ABSTRACT

Title of Dissertation: **HEATED RESISTANCE: THERMAL TREATMENT TECHNOLOGY MITIGATION OF BIOLOGICAL WASTES' ANTIBIOTIC RESISTANCE AND GENE MOBILITY IN WASTE SYSTEMS.**

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Doctor of Philosophy,
2023

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The burgeoning global threat of antimicrobial resistance (AMR) has policy makers, veterinarians, farmers, and physicians re-evaluating antibiotic stewardship. Worldwide, millions of people are affected by multidrug resistant bacteria. Human and animal waste are primary transporters of antibiotics, antibiotic resistant bacteria (ARB), and antibiotic resistant genes (ARGs) through rural and urban systems. Resistance within biological waste, as it moves through the landscape and sanitary/manure infrastructure to adjacent natural systems, is yet to be fully understood. The various environmental conditions, bacterial composition, and genetic factors result in highly complex interdependent relationships that influence the occurrence and

dissemination of ARB and ARGs. Understanding the fate of ARB and movement of ARGs is critical to evaluating environmental and anthropogenic impact.

Agricultural systems and wastewater treatment plants are target locations for quantifying connections between clinical and animal antibiotic use and environmental AMR. Waste management techniques/technologies, such as compositing and anerobic digestion (AD), have been shown to be effective in combating AMR. Studies have highlighted temperature as a key environmental determinant that could influence antibiotic degradation, ARG, and ARB abundance. The proposed research examines advanced heat-based manure and wastewater technological capacity for AMR reduction, while measuring treatment impact on ARG dissemination. Focusing on the reduction of AMR within biological waste treatment and the distribution of AMR factors into the environment.

A key metric to understanding AMR is accurate detection and quantitation of antibiotic concentration within manure and other biosolid waste products. The first phase of this dissertation research focused on the development of a liquid chromatography in tandem with mass spectrometry (LC-MS/MS) method for detecting multi-class antibiotics residuals in various manure substrates. To combat the challenges of manure heterogeneity, this work focused on novel extraction methodology to achieve higher recovery of tetracyclines, macrolides, sulfonamides, and beta lactams simultaneously in a complex manure matrix. The method includes a two-step, liquid-solid extraction using 10 mL of 0.1 M EDTA-McIlviane buffer followed by 10 mL of methanol. Reporting total antibiotic recoveries of 67–131% for tetracyclines, 56% for sulfonamide, 49–53% for macrolides, and 1.3–66% for β-lactams. This method is novel in its application to four different manure substrate and utilization for waste risk assessment. This developed method was used for antibiotic quantification throughout the three

thermal treatment studies to determine antibiotic concentrations, degradation, and monitor agricultural contributions to environmental AMR.

The following research extensively focused on the evaluation of three advanced, high temperature waste treatment technologies on the mitigation of antibiotic resistance factors, including a composting rotary drum bedding recovery unit (BRU), thermophilic (55°C) and mesophilic (35°C) AD, and thermal hydrolysis pretreatment to reduce antibiotics, ARGs, and ARB. The assessment of environmental components, such as metals, bacterial community, and nutrient composition, are also included to determine any relational trends.

The BRU study was conducted as a mass balance analysis to highlight antibiotics, ARGs and ARB partitioning within the BRU system. Dairy manure samples were collected over 24-hour period as the manure was treated with a solid-liquid separator producing two streams of substrates (liquids and separated solid), with the separated solid fraction continuing to the high temperature BRU processing. This study generated a mass flow analysis of manure and partitioning of antibiotic resistance factors throughout the manure treatment system. The study indicated that most of the manure mass containing the AMR factors goes untreated following solid-liquid separation, with 95% of the mass pumped to a storage lagoon and 5% proceeding to BRU processing. The removal of antibiotic residuals during BRU processes was insignificant, yet the BRS processing was 100% effective in removing the ARB examined. Five (*Intl1, sul1, tetQ, tetX and tetM)* of the eight ARGs were found to have significant reduction (>95%) following the thermophilic rotary drum composting portion of the BRU system. While the three other ARGs (*tetW, ermB and bla2)* remained constant despite treatment.

An AD experiment was implemented as lab-scale destructive assay, highlighting antibiotic removal at two temperature and over time. This destructive batch assay used 18 sets of

triplicate AD reactors filled with antibiotic spiked dairy manure and incubated under anerobic conditions at 35°C or 55°C for 43 days. Triplicates bottles destructively sampled at six time points (Day 0, 3, 9, 21, 36, and 43) to generate a degradation curve. The antibiotic erythromycin was more efficiently degraded under mesophilic conditions, with 100% removal by Day 36 compared to 97% reduction for thermophilic conditions during the 43-day digestion period. Though the higher temperature conditions proved better for oxytetracycline degradation, with 66% removal compared to only 22% removal for mesophilic conditions. ARG removal was dependent on the bacterial community, as the different conditions selected for various bacteria. While both conditions proved to be effective in reducing most of the ARGs (4-5 out of 8 genes tested), enrichment of other resistance genes was also documented. The *tetW* gene was found to increase >81% for both digester temperatures, highlighting the variety of bacteria harboring resistance genes and their varied responses to environmental conditions. The *ermB* genes was found to be located on the *intl1* mobile genetic element and likely resided within bacteria that was not heat tolerant. This study highlighted the role of residential digester bacteria in harboring and potentially transferring resistance genes.

The thermal hydrolysis (THP) technology ability to extensively lysis substrates was examined with subsequent AD for its impact on reducing antibiotic resistance factors. Comparative analysis of THP processing on spiked diary manure and wastewater biosolids followed by mesophilic digestion at 35°C was conducted to document substrate response to the treatment. AD was conducted as a destructive assay for 30 days with a 4-point sample curve (Day 0, 10, 20 and 30). This study can be found in the appendix.

In addition to the lab and field work described above, this body of research also included a review and a proposed communication model for antibiotic resistance education for the general

public. Lay audiences' exposure and understanding of complex natural issues, such as AMR and climate change, are essential to behavioral changes and potentially legislative actions. By surveying and evaluating various aspects of scientific communication, this research empathized five rhetorical elements of storytelling shown to influence audience reception to scientific messaging. Communication techniques, such as narrative structure, normalization of the subject using human scaling, non-agentive language, trusted experts for message delivery, and future simulation, were all analyzed and reviewed for their effectiveness and incorporated into a mock model for presenting information about AMR. Bridging gaps between research institutions and the public is key to generating more inclusive spaces for innovation and mitigating issues interwoven within the built and natural environment.

HEATED RESISTANCE: THERMAL TREATMENT TECHNOLOGY MITIGATION OF BIOLOGICAL WASTES' ANTIBIOTIC RESISTANCE AND GENE MOBILITY IN WASTE SYSTEMS.

by

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Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2023

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Dedication

Without my community, village my support system Carlton as we know him does not stand before you today. To my mom, dad, brothers, grandparents, cousins, aunts, and uncles I cannot express the amount of gratitude and appreciation I have for all you and your support on my journey. We didn't know how we were going to make me scientist, but we did and that is the results of all the lessons, tips, advice that you all have acquired on your own and have shared with me. To all my friends who basically extend family thank you for taking the chance to get to know me for allowing me to grow alongside you and provide me with your honest inputs and unwavering support. I have had so many years to learn and live beside you all who each have such amazing aspects to contribute to the world and you each choose to share them with me. Some of you all have been by my side for as long as I can remember while other, I have met later in life bond still appear to hold just as strong. My friends have help me to navigate school and things that we have called life I am just honored to know and call you all my friends. To be black in the world especially black in STEM, black in environmental science can be extremely isolating. There are not a lot if any scientist in my immediate community so to say this journey has been a challenge is grave understatement. But with everyone's help we have crafted a path to bring me here today. I must give a special dedication to my oldest brother Chris who has always been looking out for me and has agreed to read so many of my school applications, papers, scholarships, fellowships etc. not being familiar with STEM but always providing feedback that has help me become the scientist that now. To my roommate/ brother Rod neither of could fathom what connecting to living together

and attend UMD in DC would hold for us. You have been my family from the start we have gone through our respective program's life and pandemic, and we have seen highs and lows. Through it all we stuck be each other's side, I am forever grateful for the person you are and becoming I definitely would not be here without you. The universe put the right person in my corner to help me grow. Also special thanks to Ro, Breanna, Steph and Sofia you all have all been so helpful just to talk, vent, or helping with various work as well. Thank you to my village for cultivating a space and allowing me to exist with all my quirks, shenanigans, challenges and shortcoming without you I cannot do any of this.

