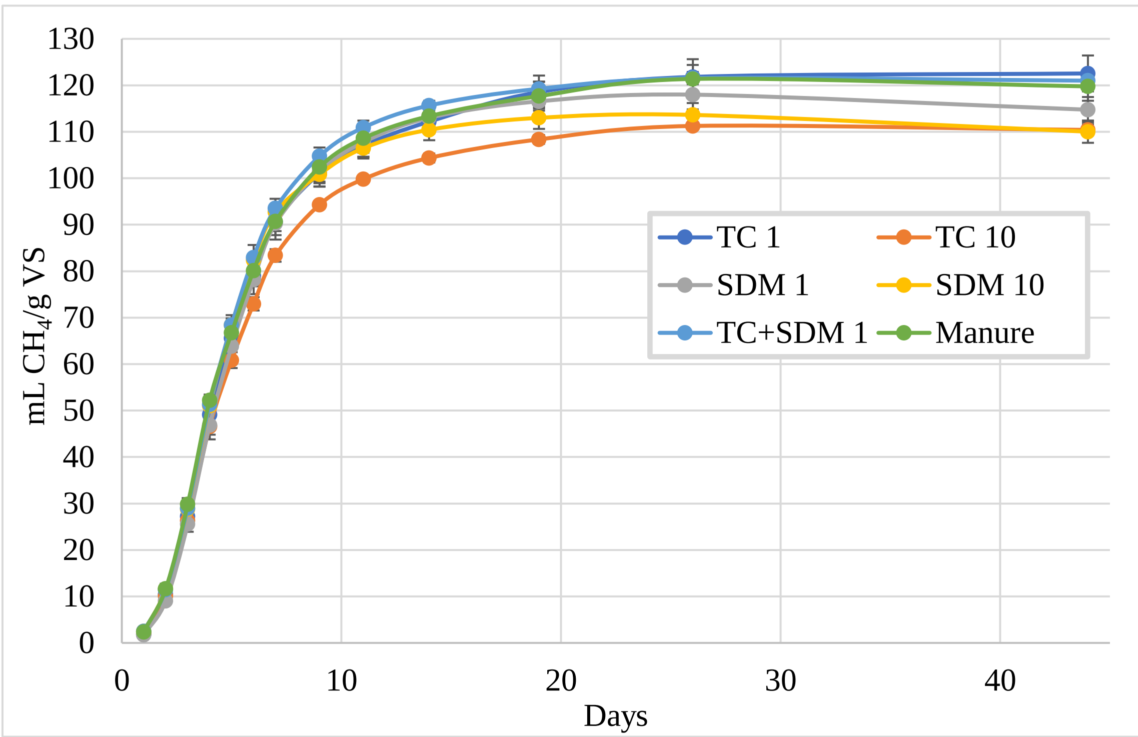

Figure 4.1: Cumulative CH_4 production over 44-days in bench scale anaerobic digestion of dairy manure normalized by volatile solids (VS). Treatments include 1 mg/L and 10 mg/L spikes of tetracycline (TC) and sulfadimethoxine (SDM), a mixture of TC and SDM at 1 mg/L, and manure-only.

Mitchell et al. (2013) examined another sulfonamide, sulfamethazine (SMZ), and found no biogas inhibition compared to the control during mesophilic AD with concentrations up to 280 mg/L of SMZ, compared to our 7.8% inhibition at 10 mg/L of

SDM and TC. The inhibitory potential of antimicrobials on CH_4 production at high dosages (30 mg/L-350 mg/L) has been documented more frequently than for lower doses representative of concentrations observed in farm manure systems (Beneragama et al., 2013; Coban et al., 2016; Mitchell et al., 2013).

Table 4.3: Average and standard error of cumulative methane (CH_4) production, total solids (TS), and volatile solids (VS) for tetracycline (TC) and sulfadimethoxine (SDM) spikes of 1 and 10 mg/L and a manure-only treatment before and after 44-days of anaerobic digestion.

Reactors	**Cumulative CH_4** (mL CH_4/g VS)	**TS (g/L)**		**VS (g/L)**	
		Pre	**Post**	**Pre**	**Post**
TC 1	122.6 ± 3.9	22.0 ± 0.3	15.6 ± 0.1	13.4 ± 0.2	9.3 ± 0.1
TC 10	110.4 ± 1.2	18.5 ± 0.5	13.7 ± 1.4	11.8 ± 0.3	8.2 ± 1.1
SDM 1	114.8 ± 2.7	19.3 ± 1.5	13.1 ± 1.7	12.4 ± 1.1	7.8 ± 1.2
SDM 10	110.0 ± 2.4	19.8 ± 0.7	13.4 ± 0.6	12.7 ± 0.5	7.7 ± 0.5
TC SDM 1	121.0 ± 1.8	17.8 ± 0.7	15.7 ± 0.2	11.5 ± 0.4	9.4 ± 0.1
Manure	119.8 ± 3.1	19.4 ± 0.7	13.8 ± 0.8	12.7 ± 0.6	8.1 ± 0.5