Acknowledgements

Thank you to my advisor and Dr. Stephanie Lansing and my dissertation committee Dr. Stephanie Yarwood, Dr. Birthe Kjellerup, Dr. Clifford Rice, and Dr. Kasey Jeong for all your guidance and support for my dissertation research.

Thank you to the United States Department of Agriculture and National Institute of Food Agricultural for providing funding for the grant to carry out this antibiotic resistance research. Also thank you to University of Maryland Global stewards' program, National Science Foundation, Southern Regional Education Board, the Science Mentoring and Diversity Program, the University of Maryland Graduate School for proving financial through fundings and fellowships support throughout my dissertation. I would also like to acknowledge my various collaborators at USDA, Virginia Tech University, The University of Nebraska-Lincoln, and Cornell University for all the support in this research and my development as a scholar and scientist.

Also thank you to the Bioprocessing and Biotechnology Lab team for all your assistance with experiments, set-up and data collection and allowing the research to be conducted in these facilities. A special thank you to Sarah Emche you have been instrumental in providing guidance, mentorship and assistance with various projects and helping to develop new skills specifically all the challenges with the gene analysis. Also, special thank you to Dr. Amro Hassanien, Danielle Delp, Theo Nimpson, Usoshie Chatterjee, Kirk Mahoney, Dr. Naresh Amradi, Derrick Sanders, Emily McCoy, Olivia Robinson, Briana Mercado, Mandy Zhang. Special thanks to

Annie Yarberry for all the support and guidance over the years and all the fun time we had working together at the USDA and attending the various conferences.

Table of Contents

Dedication ii

Acknowledgments iv

Table of Contents vi

List of Tables ix

List of Figures x

List of Illustrations

List of Abbreviations xi

Chapter 1: Introduction

1.1 Antibiotics Utilization and Antibiotic Resistance in Agriculture 1
1.2 Environmental Exposure 3
1.3 Wasterwater Treatment 6
1.4 Research Approach 9

Chapter 2: Quantifying Antibiotic Distribution in Soilds and Liquid Fractions of Manure Using a Two-step, MultiResidue Antibiotic Extraction

Abstract 11
2.1 Introduction 12
2.2 Results and Discussion 15
2.2.1 Comparative Analysis of Buffer vs Solvent Extraction Efficay 15
2.2.2 Initial Two-Step Extraction with Buffer Followed by Methanol 18
2.2.3 Two-Step Extraction on Solid and Aqueos Manure Samples Based on Total Solids 19
2.2.4 Optimizing the Two-Step Method to combine Extracts fro One Injection. 21
2.2.5 Extraction Method Performance Using Method A 25
2.3 Material and Methods 26
2.3.1 Standaards and Reagents 26
2.3.2 Collection of Diary Manure Samples 28
2.3.3 Sample Preparation 28
2.3.4 Sample Cleanup 30
2.3.5 UPLC-MS/MS 30
2.3.6 Method Trials 31
2.3.6.1 omparative Anlysis of Buffer vs Solvent Extraction Efficacy 31
2.3.6.2 Intial Two-Step Extraction with Buffer Followed by Methanol 32

2.3.6.3 Two-Step Extraction on Solid and Aqueous Manure Samples Based on Total Solids 33
2.3.6.4 Optimizing the Two-Step Method to Combine Extracts for One Injection 33
2.3.7 Evaluation of Performance of Method Trials 34
2.4 Conclusion 36
Chapter 3: Antibiotic Resistance Partitioning in Dairy Manure Processed Through a Continuous High Temperature Rotrary Drum Bedding Recovery Unit
3.1 Introdution 37
3.2 Material and Methods 41
3.2.1 Experimental Design 41
3.2.1.1 Study Location 41
3.2.1.2 Residence Time Tracing in Full-Scale BRU 42
3.2.1.3 Mass Flow Experiment 43
3.2.2 Antibiotic Quantification 44
3.2.3 Sample DNA Isolation and qPCR 45
3.2.4 Bacterial Culturing for Fecal Indicators 46
3.2.5 Sample Characterization 47
3.2.6 Statictical Analysis 48
3.3 Results and Discussion 48
3.3.1 Fate of Antibiotics in BRU Processing 48
3.3.2 Fate of Antibiotic Resistance Genes and Gene Partitioning 54
3.3.3 Fecal Indicator Bacterial Analysis 58
3.3.4 Nutrient and Heavy Metal Analysis 51
3.4 Conclusions 64
Chapter 4: Time Series Analysis of Antibioitc Resistance within Diary Manure under Mesophilic and Thermophilic Anaerobic Digestion
4.1 Introduction 66
4.2 Material and Methods 70
4.2.1 Experimental Design 70
4.2.2 Antibiotic Extraction and Quantification 72
4.2.3 DNA Isolation and qPCR Resistance Gene Quantitation 73
4.2.4 Detection and Quantification of Fecal Indicator Bacteria 74
4.2.5 Digestate Pysicochemical Chacterization 75
4.2.6 Statistical Analysis 76
4.3 Results and Discussion 77
4.3.1 Methane Production 77
4.3.2 Fate of Antibiotics during Digestion 80
4.3.3 Fate of Antibiotic Reistance Genes (ARGs) during Digestion 84
4.3.4 Fecal Indicator Bacteria Growth during Digestion 90
4.3.5 Correlation Analysis between AD Characteristics and Co-occurrence of ARGs 93

5.4 Conclusion 97
Chapter 5: Improving Public Engagment with Complex Scientific Issues Exmplified

by Antimicrobial Resistance

Abstract 99
5.1 Introduction 100
5.2 Message Design 102
5.2.1 Narrative Structure 103
5.2.2 Normalizatoin of Subject Using Metaphors and Hman Scaling 104
5.2.3 Non-Aentive Language 105
5.2.4 Message Delivery by a Trusted Expert 106
5.2.5 Future Simulation 106
5.3 Message Design and Delivery Flowchart 108
5.4 Conclusion 114

Chapter 6: Conclusion
6.1 Intellectual Merit 116
6.2 Future Work 119

Appendices 120

Bibliography 142

List of Tables

Table 2.1 Antibiotic Physiochemical Poperties and Analytical Method Parameters 17
Table 2.2 Total Solids of Manure and Extrraction Mass 21
Table 2.3 Antibiotic Recovery Results of Method A and Method B Comparision 23
Table 2.4 Method Performance Parameters 26
Table 3.1 Concentation of Antibiotics detected in Manure from farm-scale BRU experiment 51
Table 3.2 Average Mass Flow of Antibiotics 52
Table 3.3 Correaltion Analysis of Antibiotic Reistance Genes (ARGs) and Heavy Metals 63
Table 4.1 Correaltion Analysis between the relative abudance of Antibiotic Reistance Genes (ARGs) and Digestate Characteristics 94
Table A1 Primer and PCR Conditions 120
Table A2 Nutrient and Metal Concentrations in Manure 121
Table B1 Mesophillic Anaerobic Digestion Antibiotic Removal Rates 122
Table B2 Thermophilic Anaerobic Digestion Antibiotic Removal Rates 122
Table B3 Pre and Post BMP pH and Reductions 123
Table C1 Substrate Charaterization and Biochemcal Methane Potential (BMP) Loading Measurements 130

List of Figures

Figure 2.1 Antibitoic Recoveres from extraction of spiked manure with either EDTA MCIlvaine buffer of 50:50 MeOH:ACN 16
Figure 2.2 Antibiotic recoveries from intitial two-step extraction experiment with EDTA MCIlvaine as first extractant followed by MEOH 19
Figure 2.3 Extraction Recoveries of anitbiotics from four types of manure proceesed through a manure treatment system 20
Figure 2.4 EDTA-MCIlvaine and MeOH extratction methodology using method A and Method B 22
Figure 3.1 Averages samples of Antibiotic Resistance Gene relative abudance normalizeed per gram maure and based on the mas flow 56
Figure 3.2 *E.coli* and *Enterococci* Bacterial counts during Bedding Recovery Unit (BRU) Processing 59
Figure 4.1 Average and Cumulative CH_4 Production durin Digestion 77
Figure 4.2 Quantified Concentration of Spiked Antibitoics though Mesophilic and Thermophilic 43-day Digestion 81
Figure 4.3 Relative Abundance of Antibiotic Resitance Gene copies under Mesophilic and Thermophilic Conditions 86
Figure 4.4 Bacterial Colony Forming Units Quaitifcation of *E.coli* and *Enterococci* reistant to Erythromycin 91
Figure 6.1 Decision-Making Flowchart for Developing Science Communication Plan 109
Figure 6.2 Media Model for Communcation Principles of Amr to the public 110
Figure 6.3 Metaphorical decpiction of bacterial interaction as a Bee Hive 113
Figure A1 Relative Abundance of 16S based on Manure Mass Flow 121
Figure B1 Relative Abudance of 16S during Anaerobic Digestion 121
Figure C1 Average and Cumlative CH_4 Production Post Thermal Hydroylsis 111

List of Abbreviations

AD anaerobic digestion

AMR antimicrobial resistance

AR antibiotic resistance

ARG antibiotic resistant gene

ARB antibiotic resistance bacteria

AMP ampicillin

ERY erythromycin

FIB fecal indicator bacteria

OXY oxytetracycline

MGE mobile genetic element

RA relative abundance

TET tetracycline

THP thermal hydrolysis

UPLC-MS ultra-high pressure liquid chromatography tandem mass spectrometry

WWTP wastewater treatment plant

Chapter 1: Introduction

1.1 Antibiotics Utilization and Antibiotic Resistance in Agriculture

The agricultural sector is a major contributor to antibiotics pollution worldwide (Kumar et al. 2005b; Kraemer et al. 2019). Antibiotics can be transferred to the surrounding environment via livestock manure/sludge, resulting in local cycling of antibiotic transfers (Manzetti and Ghisi, 2014; Gothwal and Shashihar 2015; Manyi-Loh et al. 2018). The intensive application of antibiotics as a growth promoter, or as prescribed medication for treatment for disease, occurred early in the golden antibiotic age. The Swann Report (1962) recommended that antibiotics suitable for addition to animal feed without prescription be restricted to: 1) those of economic value in livestock production, 2) those that have little or no application as therapeutic agents in man or animals, and 3) those that will not impair the efficacy of a prescribed therapeutic antibiotic or antibiotics the development of resistance strains or organism (Woodbine, 1977). The reported beneficial usage of feed additives or sub-therapeutics antibiotics (broad spectrum activity, and narrow spectrum gram positive activity) enhanced growth and feed efficiency, which concurrently developed with the intensification of use in animal husbandry (Elaine et al. 2012). The increased feed efficiency allowed for the same amount of meat production with a smaller number of animals, resulting in smaller cropland usage, decreased manure production, and other economic benefits (Durso and Cook, 2014). While antibiotic and antimicrobial consumption patterns have geographical variations dependent on farming systems (animal type, purpose, scale, etc.), the greatest concern stems from

the use, types, and mode of action of the antibiotics employed that closely relate to antibiotics with high clinical relevance.

The 2014 Summary Report on Antimicrobials Sold or Distributed for use in Food Producing Animals states that of all the antibiotic sold in the United States, approximately 80% were sold for use in animal agriculture, with 70% of these deemed medically important (Martin et al. 2015). The excessive usage of antibiotics overall can also be attributed to the absence of strict regulation over the types of antibiotics that are accessible to the animal husbandry industry via balanced supplementation or premixes processed and sold through the feed manufacturer industry, farms, and veterinary supply centers (Chang et al. 2015). The 2015 Global Trends in Antimicrobial Use in Food Animals (Van Boeckel et al. 2015) publication reported that globally the average antimicrobial agent consumed per annum of animal produced (per kg) differed across animal species documenting cattle (45 mg/kg), chicken (148 mg/kg) and pigs (172 mg/kg). Modes of antibiotic administration should be factored in as well, given that residence time of antibiotics given through water/feed consumption accumulate differently than intravenous methods, which can lead to larger build up in adipose tissue. This constant low-level dosage in addition to higher concentrations spikes for disease management poses the greatest threat in concerns to the spreading of antibiotics, ARB, and ARGs. Antibiotic-induced selective pressure on commensal bacteria can lead to selection of resistant bacteria that eventually reach the environment. Multiple studies have supported the principle of co-evolution of gut microbiota as a consequence of antibiotic administration in swine at subtherapeutic levels (Looft et al. 2012; Kim et al. 2012), with reported

increased prevalence of specific bacterial taxonomic groups, antimicrobial resistance genes related to the administered antibiotics, and genes for other types of antibiotic resistance.

Over the past several years, the FDA has taken steps towards more judicious use of medically important antibiotics use in animal feed and/ or water. In 2017, the Veterinary Feed Directive (VFD) was passed in the United States. The VFD effectively eliminated the use of medically important antibiotics as growth promoters and restricted use for therapeutic purposes under verified veterinarian provisions and with an established veterinary-client partnership (Schultz, 2017; Veterinary Feed Directive Final Rule and Next Steps [Factsheet], 2020). Despite recent legislation, animal agriculture remains an important sector in terms of diffusion of antibiotics and their corresponding metabolites into the soil and natural water.

1.2 Environmental Exposure

Livestock metabolism can leave 30-90% of antibiotics unmetabolized, or as biologically active metabolites. Studies focusing on the gut microbiota and digestion have shown antibiotics in the human and animals generate conditions selective for antibiotic resistant bacteria within the intestines, which can be transported through excrement (Kim et al. 2017). These antibiotics and associated ARB accumulate in the animal/human waste and can be exposed to the environment. Other exposure routes include pharmaceutical production and other industry effluents. Studies from around the world have showed antibiotics in the environmental mainly originate from veterinary and agricultural industries, wastewater plants, landfills, effluent from

hospitals, and industrial sites (Manzetti and Ghisi 2014; Gothwal and Shashihar 2015; Manyi-Loh et al. 2018; Kraemer et al. 2019). The compounding effects of antibiotics and their partially metabolized metabolites with antibiotic resistant bacteria result in contamination of soils, water systems, and associated ecosystems. This group of contaminants have increased in detected concentrations across a wide range of environments (Mohaptra et al. 2016;). Some contaminants sources can be as high as 216 mg/ L (manure slurry), resulting in contaminated soils with known concentration reaching 400 g antibiotics/ha (Kumar et al. 2005b; Manzetti et al. 2014). While compared to other contaminants, these are low concentrations, but antibiotics can circulate through the environment and contaminate groundwater (Larsson 2014). Multiple studies have stated that aquatic ecosystems, such as lakes, ponds, and closely associated soils, are the most vulnerable to antibiotic contamination (De Liguoro et al. 2003; Grenni et al. 2018).

The gut microbiota of livestock is transferred into animal excreta, thus, the first nexus in the environmental pathways of the dissemination of animal based antibiotic resistance starts in the cesspits and other waste collection vessels (You and Silbegeld 2014). Livestock waste is initially released to the environment through on-site storage, such as open-air pits, followed by broader discharge via the land application of manure. The agricultural practice of manure application as a fertilizer is implemented world-wide and has historically been used since the early stages of agriculture; resulting in highly variable research connecting this practice to the prevalence of antibiotic resistant bacteria and antibiotic resistant genes in amended soils and adjacent natural systems (Heuer et al. 2012; Peng et al. 2017; Xie et al

2018). Once manure is applied, the microbial and chemical constituents can be widely dispersed through dust, air, rainfall, and the animal movement itself. In addition to global inconsistency in the documentation, utilization, and regulation of antibiotics based on region and type of animal husbandry, the overall lack of uniform practices for animal manure treatment and application results in uncertainties in the exact effect of manure application as a direct source of environmental contamination. Animal waste can act as reactors that produce new genotypes/phenotypes for resistance, and with the large production volume of animal waste, broad contact with the environment, and insufficient waste management, downstream risks can arise for animal and human populations (Banquero et al. 2008). This emphasizes the importance of understanding the emergence and dissemination of AMR, driven by antibiotic use in animal agriculture, corresponding waste management, and the environment.