While no lag phase was observed in CH_4 production in this study, Shi et al. (2011) found a lag phase during AD of swine manure at 25 ºC with TC added at higher dosages (25 and 50 mg/L), indicating that TC could delay biogas production at higher dosages. Although the 1 mg/L SDM and TC spikes in this study did not significantly lower CH_4 production, another microcosm study (Loftin et al., 2005) using anaerobic lagoon swine slurry incubated at 22 ºC showed significant inhibitions in CH_4 production for both TC and SDM at concentrations as low as a 1 mg/L. Inhibition percentages of 40-45% at 5 and 25 mg/L spikes of TC and SDM were observed in the Loftin et al. (2005) study. Operating conditions, such as mixing rate and VS content, have been shown to affect microbial community dynamics associated with CH_4 production, so the differences in manure/inoculum sources and experimental operating parameters might account for the differences observed in CH_4 inhibition (Turker et al., 2016).

4.3.2: Volatile Fatty Acids (VFAs) reduction during digestion

The TC 10 had slightly lower acetic acid reductions (98% reduction) during AD compared to the other reactors (100% reduction), which could explain the lower CH_4 production in this reactor (Table 4.4). The acetic acid concentrations did not affect the final pH of the reactors, with all reactors maintaining a stable pH of approximately 7.5 before and after AD.

The pre-digestion acetic acid concentrations in the reactors were similar (900-978 mg/L as COD), except the SDM 1 reactor (851 mg/L as COD), which was significantly less than the manure-only and TC 10 reactors (*p*-value of 0.0265 and 0.0087, respectively). All VFAs decreased 100% in the manure-only reactors during AD (Table 4.5). The TC 1, SDM 1, and SDM 10 reactors had 100% degradation of acetic acid in all triplicates. Propionic acid and butyric acid degraded 100% in all reactors.

Table 4.4: Average and standard error of volatile fatty acids, presented as mg/L chemical oxygen demand (COD) for tetracycline (TC) and sulfadimethoxine (SDM) spikes of 1 and 10 mg/L and manure-only reactors before and after 44-day anaerobic digestion of dairy manure at 35°C.

	Acetic Acid (mg/L)		**Propionic Acid** (mg/L)		**Butyric Acid** (mg/L)		**Valeric Acid** (mg/L)	
Treatment	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**	**Pre**	**Post**
TC 1	924 ± 21	0 ± 0	408 ± 7	0 ± 0	295 ± 5	0 ± 0	0 ± 0	685 ± 50
TC 10	978 ± 28	20 ± 17	459 ± 31	0 ± 0	364 ± 44	0 ± 0	0 ± 0	0 ± 0
SDM 1	851 ± 7	0 ± 0	381 ± 6	0 ± 0	278 ± 11	0 ± 0	0 ± 0	0 ± 0
SDM 10	911 ± 17	0 ± 0	410 ± 11	0 ± 0	299 ± 12	0 ± 0	0 ± 0	684 ± 46
TC+SDM 1	900 ± 15	45 ± 34	389 ± 9	0 ± 0	280 ± 11	0 ± 0	0 ± 0	664 ± 40
Manure	958 ± 23	0 ± 0	450 ± 25	0 ± 0	340 ± 27	0 ± 0	649 ± 24	0 ± 0

Labatut and Gooch (2014) stated that accumulation of VFAs during AD can be a sign of process instability, and VFA concentrations above 1200 mg/L could inhibit biogas production. The SDM 10 reactor had an accumulation of valeric acid post-digestion (684 mg/L), which could indicate some process instability corresponding to the decreased CH_4 production in this reactor, however, the TC 1 and the TC+SDM 1 reactor also showed an accumulation in valeric acid during AD without any inhibition to CH_4 production (Table 4.4). The overall process stability in the reactors was shown through the significant reductions post-AD in acetic and propionic acid concentrations, with reductions ranging from 95 to 100%.

4.3.3 Total solids (TS) and volatile solids (VS) reduction during digestion

VS and TS reduction during AD ranged from 26% to 40%, except the TC+SDM 1 reactor, which had an 18% VS reduction and a 12% TS reduction. Decreased VS reduction in the TC+SDM1 reactor did not correspond to a significant decrease in CH_4

production (Table 4.3). While reductions in the TC 1, SDM 10, and manure only reactors were significant (*p*-value ranging from 0.001 - 0.02), reductions in the TC 10, SDM 1, and TC+SDM 1 reactors were not significant (*p*-value ranging from 0.1 - 0.2).

Reactors had average TS and VS pre-digestion values ranging from 17.8 - 22.0 g TS/L and 11.5 - 13.4 g VS/L, and post-digestion values ranging from 13.10 - 15.67 g TS/L and 7.73 - 9.37 g VS/L (Table 4.3). While the TC+SDM 1 reactor had lower VS reduction and an accumulation of valeric acid during AD, there was not a significant impact on CH_4 production. Taken together, these values could be indicative of potential process instability if further disturbance occurs. There was no significant difference in the TS or the VS between reactors before AD (*p*-values of 0.0792 and 0.4195, respectively) or after AD (*p*-values of 0.3312 and 0.4330, respectively), indicating that the antimicrobial additions did not significantly impact solids degradation.

4.3.4 Fate of sulfadimethoxine (SDM) and tetracycline (TC) during digestion

SDM decreased by >99% during AD for all reactors when compared to the expected SDM concentration following the SDM spike (*p*-value = 0.002) (Table 4.5). Additionally, no SDM was detected in the manure-only reactors or inoculum controls, indicating that there was no background concentration of SDM in the manure or digester inoculum used in the experiment, and therefore, all SDM detected in the pre-AD samples can be attributed to the antimicrobial spikes performed in the lab. This result showing >99% degradation differed from the findings in Mitchell et al. (2013), where another sulfonamide, sulfamethazine (SMZ), was examined at concentrations of 0.28 - 280 mg/L during the digestion of cattle manure with wastewater sludge inoculum and SMZ did not significantly degrade during the AD process.

Wang et al. (2006) cited biodegradation by the microbial community as the main elimination pathway for sulfonamides. The difference in degradation patterns from the present study to Mitchell et al. (2013) could be attributed to differences in the microbial communities of the inoculums, since the current study uses dairy manure as a source and Wang et al. (2006) sourced inoculum from wastewater treatment. The microbial community of the manure source in this study was characterized in a prior study (Witarsa et al. 2016) and not quantified again for this study. In the prior study, the terminal restriction fragment (TRF) 302 represented 85% of the relative gene abundance and this TRF was identified as belonging to the *Methanosaetaceae* family.

In the TC+SDM 1 reactor and the TC 10 reactor, the TC concentrations decreased >85% through digestion, when post-AD concentrations were compared to the expected concentrations in the pre-AD samples due to the spiked antimicrobial addition (Table 4.5). The manure reactor and inoculum control had 0 - 70 ng/g DW TC, indicating some background levels of tetracyclines in the manure substrate. The triplicates for the TC 1 reactor did not present a uniform pattern of degradation, leading to an average percent increase in antimicrobials when post-AD samples were compared with the antimicrobial spike.

Table 4.5: Average and standard error of sulfadimethoxine (SDM) and tetracycline (TC) concentrations before and after 44 days of mesophilic anaerobic digestion, along with percent reduction calculated between the antimicrobial spike and the post-digestion concentration.

Reactors	**Spike (mg/L)**		**SDM**			**TC**		
	SDM	TC	Pre (mg/L)	Post (mg/L)	% reduction	Pre (mg/L)	Post (mg/L)	% reduction
TC 1	-	1	ND*	ND*	-	1.08 ± 0.56	1.07 ± 0.57	-6.8#
TC 10	-	10	ND*	ND*	-	0.09 ± 0.03	1.16 ± 0.24	88.4
SDM 1	1	-	0.06 ± 0.02	0.01 ± 0	99.3	ND*	ND*	-
SDM 10	10	-	1.02 ± 0.05	0.03 ± 0	99.7	ND*	ND*	-
TC+SDM 1	1	1	0.08 ± 0.01	ND*	100.0	0.16 ± 0.02	0.04 ± 0.02	95.7
Manure	-	-	ND*	ND*	-	ND*	0.01 ± 0.00	-

*ND = not detectable.
#A negative value indicates a percent increase.

Tetracyclines transform readily between pre-cursor and transformation products, depending on pH and other environmental conditions, and this could explain the variability in the triplicates for the TC 1 reactor (Chen and Huang, 2009; Kühne et al. 2000). Liu et al. (2018) examined TC degradation in pilot scale AD system during two seasons (summer and fall) and found TC degradation (up to 18%) post-digestion in the summer season as well as TC accumulation in the AD effluent during the fall season, during their two experiments, supporting the variability of tetracycline transformation in varying environmental conditions.

The method recovery rates for tetracycline were 10 - 35% higher in the post AD samples compared to the pre-AD samples, likely due to the significantly lower solids contents in the post-AD samples and increase of particle surface area and sorption sites in the post AD particles (Table 4.3). Solids content significantly decreased in the effluent compared to the influent in our study due to microbial degradation of organics (Table 4.3), likely contributing to the higher extraction efficiencies in the post-AD samples. Additionally, tetracyclines sorb strongly to solids, therefore, sampling a heterogeneous manure matrix can still result in solid particle variability and TC sorption variability (Wallace et al., 2018; Wu et al., 2009).