Previous literature has extensively observed the direct and indirect impacts of animal manure amendments to agricultural fields and adjacent ecosystems in relation to corresponding antibiotic concentrations, overall resistance bacteria, and gene abundance in various environments (Kumar et al. 2005b; Pruden et al. 2013, Xie et al. 2018). The loading of biologically active compounds and metabolites range from ug/kg to as high as hundreds of mg/kg depending on the substrate, compound characteristics and species, and region (Kemper, 2008a). Some antibiotics, such as beta-lactams and macrolides, easily degrade and have a shorter half-life, while others, such as tetracyclines and sulfonamides, have half-lives that can last months in manure (Albero et al. 2018). However, measured concentrations of these compounds in

manure amended soils are significantly lower or undetectable. Little to no detection of compounds are likely inaccurate due to analytical barriers that exceed current technological capabilities, which can lead to the large under estimation of antibiotics in soil and aquatic habitats (Seifrtová, et al. 2009; Carvalho et al. 2016) . Similar to the sublethal concentration of animal feed, there is a greater understanding that these lower concentrations are more favorable drivers of generating resistance (Jutkina et al. 2018). The relationship between the simultaneous burdening of both genes/bacteria and pollutants into the environment involve multiple mechanisms for *in situ* selection for AMR in the environment due to inputs of antimicrobials, metals, and other residues (You and Silbegeld 2014). The linkage between agricultural antibiotic use and the expansion of the environmental resistome have been established (You and Silbegeld 2014; Chee-Stanford et al. 2009). The exposure of the compounds and resistant bacteria can reshape the microbial composition of the surrounding environments. The disposition and dissemination of these new bacteria is primarily spread through manure (Durso and Cook 2014), which can lead to increasing the overall resistance and reduce effectiveness of medically important antibiotics.

1.3 Wastewater Treatment

Bacterial antibiotic resistance is a global issue that can result in untreatable infections, death and increasing cost to the health care system. Recent research has focused on establishing connections between antibiotic use for human health and in agricultural settings to document relevant ARB and ARGs. Along with agricultural concerns, efforts have been especially focused on wastewater treatment plants (WWTPs). WWTPs offer a unique situation that brings human society and the

environment together through sewage flow that combines residential, industry and hospital waste lines together. Sewage treatments are the final sites for treating antibiotic contamination before entering natural systems. Yet WWTPs were not designed for antibiotic removal, thus, the effluent is the main anthropogenic source of contamination (Kim and Aga, 2007; Michael et al. 2013; Manaia et al. 2018). These waste streams hold various amounts of compounds and pollutants at sub-inhibitory level that can promote the occurrence of horizontal gene transfer and proliferation of resistant bacteria (Karman et al. 2017). Waste treatment typically includes three stages of treatment: primary treatment (solids are removed by physical operations), secondary treatment (biological and chemical processes are used to remove organic matter), and tertiary (nutrient removal, toxic pollutant removal, suspended solids removal etc.). Throughout these processes, there are considerable changes to the distribution of bacteria and overall population dynamics. Differences in treatment plant design and operations (e.g. mechanical, biological, physical, chemical, and physical-chemical) may affect the fate of antibiotics, ARGs and ARB in various ways that consequent development and spread of resistance (Rizzo, 2013). Most full-scale WWTPs include sedimentation processes to remove sludge, and this sludge substrate is commonly associated with ARB and ARGs (Barancheshme and Munir, 2018). WWTP sludge is nutrient-rich and a microbially dense biomass that could correspond to increased concentrations of antibiotics and other selection factors linked to elevated ARGs. Therefore, WWTPs have been deemed hotspots, because of the ability of WWTP to harbor large quantities of ARB, ARGs and the facilitation of horizontal gene transfer (Manaia et al. 2018; Nguyen et al. 2021). Biological

wastewater treatment systems using active sludge is a common technique that may reduce the concentration of antibiotics and other pharmaceuticals (Manaia et al. 2018). The aeration process of biological treatment is the key step in the removal of most other pollutants, yet nonvolatile pollutants, such as antibiotics, are removed via mechanisms of biodegradation and/or sorption (Kim and Aga, 2007). The biological treatment process creates a suitable environment for resistance development due to the continuous mixing of bacteria and antibiotics (Ferreira de Silva et al. 2006; Rizzo et al. 2013; Pazada et al. 2019). Antibiotic removal efficiencies of conventional sewage treatment vary substantially, as stated previously these systems were not designed to deal with these types of emerging pollutants (Gothwal and Shashidhar, 2014). Some studies have showcased the benefits of advanced treatment, such as membrane bioreactors, which have been shown to have higher levels of antibiotic removal then conventional treatments (Le-Minh et al. 2010: Michael et al. 2013 and Nguyen et al. 2021).

Research establishing the link between excessive human and animal antibiotic consumptions and the ecological impact of environmental exposures is ongoing. Researchers do state that biological waste could be a predominant variable that facilities transport and resistance development (Michael et al. 2013; Manaia et al. 2018;). This highlights the importance of biological waste management and distribution through the built and natural environment. Though antibiotic stewardship is the key to long-term AMR mitigation, the evaluations of waste and manure management systems and treatments are a critical component to reducing AMR's expansion (Ricker et al. 2020). The proposed research is set to evaluate high

temperature biosolids and waste technologies that are used in agricultural and wastewater treatment plants. By determining the effectiveness of rotary drum composter, anaerobic digestion, and thermal hydrolysis on antibiotic removal, ARG, and ARB reduction, this study will expand beyond reduction analysis by surveying antibiotic resistance throughout the treatment process and attempting to pinpoint specific conditions and variables that influence treatment efficacy. In addition to treatment evaluation, the research is set to address the uncertainties of resistance genes transferences and occurrence during waste treatment. Through waste sample characterization and lab-based gene transfer experiments, the study will draw connections that validate treatment efficacy to microbial dynamics.

1.4 **Research Approach**

To combat the emerging pollution of antibiotics and corresponding increase in AMR in the environment, within livestock animals and human there has to be a better understanding of antibiotic resistance. Examination of the role manure and biological waste play in the transport of antibiotics themselves and resistance genes is key to combating point and nonpoint sources of resistance dissemination. Under the guide of the CDC “One Health” model, this research is structured to investigate antibiotic resistance movement through the landscape via biological waste and the evaluation of current technologies mitigation capabilities.

The first aim of this research is to determine the efficiency of advanced, high temperature AD and waste treatments on the mitigation of antibiotics resistance factors**.** The research will focus on the effect of a rotary drum bedding recovery unit

(BRU), thermophilic and mesophilic AD, and thermal hydrolysis pretreatment on the reduction of antibiotics, ARGs, and ARB. The assessment of environmental components, such as the presence of metals, bacterial community structure, and nutrient composition will also be included to determine any relational trends.

Furthermore, this study seeks to investigate the impact of waste treatment on the movement of mobile genetic elements in the dissemination of ARGs. Combing metagenomic analysis, bacterial culture-based methodology, and lab-scale waste simulations, the research is set to expand previous literatures correlational notions of nutrients, pH, bacterial community composition influence on mobilization of mobile genetic elements (MGEs) and antibiotic resistance genes (ARG) abundance.