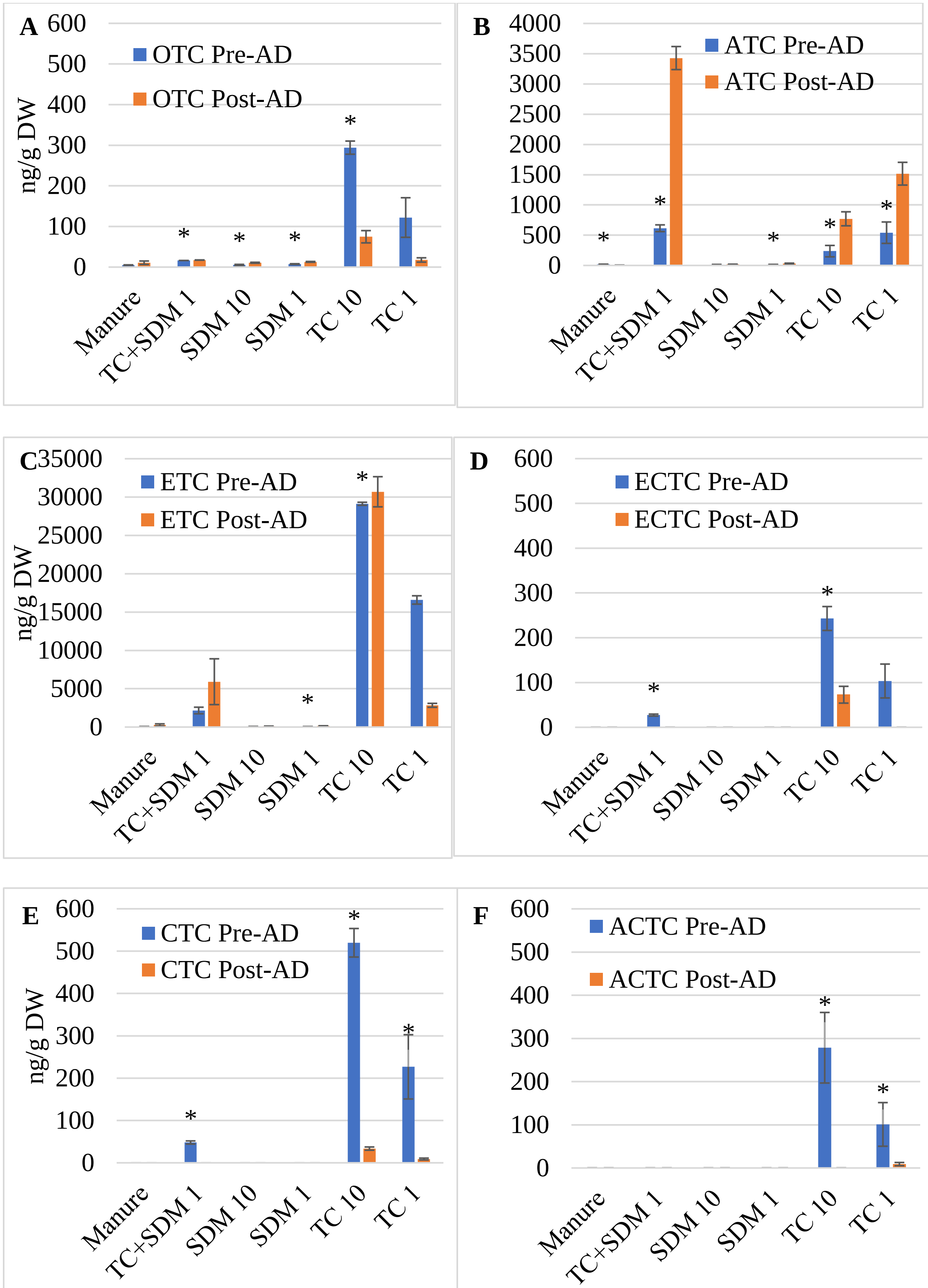
A
B
C
D
E
F
ng/g DW
OTC Pre-AD
OTC Post-AD
ATC Pre-AD
ATC Post-AD
ETC Pre-AD
ETC Post-AD
ECTC Pre-AD
ECTC Post-AD
CTC Pre-AD
CTC Post-AD
ACTC Pre-AD
ACTC Post-AD
Manure
TC+SDM 1
SDM 10
SDM 1
TC 10
TC 1

Figure 4.2: Concentrations of tetracycline metabolites in ng/g dry weight (DW) before and after anaerobic digestion: (A) oxytetracycline (OTC), (B) anhydrotetracycline (ATC), (C) 4-epitetracycline (ETC), (D) 4-epichlorotetracycline (ECTC), (E) chlorotetracycline (CTC), and (F) anhydrochlorotetracyline (ACTC). *Indicate treatments that are significantly different before and after digestion at $\alpha = 0.05$.

Degradation products of TC were measured in the pre- and post-AD samples, and the concentrations were variable (Figure 4.2). Concentrations of epi-tetracycline (ETC) and anhydrotetracycline (ATC) were the highest pre-AD, ranging from 236 - 29,000 ng/g DW in the TC-spiked reactors. Oxytetracycline (OTC), 4-epi-chlortetracycline (ECTC), chlortetracycline (CTC), and anhydrochlortetracycline (ACTC) were present in trace concentrations pre-AD (<550 ng/g DW) and decreased 70 - 100% post-AD in the TC 1 and TC 10 reactors. Arikan et al. (2008) observed a 75% reduction in CTC and 33% reduction in ECTC after a 33-day mesophilic AD period, which is comparable to the degradations of CTC and ECTC in our study.

Although the SDM 10 and TC 10 reactors inhibited CH_4 production by 7.8% relative to the manure only reactor, the TC and SDM degradation during AD were still high (88% and 99%, respectively). ETC and ATC metabolites increased 5.4% and 225%, respectively, post digestion. These observed variabilities in the TC and TC degradation product concentrations and the significant increase in ATC are not surprising, as it has been well documented that TC undergoes reversible epimerization to ETC and irreversible dehydration to ATC (Yuen et al., 1977). Increases in metabolites post-AD was also documented by Arikan et al. (2008), with a two-fold increase post-AD of the tetracycline metabolite, iso-chlortetracycline. Fedler and Day (1985) found that accumulation of antimicrobial metabolites could be the cause of CH_4 inhibition, rather than the parent compounds. Degradation products for sulfadimethoxine were not measured during this study.

4.3.5 Fate of antimicrobial resistance genes during digestion

The declines in TetM and Sul1 absolute gene concentrations corresponded to declines in 16S rRNA gene copies per mg, which suggests that the gene decline could be related to a decline intotal biomass resulting from the digestion process. Sul1 and TetM gene copies ranged from 400 - 1200 gene copies/mg of sample across all reactors. TetM gene copies decreased by 23 to 55% in all reactors after 44 days of digestion. When TetM gene copies were normalized by 16S rRNA gene copies (Figure 4.3), the relative abundances of the genes decreased (18 - 30%), although not significantly (p-value = 0.2801), except for the TC+SDM 1 treatment (p-value = 0.0012).

Palmer et al. (2010) reported that while TC strongly selects resistant organisms, its numerous degradation products select for sensitive organisms. Solutions of TC applied at high concentrations (1000 ng/mL) strongly favored the growth of resistant bacteria. Following substantial degradation, not only did the loss of TC abolish selection of resistance, but the accumulation of its degradation products caused strong selection against resistance. Hence, accumulation of ATC in AD-treated manure may have beneficial consequences in terms of ARG removal.

Sul1 copies/mg decreased by 13 - 32% in all reactors after AD, except for the TC+ SDM 1 reactor, which increased by 5% in the post-AD samples. Additionally, this reactor had the lowest VS reduction (18%) and acetic acid removal (95%) during AD of all reactors When the Sul1 gene copies were normalized by 16S rRNA gene copies (Figure 4.3), the relative Sul1 gene abundancies increased by 4 - 20% post-AD in all reactors, however, changes in Sul1 were not statistically significant (*p*-value = 0.1145).

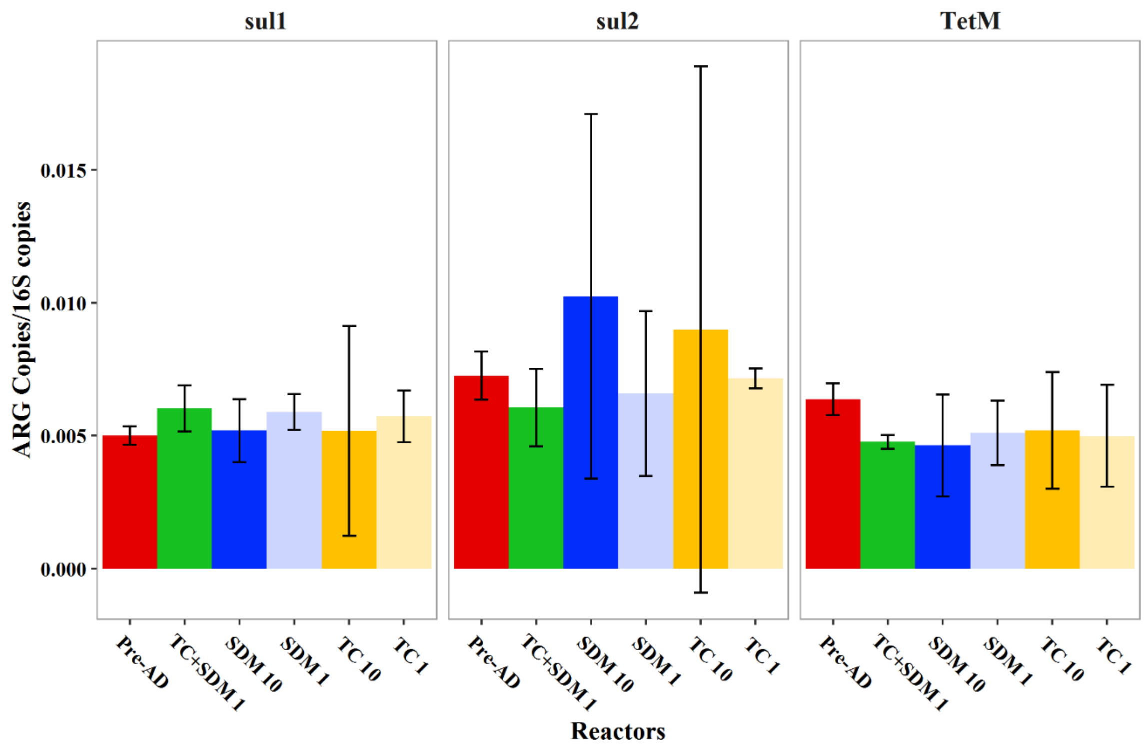

Figure 4.3: Sul1, Sul2, and TetM gene copies normalized by 16S rRNA gene copies before and after 44 days of anaerobic digestion at 35°C. Pre-AD samples from all reactors were pooled for pre-AD gene copy analysis. Treatments include 1 mg/L and 10 mg/L spikes of tetracycline (TC) and sulfadimethoxine (SDM), a mixture of TC and SDM at 1 mg/L, and manure-only. N=5 for pre-AD; N=3 for each post-AD spiked reactor.