Chapter 2: Quantifying Antibiotic Distribution in Solid and Liquid Fractions of Manure Using a Two-Step, Multi-Residue Antibiotic Extraction

As published in Antibiotics
https://doi.org/10.3390/antibiotics11121735

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Keywords: UPLC-MS/MS; dairy manure; tetracyclines; β-lactams; sulfonamides; macrolides

Abstract:

Antibiotic distribution and analysis within liquid and solid fractions of manure are highly variable due to each compound's respective physiochemical properties. This study developed and evaluated a uniform method extracting 10 antibiotics from 4 antibiotic classes (tetracycline, sulfonamides, macrolides, and β-lactam) from unprocessed manure, solid–liquid separated manure, and composted solids. Through systematic manipulation of previously published liquid chromatography tandem mass spectrometry methods; this study developed an extraction protocol with optimized recovery efficiencies for varied manure substrates. The method includes a two-step, liquid-solid extraction using 10 mL of 0.1 M EDTA McIlviane buffer followed by 10 mL of methanol. Antibiotics recoveries from unprocessed manure, separated liquids, separated solids, and heat-treated solids using the two-step extraction method had relative standard deviations < 30% for all but ceftiofur. Total antibiotic recoveries were 67–131% for tetracyclines, 56% for sulfonamide, 49–53% for macrolides, and

1.3–66% for β-lactams. This is the first study to use one protocol to assess four classes of antibiotics in liquid and solid manure fractions. This study allowed for more precise risk assessment of antibiotic transport in manure waste stream applied to fields as a liquid or solid compost.

2.1 Introduction

The practice of antibiotic administration for livestock welfare can leave as much as 90% of the administered antibiotics excreted unmetabolized or as biologically active metabolites in animal manure and urine (Spielmeyer et al. 2018). The presence and release of antibiotics within agroecosystems influences the development of global antibiotic resistance. Recent legislation within the United States (US) has regulated the use and distribution of medically important veterinary antibiotics (CVM, 2019; 2021). While more responsible administration of antibiotics is on the rise, there is a need to determine how antibiotics that are used persist through current manure management treatment systems in place at livestock farms.

Though there are numerous manure management technologies, solid–liquid separation has been increasingly adopted by larger US dairy farms over the past 10 years (Wallace and Aga et al. 2016). This process can produce multiple effluent streams with unique physical/chemical properties that influence antibiotic concentrations and distribution. Rico et al. (2007) reported that the liquid fraction of manure contained 54% of the total solids (TS) and 48% of the volatile solids (VS) of the raw manure. The changes in the manure properties due to management and treatment affect the interactions between the manure and antibiotic residuals, solubility, and microbial degradation, which subsequently influences fate and transport of antibiotics, antibiotic

resistant genes, and antibiotic resistant bacteria in the environment (Wallace and Aga et al. 2016). Due to the variability in manure composition before and after manure treatment, it is necessary to develop an antibiotic extraction method that is designed to extract multiple antibiotics from manure matrices with vastly different solids concentrations to maintain a baseline metric for comparison. While numerous multi-class antibiotic residual studies have focused on antibiotic extraction and quantitation from manure (Berendsen et al. 2015; Jansen et al. 2019; Popova et al. 2019; Wang et al. 2019b) , each of these methods have been fine-tuned for a specific type of manure; either manure solids (Jacobsen and Halling-Sorensen, 2006; Pan et al. 2011; Ho et al., 2012, 2014; Berendsen et al., 2015; Feng et al. 2016; Van den Meersche et. Al 2016b; Karaca et al. 2018; Jansen et al. 2019) , manure liquids , or manure slurries (McKinney et al. 2010; Zhou et al. 2012; Wallace et al. 2018).The few studies that have investigated multiple manure substrates have used different extraction protocols for the liquid and solid manure fractions (Xian-Gang et al., 2008; Zhou et al., 2012; Wallace and Aga, 2016; Hurst et al., 2018; Gros et al., 2019). The ability to assess antibiotics concentrations in the liquid and solid fractions of manure allows for the capacity to detect both dissolved and sorbed antibiotic residues for more precise risk assessment. Prior to this study, a one-size-fits-all method to extract antibiotics from both liquid and solid manure had not been developed.

Classes of antibiotics with clinical relevance administered to dairy cows include tetracyclines, macrolides, sulfonamides, and β-lactams. While each antibiotic class exhibits different physicochemical properties, several studies have successfully extracted multiple antibiotic classes from solid or liquid manure (not both) using a

single extraction protocol, predominantly focusing on tetracyclines, sulfonamides, macrolides, and quinolones (Pan et al. 2011; Berendsen et al. 2015; Gao et al. 2016; Guo et al. 2016; Karaca et al. 2018) , while few studies reported on β-lactams (Wang et al., 2019a) . These studies did not extract multiple antibiotic classes from both liquid and solid manures using the same protocol. A study by Wang et al. (2019a) concluded that 94–99% of the antibiotics detected in swine manure were in the solid fraction, with sulfonamides predominantly found in the liquid fraction, yet they used separate techniques to assess these fractions. To account for the fact that antibiotic concentrations tend to partition based on the solids in the manure, the current study based the sample extraction mass on the total solids (TS) measurements of the manure for the varied manure substrates, which accounted for settled and dissolved solids, as the presence of organics affects antibiotic retention.

To date, there have not been studies that have successfully conducted a single extraction protocol for multiple antibiotic classes that included β-lactams and manure substrates with varying TS concentrations. In this study, several extraction procedures were systematically evaluated to determine the most reliable method for multi-residue antibiotic extraction from four manure types within an on-farm manure management system. The developed protocol created an extraction method for the detection and quantification of four β-lactams, one sulfonamide, three tetracyclines, and two macrolides using ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) in raw manure, solid and liquid separated manures, and heat-treated manure solids. This work provides further understanding of antibiotic distribution

throughout a manure management system, allowing for a more holistic interpretation of the environmental impact of manure utilization on fields as a fertilizer.

2.2 Results and Discussion

2.2.1. Comparative Analysis of Buffer vs Solvent Extraction Efficacy

In this extraction trial, recovery efficiencies of the EDTA-McIlvaine buffer and methanol: acetonitrile (MeOH:ACN) were compared. The recoveries of antibiotics in the cleaned-up EDTA-McIlvaine fraction were between 66% and 88% for the tetracyclines, between 0% and 50% for the β-lactams, 44% for SUL, and 34% and 42% for TUL and TYL, respectively (Figure 2.1). The recoveries for samples extracted using 50:50 MeOH:ACN were 4 to 15% for the tetracyclines, 0 to 33% for the β-lactams, 69% for SUL, 21% for TUL, and 45% for TYL, respectively (Figure 2.1). Separate analysis of the extractants indicated strong preferences of some of the antibiotics to either the EDTA-McIlvaine buffer or the organic solvent. Thus, it was decided that a phased two-step extraction procedure would be the most beneficial for the suite of antibiotics in this study. This trial indicated that analytes with higher log K_{OW} coefficients (Table 2.1) generally exhibited lower recoveries in the aqueous buffer extraction solvent, seen below in Figure 2.1.

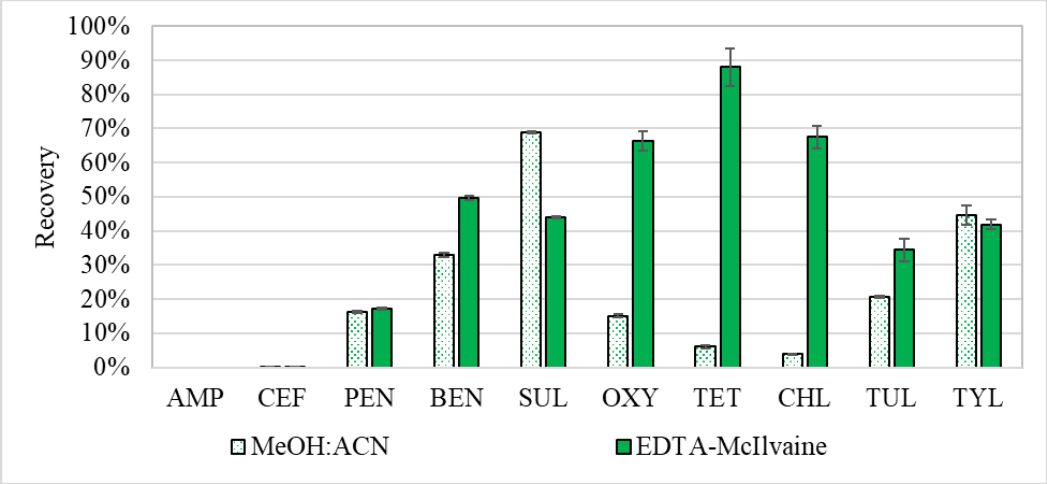


Figure 2.1. Recoveries from extraction of spiked manure with either EDTA McIlvaine buffer (solid bar) or 50:50 MeOH:ACN (the dotted bar). Recoveries are treatment averages (n = 3), with error bars based on ± standard error. The antibiotics examined include Ampicillin (AMP), Ceftiofur (CEF), Penicillin-G (PEN), Benzylpenicilloic Acid (BEN), Sulfadimethoxine (SUL), Oxytetracycline (OXY), Tetracycline (TET), Chlortetracycline (CHL), Tulathromycin-A (TUL), and Tylosin Tartrate (TYL).