Zhang et al. (2015) recorded total abundance of tetracycline and sulfonamide ARGs during mesophilic digestion and found an overall reduction of tetracycline ARGs of

42.7% but increases in sulfonamide ARGs of 26.3%. Interestingly, in the Zhang et al. (2015) study, TetM and Sul1, specifically, were both enriched after AD by 39.5% and 48.2%, respectively. Anaerobic digestion could be an effective method of removal for TC ARGs, however, further research is needed to clarify conflicting results from previous literature. The removal efficiency of sulfonamide ARGs during AD is less effective, even though the removal of the sulfonamide antimicrobial residuals was > 99%.

4.4 Conclusions

Significant decreases in CH_4 production (7.8%) with 10 mg/L TC and SDM spikes indicate AD performance was affected at higher concentrations of antimicrobials, with no inhibition observed at 1 mg/L. There was > 99% degradation of SDM during AD, but this did not correspond with decreased Sul1 abundance post-AD. Tetracycline degradation and TetM decreases were observed during AD. Further exploration is needed to understand TC detection accuracy and the relationship between sulfonamide concentrations and Sul1 concentrations during AD. The study results can be used to understand antimicrobial resistance mitigation using manure-based digestion with concentrations observed on-farm.

5.0 Conclusions

The goal of this research was achieved through furthering the understanding of antimicrobial degradation and nutrient transformations under various manure management conditions at the field and bench scale. The impact of farm, digester, and compost management practices on nutrient transformations, antimicrobial perpetuation, and antimicrobial resistance was explored and cultivated a better understanding of the effect of manure management practices on reducing pollution from agricultural manure fertilizers to surrounding watersheds.

The concentrations of TC were inconsistent in AD effluent at the field scale, with concentrations ranging from below detection to 34,000 ng/g DW, and the effluent concentrations were not significantly different than the AD influent, indicating limitations of antimicrobial degradation during field scale AD. TC degradation at the field scale could be limited due to short HRTs (13-16 days) or by increased sorption of TCs due to increased particulate surface area after digestion. Longer HRTs may be optimal for antimicrobial reductions during field scale digestion.

While antimicrobials degraded significantly during bench scale anaerobic digestion (>85% of TCs and sulfonamides) in this study, and in multiple other bench scale and pilot scale studies in the literature, AD at the field scale did not significantly reduce TC concentrations in the AD effluent compared to concentrations in the AD influent. Additionally, removal of antimicrobial resistance genes was explored at the bench scale. One reactor showed a reduction of tetM genes during bench scale AD, suggesting that AD could be an effective treatment for removing tetracycline ARGs from manure. Conversely, the 100% reduction of sulfadimethoxine antimicrobials during bench scale

AD did not correspond with Sul1 reduction, illustrating differences in antimicrobial versus gene reductions during manure treatment. ARGs are of critical importance due to the potential effects that horizontal gene transfer poses to medically relevant antimicrobials for human health. Understanding the reductions of ARGs and how they relate to persisting concentrations of antimicrobials in manure is critical. Field scale studies looking at antimicrobial degradation and ARG persistence during AD are not well explored, so this reveals a considerable gap in the literature and our understanding of digestion systems.

A unique feature of this study was the collection of available antimicrobial administration data, and an attempt was made to compare administration to concentrations seen in digester influent and effluent. It was difficult to make comparisons between antimicrobials concentrations in the influent and the effluent of digestion systems, as farms were sampled approximately every six weeks, which did not align with the HRTs of the farm digesters. The disconnect between sampling times and HRTs of the digesters also made it difficult to make detailed comparisons between farm administration data and concentration trends in the effluent. A more intensive monitoring process of field scale digesters, given consideration of the digester's HRT, could better reveal relationships between antimicrobial administration and concentrations in the influent and effluent.

TCs during dairy manure composting at the field scale did not follow a first order kinetic degradation, like most antimicrobials in the pilot scale and field scale composting literature. This could be closely related to pile management, physio-chemical properties, and duration of composting. The present study, which was conducted for 33-days did not see significant reductions in antimicrobials, however, previous field scale studies that ran for 80 or 180-days have observed significant reductions. Longer composting times are likely needed at the field scale to achieve similar degradation patterns of antimicrobials in bench scale composting studies.

Additionally, antimicrobials could have inhibited manure nitrogen loss during composting, therefore antimicrobial persistence in compost could have significant implications for nutrient management on farms. More intensive research during field scale composting is required to examine the impacts that aeration, pile temperature, turning, and duration of composting have on antimicrobial trends, nutrients transformations during the composting process, and any relationships between nutrients and antimicrobials.

AD and composting systems are different from farm to farm in terms of size, scale, and management practices. Degradation of antimicrobials at the bench scale, both in this study and in the literature, was not reflective of the degradation patterns monitored during field scale anaerobic digestion or composting. This highlights the need for more field scale studies to better understand factors influencing manure management on-farm, which is essential to provide accurate information to farmers for digester management, antimicrobial mitigation , and nutrient management. The role of agriculture in environmental pollution is a growing concern, especially in the Chesapeake Bay watershed, and understanding the role of manure management is key towards mitigating antimicrobial persistence and effectively managing manure nutrients and field fertilization to meet the 2025 TMDL of pollution reductions for the Chesapeake Bay.

Appendices

Appendix A: Monthly antimicrobial administration data, nutrient data, and volatile fatty acid data from field scale digestion systems.

Table A.1: Total mg of antimicrobial administration on farm at CF1

CF1	**Ceftiofur (mg) as Spectramast**	**Dihydrostreptomycin Sulfate (mg) as Quartermaster**	**Procaine Penicillin G (mg) as Quartermaster**
Jun-16	6,750	68,000	68,000
Jul-16	10,750	156,000	156,000
Aug-16	11,750	160,000	160,000
Sep-16	12,000	124,000	124,000
Oct-16	10,125	68,000	68,000
Nov-16	10,125	64,000	64,000
Dec-16	8,375	128,000	128,000
Jan-17	12,000	88,000	88,000
Feb-17	12,500	56,000	56,000
Mar-17	8,750	72,000	72,000
Apr-17	3,625	72,000	72,000
May-17	10,250	124,000	124,000
Jun-17	13,500	152,000	152,000
Jul-17	8,125	136,000	136,000
Aug-17	16,125	72,000	72,000

Table A.2: Antimicrobial administration at CF2 (Part 1)

CF2	**Oxytetracycline (mg) as Vetrimycin**	**Enrofloxacin (mg) as Baytril**	**Ceftiofur (mg) as Excede or Ceftiflex**	**Pirlimycin (mg) as Pirsue**	**Ampicillin (mg) as Polyflex**
Jun-16	350,000	75,000	233,000	3,000	1,200,000
Jul-16	400,000	0	205,000	0	1,500,000
Aug-16	0	0	205,000	1,200	300,000
Sep-16	0	0	29,000	0	600,000
Oct-16	0	0	5,000	0	600,000
Nov-16	0	0	29,000	0	900,000
Dec-16	0	0	5,000	0	600,000
Jan-17	0	0	205,000	600	900,000
Feb-17	100,000	0	170,000	3,000	1,200,000
Mar-17	0	50,000	90,000	1,200	900,000
Apr-17	0	0	105,000	0	600,000

May-17	0	0	65,000	1,200	1,500,000
Jun-17	0	25,000	133,000	0	1,200,000
Jul-17	400,000	0	154,000	0	900,000
Aug-17	0	0	149,000	1,200	600,000
Sep-17	0	0	109,000	0	600,000

Table A.3: Antimicrobial administration at CF2 (Part 2)

CF2	**Procaine Penicillin G (mg) as Quartermaster**	**Dihydrostreptomycin (mg) as Quartermaster**	**Ceftiofur (mg) as Spectramast**	**Tetracycline (mg)**
Jun-16	576,000	576,000	90,000	0
Jul-16	1,152,000	1,152,000	54,000	0
Aug-16	864,000	864,000	18,000	0
Sep-16	1,152,000	1,152,000	18,000	6,480,000
Oct-16	1,152,000	1,152,000	54,000	0
Nov-16	576,000	576,000	18,000	0
Dec-16	576,000	576,000	36,000	0
Jan-17	864,000	864,000	54,000	0
Feb-17	576,000	576,000	18,000	0
Mar-17	1,152,000	1,152,000	36,000	0
Apr-17	1,152,000	1,152,000	18,000	0
May-17	576,000	576,000	54,000	0
Jun-17	1,152,000	1,152,000	72,000	0
Jul-17	1,008,000	1,008,000	18,000	0
Aug-17	720,000	720,000	18,000	0
Sep-17	720,000	720,000	18,000	0

Table A.4: Antimicrobial administration at CF3

CF3	**Ampicillin Trihydrate (mg) as Polyflex**	**Oxytetracycline (mg) as LA 200**	**Penicillin (IU) as Albadry plus**	**Novobiocin (mg) as Albadry plus**	**Ceftiofur (mg) as Excede**
Jun-16	3,660,000	252,000	35,200,000	70,400	26,400
Jul-16	4,050,000	126,000	38,400,000	76,800	343,200
Aug-16	3,510,000	252,000	31,200,000	62,400	140,800
Sep-16	3,330,000	84,000	37,600,000	75,200	114,400
Oct-16	2,340,000	126,000	20,800,000	41,600	105,600
Nov-16	3,450,000	42,000	32,800,000	65,600	61,600
Dec-16	1,620,000	84,000	34,400,000	68,800	132,000
Jan-17	3,090,000	126,000	33,600,000	67,200	132,000
Feb-17	2,100,000	714,000	23,200,000	46,400	149,600

Mar-17	1,530,000	42,000	28,000,000	56,000	88,000
Apr-17	3,150,000	84,000	29,600,000	59,200	70,400
May-17	3,420,000	252,000	37,600,000	75,200	35,200
Jun-17	4,440,000	168,000	52,800,000	105,600	44,000
Jul-17	4,470,000	126,000	39,200,000	78,400	79,200
Aug-17	4,110,000	84,000	43,200,000	86,400	123,200
Sep-17	3,660,000	84,000	40,800,000	81,600	44,000

Table A.5: Total ammonia nitrogen (TAN), total Kjeldahl nitrogen (TKN), and total phosphorus (TP) in anaerobic digestion influent and effluent at each collaborating farm on a monthly basis.