Table 2.1. Select physiochemical properties and analytical method parameters of antibiotics investigated in this study, including their chromatographic retention times (RT).

Antibiotics	Acronym	Internal Standard	Molecular Weight (g/mol)	Precursor Ion (m/z)	Product Ion (m/z)	RT (min)	Log Kow[b]	pKa
β-Lactams								
Ampicillin	AMP	NA	349.41	350.1	106.1 [a], 114.0	1.88	1.35	3.07, 7.12 [c]
Ceftiofur	CEF	NA	523.56	524.0	241.0 [a], 125.2	2.03	1.22	2.64, 3.44, 10.7 [d]
Penicillin-G	PEN	NA	334.39	335.1	217.1 [a], 160.0	2.13	1.83	2.97, 4.75 [c]
Benzylpenicilloic Acid	BEN	NA	352.4	353.1	160.0 [a], 128.0	2.12	ND	ND
Sulfonamides								
Sulfadimethoxine	SUL	SUL-d6	310.33	311.1	156.1 [a], 92.1	2.11	1.63	1.62, 6.13 [e]
Tetracyclines								
Oxytetracycline	OXY	DEM	460.44	461.2	201.1 [a], 98.1	1.89	−0.9	3.71, 8.08, 10.15 [b]
Tetracycline	TET	DEM	444.3	445.0	410.2 [a], 154.1	1.89	−1.37	3.56, 7.09, 9.28 [c]
Chlorotetracycline	CHL	DEM	478.88	479.1	154.1 [a], 98	1.94	−0.62	3.49, 7.14, 9.28 [c]
Macrolides								
Tulathromycin-A	TUL	NAL	806.1	403.9	72.1 [a], 116.1	1.86	3.69	8.6–9.6 [e]
Tylosin Tartrate	TYL	ROX	916.112	916.5	174.1 [a], 101.0	1.97	1.95	7.71[c]
Internal Standards								
Sulfadimethoxine-d6	SUL-d6	NA	316.37	317.95	108.0 [a]	2.11		
Demeclocycline	DEM	NA	464.86	465.1	154.1 [a]	1.92		
Nalidixic acid-d5	NAL	NA	237.27	238.24	104.2 [a]	2.23		
Roxithromycin	ROX	NA	837.06	837.54	158.1 [a]	2.07		

[a] Indicates quantitative ion [b]EPA (2019), [c] Zrncic et al(2015), [d] Ribeiro and Schmidt (2017), [e] Geiser et al. (2005), [e] Villarino et al. (2013)

2.2.2 Initial Two-Step Extraction with Buffer Followed by Methanol

In this trial, each manure sample was first extracted using EDTA-McIlvaine followed by extraction using MeOH. In this initial extraction method, the two extracts were analyzed individually to show how much of each antibiotic was recovered by each solvent. When the results for each extraction were combined, recoveries varied between 12 and 77% for all antibiotics (Figure 2.2), with lower recoveries for the two β-lactams (AMP 12% and CEF 19%) and higher values for PEN (77%). Previous studies have found it difficult to extract β-lactam antibiotics due to the unstable β-lactam ring that is easily hydrolyzed in samples with high bacterial activity (Berendsen et al. 2015). This initial two-step method was successful for the β-lactam PEN and metabolite BEN, which may be due to the separation of the two extraction solvents that operate on different physicochemical properties of the PEN. Kheirolomoom et al. (1999) conducted a Penicillin G stability study and found this compound to be the most stable at pH values ranging from 5 to 8 under laboratory conditions. The EDTA-McIlvaine buffer solution used for the extraction had a pH of 5, which may be favorable to the stability of PEN and BEN within the manure matrix. Furthermore, the inclusion of hexane in the extraction protocol to reduce matrix interference (Pan et al. 2011) proved inefficient (results not shown), thus resulting in the exclusion of this step in the subsequent experiments.

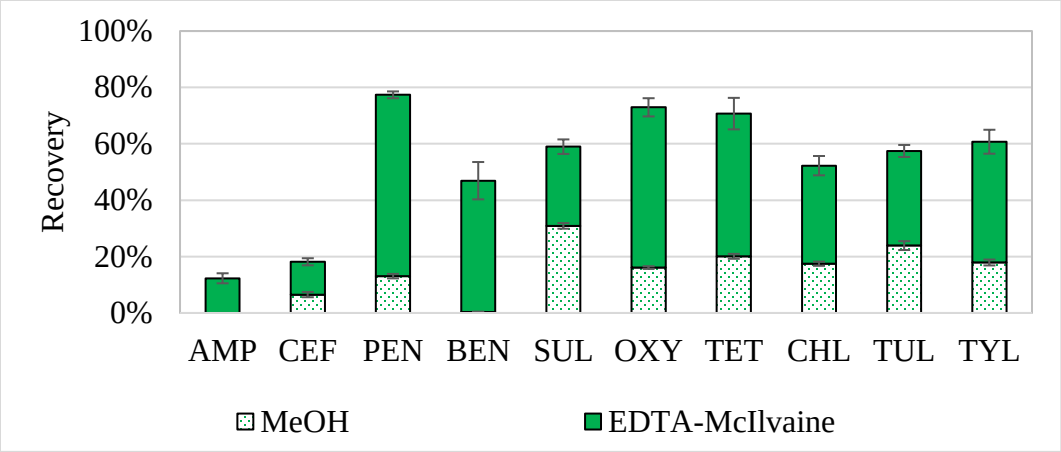


Figure 2.2. Recoveries from the initial two-step extraction experiment with EDTA-McIlvaine (solid bar) as the first extractant followed by MeOH (dotted bar) as the second extractant. Resulting recoveries were averages (n = 3), with error bars based on ± standard error. The antibiotics examined included Ampicillin (AMP), Ceftiofur (CEF), Penicillin-G (PEN), Benzylpenicilloic Acid (BEN), Sulfadimethoxine (SUL), Oxytetracycline (OXY), Tetracycline (TET), Chlortetracycline (CHL), Tulathromycin-A (TUL), and Tylosin Tartrate (TYL).

2.2.3 Two-Step Extraction on Solid and Aqueous Manure Samples Based on Total Solids

The initial two-step extraction from above, without the hexane step, was used as the extraction method for manure collected at four different points at a NY Farm: (1) unprocessed manure pit (UPM), (2) separated liquid manure pit (SL), (3) separated solids (SS) from the top of a screw press, and (4) solids heat treated using a bedding recovery unit (BRU). The average antibiotic recoveries over the four manure types for the EDTA-McIlvaine extraction fraction for the antibiotics that were recovered were 10.5–62.4%, and the average recoveries for the MeOH extraction fraction were between 2.3 and 31.3%. Ampicillin was not recovered from any of the manures using this extraction. The total antibiotic recoveries for the four manure types were calculated by summing the recoveries over the two extraction fractions with average total recoveries for each antibiotic class across the four manure types of: 24% ± 12% to 76%

± 3% for the β-lactams, 50% ± 3% for SUL, 56% ± 2% to 74% ± 2% for the tetracyclines, and 51% ± 5% to 59% ± 9% for the macrolides (Figure 2.3).

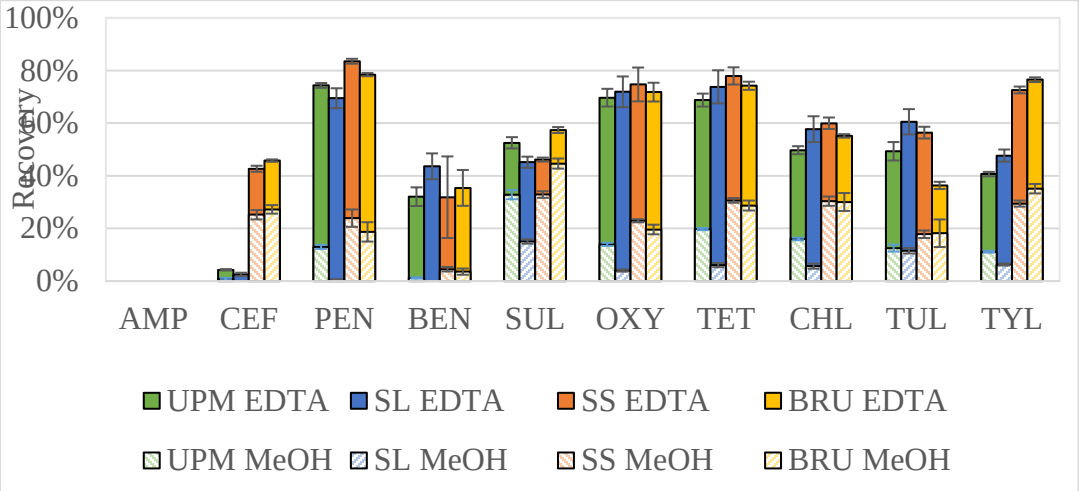


Figure 2.3. Extraction recoveries of antibiotics from four types of manure processed through a manure treatment system (graphed from left to right within each antibiotic): unprocessed manure (UPM), liquid separated manure (SL), solid separated manure (SS), and manure treated using a bedding recover unit (BRU). The extraction was a two-step process with an EDTA-McIlvaine buffer as the first extractant (EDTA, top half of the bar) and MeOH as the second (bottom half of the bar). Values from each extraction fraction are averages (n = 3) with ± standard error bars. The antibiotics examined include Ampicillin (AMP, not recovered), Ceftiofur (CEF), Penicillin-G (PEN), Benzylpenicilloic Acid (BEN), Sulfadimethoxine (SUL), Oxytetracycline (OXY), Tetracycline (TET), Chlortetracycline (CHL), Tulathromycin-A (TUL), and Tylosin Tartrate (TYL).

While most of the antibiotics in the SL manure were extracted in the first extraction step using the EDTA-McIlvaine buffer (>67% for all antibiotics), the extraction recoveries for manure with higher solids content ranged from 22–96%, with an average of 63% ± 3.6%. The heightened recovery in the first extraction step (EDTA-McIlvaine buffer) for the SL manure over the other manure types indicates that the antibiotics in the SL manure are mainly in the aqueous phase and not associated with any remaining fine solids in the sample; even though the TS in all samples was held constant (~0.25 g TS/g manure) when samples were prepared for extraction (Table 2.2). The RSDs for the recoveries of all antibiotics across the four manure types were 3–21% except for CEF (RSD 99%) and TYL (RSD 30%), indicating that this initial two-

step method was appropriate as a one-size-fits all extraction approach for most of the antibiotics in this study. The reduced recoveries of CEF and TYL in the UPM and LS samples could be due to the rapid degradation of CEF and TYL in the presence of microorganisms, which would be present in higher bacterial loads in the liquid samples compared to the treated manures (Van den Meersche et al. 2016; Jansen et al. 2019). Even though CEF and TYL had reduced extraction efficiencies for the more liquid substrates, average recoveries for all antibiotics in all samples were >32%, except CEF (4–46%) and AMP (not recovered). Lower recovery for CEF and AMP is likely due to β-lactam antibiotics being unstable in manure matrices (Berendsen et al. 2015) . The performance of this single extraction method on a range of manure types was consistent for most antibiotics and indicates that basing the mass of manure extracted on the TS concentration was a viable sample preparation approach and eliminates the need for multiple antibiotics extraction methods for different manure matrices moving forward.

Table 2.2. Total solids (TS) of the manure used in this study, and the wet mass used in the extraction experiments based on a TS of 0.25 g/g manure for extraction. Solids are reported as average values (n = 3) with ± standard error.

Manure	Total Solids ($g_{solids}/g_{wet\ manure}$)	Mass Extracted (g_{wet})
Blank Dairy Manure (BDM)	0.134 ± 0.005	2
Unprocessed Pit Manure (UPM)	0.074 ± 0.004	3.61
Separated Liquid (SL)	0.064 ± 0.0002	4.20
Separated Solids (SS)	0.347 ± 0.010	0.770
Bedding Recovery Unit (BRU)	0.370 ± 0.120	0.722

2.2.4 Optimizing the Two-Step Method to Combine Extracts for One Injection

This set of extractions was conducted to determine the most effective way to combine the two extraction fractions using either Method A or Method B (Figure 2.4).

To compare the extract combination method, the volumes of EDTA-McIlvaine and MeOH were kept at 10 mL in both treatments. The results of the extraction comparison showed that Method A had a significantly higher average recoveries (54%), except for AMP and BEN, which had consistently lower recoveries throughout this study. Recoveries of SUL and the tetracycline drugs were all significantly higher using Method A (extracts combined before SPE) than using Method B (EDTA-McIlvaine fraction cleaned up via SPE and eluted into MeOH fraction) (p-values < 0.014), while the macrolide recoveries were not significantly impacted by the extract combination method, and the β–lactam recoveries were drug-dependent (Table 2.3).