	TAN (mg N/L)		**TKN (mg N/L)**		**TP (mg P/L)**	
	Influent	**Effluent**	**Influent**	**Effluent**	**Influent**	**Effluent**
CF1						
Jun-16	374	260	470	315	64	38
Sep-16	321	421	265	453	36	53
Oct-16	38	129	461	273	59	39
Feb-17	75	246	337	584	98	101
Jun-17	274	246	736	645	53	90
Aug-17	342	367	587	386	61	33
CF2						
Jun-16	643	1749	2354	2088	374	320
Sep-16	1320	1530	2559	2650	364	405
Oct-16	852	1660	1959	2684	297	410
Feb-17	1196	1074	3009	2994	80	374
Jun-17	1439	1753	3435	3323	74	446
Aug-17	1037	1461	2494	2295	37	321
CF3						
Jun-16	1950	2660	3190	2725	750	643
Sep-16	1790	2740	3990	3650	930	908
Oct-16	1240	2370	4340	3650	1010	1086
Feb-17	1481	3258	4010		860	
Jun-17	2156	2782	4021	4230	1036	676
Aug-17	1672	2407	2575	2748	771	641
CF4						
Jun-16	1860	1800	3680	2675	690	523
Sep-16	1440	1560	3450	2850	675	539
Oct-16	1610	741	3751	3238	679	600
Feb-17	1776	1826	3431	3787	566	618
Jun-17	1580	2016	2874	3066	462	503
Aug-17	1318	1664	3068	3142	578	547
CF5						
Jun-16	1500	1190	2253	3175	330	423
Sep-16	1540	1740	3325	2675	490	413

Oct-16	1480	1030	3126	3103	504	405
Feb-17	1833	1527	3726	3360	506	421
Jun-17	1407	2082	2043	3405	357	464
Aug-17	969	1514	2086	2689	326	415
			CF6			
Jun-16	1520	2040	2825	2525	768	700
Sep-16	1220	2300	3100	3025	1168	848
Oct-16	189	2220	3107	3356	1071	842
Feb-17	723	1337	2747	3191	1006	757
Jun-17	907	1678	3328	3814	479	812
Aug-17	768	375	2190	1843	614	444

Table A.6: Monthly volatile fatty acid concentrations at each collaborating farm before and after digestion.

	Acetic (mg/L COD)		**Propionic (mg/L COD)**		**Butyric (mg/L COD)**		**Valeric (mg/L COD)**	
	Influent	**Effluent**	**Influent**	**Effluent**	**Influent**	**Effluent**	**Influent**	**Effluent**
	CF1							
Jun-16	415	47	308	33	74	50	160	0
Sep-16	48	88	60	66	44	49	0	67
Oct-16	94	0	55	0	30	0	0	0
Feb-17	0	549	0	647	0	146	0	90
Jun-17	6120	10	1899	125	957	83	466	110
Aug-17	206	30	175	122	238	0	435	0
	CF2							
Jun-16	3109	1468	874	1725	488	117	287	268
Sep-16	2188	446	781	690	485	105	241	68
Oct-16	1488	555	457	722	262	82	98	73
Feb-17	2269	3407	1211	1768	519	689	272	305
Jun-17	1216	535	393	1481	220	67	272	151
Aug-17	1621	550	429	939	389	74	338	460
	CF3							
Jun-16	10383	2019	3895	565	4590	123	1172	252
Sep-16	6416	1128	2952	253	1849	146	572	192
Oct-16	4357	5234	3116	3012	3113	2231	375	766
Feb-17	6290	1451	2343	326	707	88	324	0
Jun-17	5264	10115	1476	1922	1739	2305	796	1348
Aug-17	5632	2271	2394	416	1184	228	423	457
	CF4							
Jun-16	8915	244	4175	62	1843	27	530	79

Sep-16	2995	197	1083	110	525	89	257	79
Oct-16	3657	186	1306	40	595	22	151	0
Feb-17	5514	619	3768	271	827	158	348	243
Jun-17	5356	171	1509	58	694	24	480	0
Aug-17	4436	156	1512	92	805	64	675	0
	CF5							
Jun-16	4654	274	1586	61	782	32	401	80
Sep-16	2894	144	1154	30	628	0	301	0
Oct-16	2407	394	1026	75	552	21	209	0
Feb-17	2938	430	1964	54	553	41	293	0
Jun-17	4811	399	1528	70	769	75	513	111
Aug-17	1900	158	699	31	413	62	420	0
	CF6							
Jun-16	4113	207	1864	67	3723	49	3038	78
Sep-16	2612	180	731	62	993	42	606	0
Oct-16	3645	127	1143	25	2486	20	1193	0
Feb-17	3968	133	3521	0	1494	0	981	84
Jun-17	3984	18	1117	55	729	67	497	223
Aug-17	2938	129	1699	29	1007	70	772	455

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