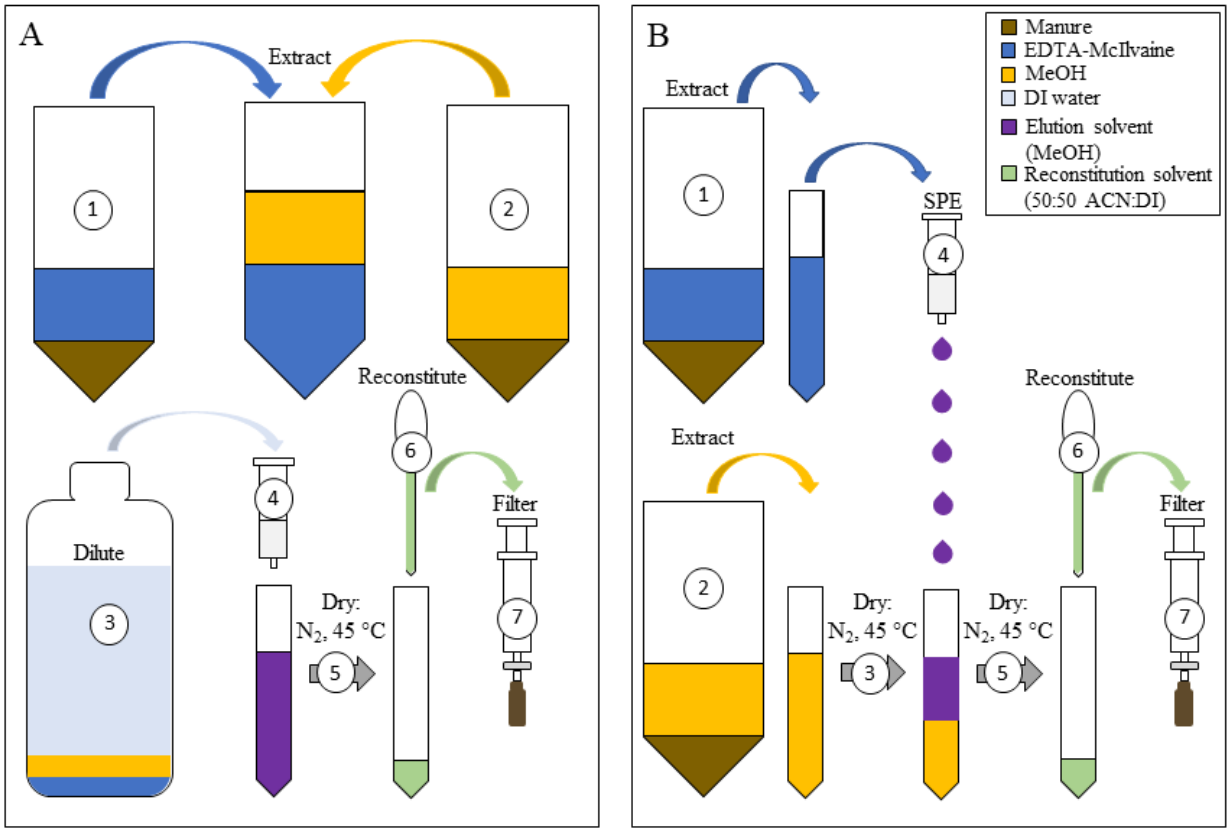


Figure 2.4. EDTA-McIlvaine and MeOH extracts based on methods in Section 3.6.3 were combined using two methods: (**A**) the two extract fractions were mixed, then diluted to 500 mL with DI water prior to cleanup using solid phase extraction (SPE), and (**B**) the EDTA-McIlvaine fraction was cleaned up using SPE and then eluted into the MeOH fraction. Circled numbers refer to sequence of steps in each method.

Table 2.3. Results for optimization experiment that combined extracts for one injection per sample. Method A was compared to Method B as diagrammed in Figure 4.

Antibiotic	Method A	Method B	*p*-Value
	Recoveries (%) ± SD		
Oxytetracycline	131 ± 13	45 ± 4	0.0004
Tetracycline	114 ± 7	68 ± 2	0.0004
Chlorotetracycline	67 ± 3	54 ± 1	0.0021
Penicillin-G	66 ± 1	58 ± 1	0.0006
Sulfadimethoxine	56 ± 3	31 ± 2	0.0003
Tylosin	53 ± 2	47 ± 6	0.18
Tulathromycin-A	49 ± 9	43 ± 2	0.32
Ceftiofur	11 ± 0.3	6.4 ± 0.5	0.0002
Ampicillin	2.3 ± 0.1	7.3 ± 0.4	0.00003
Benzylpenicilloic Acid	1.3 ± 0.04	5.6 ± 0.4	0.0001

SUL recoveries in Method A (56% ± 3%) were within reported recoveries from manure (64%) and lagoon wastewater (54%) using SPE for cleanup (Zhou et al. 2012). SUL recovery was likely highest when combining and diluting the extracts prior to SPE due to its hydrophobicity, which acted as a driving force for SUL to sorb to the SPE cartridge resulting in a higher recovery.

Tetracycline drug recoveries were highest (67–131%) in Method A, which combined and diluted the extracts prior to SPE. The recoveries from Method A were 65%, 41% and 19% higher for OXY (131% ± 13%), TET (114% ± 7%), and CHL (67% ± 3%) in comparison to Method B, respectively (*p*–values < 0.003) (Table 3). Utilization of high volumes of EDTA-McIlvaine buffer has been shown to increase tetracycline recoveries; as a result of the increased concentration of chelators in the solution binding to the cationic solids in the manure and preventing tetracyclines from adsorbing to them [4]. Recoveries of tetracyclines in Method A (67–131%) were within values reported for extraction in manure using liquid-solid extraction followed by SPE (96–170%) (Zhang et al. 2019). The macrolide recoveries were not significantly

impacted by the extract combination method. The lower recoveries for TUL in this study are likely due to the non-extractable portion of antibiotics irreversibly bound to the manure matrix compared to surface water. Jansen et al. (2019) observed similar TYL recoveries (~60%) to this study in spiked manure extracted using trifluoric acetic acid in ACN and cleaned up using SPE. Antibiotic interaction within manure differs in comparison to soils or other aqueous matrices due to a larger concentration of natural organic matter, which can provide more sites for sorption (Wallace and Aga, 2016; Spielmeyer et. al 2018)

The β–lactam recoveries, with exception of PEN, were low throughout the study, which was not surprising, considering β-lactams have proven difficult to recover in previous studies (Berendsen et al. 2018; Jansen et al. 2019). While CEF recovery was significantly higher in Method A than in Method B (p–value < 0.001), AMP and BEN recoveries were significantly lower (p–value < 0.04) in Method A than in Method B, and the recovery of PEN was not significantly affected by either of the combination methods (p–value = 0.121). β-lactams still prove to be a challenging class of analytes. Consistent recovery of PEN using this method is a new development not yet reported in previous extraction studies and is likely due to separation of the solvents during extraction. Since β-lactams (penicillins and cephalosporins) are one of the most used therapeutic drugs for dairy operation (second to tetracyclines) (Oliver et al. 2020), the ability to track penicillin through manure treatment systems and determine their fate in the environment is important. The final two-step extraction using 10 mL each of EDTA-McIlvaine buffer and MeOH and combined with Method A is the recommended

method that was proven to be a valuable protocol for multiclass antibiotic analysis and beneficial for the ability to recover penicillin consistently.

2.2.5 Extraction Method Performance Using Method A

The percent recovery, RSD, matrix effect (ME), linearity, limit of detection (LOD), and limit of quantitation (LOQ) were calculated for this method (Table 2.4). Using the recommended extraction method, acceptable recoveries (> 50%) for most antibiotics were shown when spiked at a concentration of 350 µg/kg. The RSD calculated for the recovery study was < 25% for all analytes. The ME ranged from 57 to 89% for all antibiotics, which indicates major signal suppressions from manure extractions for all analytes except TUL due to residual material in the samples. The MEs in these samples were corrected by using an IS for each drug class and labelled analogs, when available, except for β-lactams. The linearity was >0.99 for most analytes obtained from a concentration range of 0.01–1 µg/mL. The LDLs ranged from 0.229–8.05 µg/kg wet weight, and the LOQs ranged from 0.694–24.4 µg/kg wet weight, which illustrates the ability of this method to detect low concentrations of antibiotics in manure.

Table 2.4. Method performance parameters for the two-step extraction of antibiotics from manure using Method A.

Antibiotics	Recovery (%RSD), n = 3	Matrix Effect	Linearity Fit (R^2)	LOD [a] (µg/kg)	LOQ [b] (µg/kg)
AMP	2% (4%)	88%	0.995	3.58	10.8
CEF	11% (21%)	88%	0.996	0.893	2.71
PEN	57% (22%)	79%	0.986	2.53	7.68
BEN	0.5% (31%)	85%	0.988	4.83	14.6
SUL	56% (8%)	68%	0.999	0.606	1.84
OXY	131% (17%)	88%	0.999	8.05	24.4
TET	114% (10%)	88%	0.999	2.02	6.11
CHL	66.2% (6%)	89%	0.999	7	21.2
TUL	47% (25%)	57%	0.989	3.18	9.64
TYL	55% (3%)	86%	0.996	0.229	0.694

[a] Limit of detection, [b] Limit of quantitation.

2.3. Materials and Methods

2.3.1 Standards and Reagents

Reference standards used in the extraction experiments encompassed four groups of antibiotics: tetracyclines, sulfonamides, macrolides, and β-lactams. Standards purchased from Sigma-Aldrich (St. Louis, MO, USA) included tetracycline hydrochloride (TET) at >95%, oxytetracycline hydrochloride (OXY) at >95%, sulfadimethoxine (SUL) at >98.5%, tulathromycin A (TUL) at >95%, tylosin tartrate (TYL) at >80%, penicillin G sodium salt (PEN) at 96–102%, benzylpenicilloic acid disodium salt (BEN) at >95%, ampicillin (AMP) at >95%, and ceftiofur hydrochloride (CEF) at >95%. Chlorotetracycline hydrochloride (CHL) was a United States Pharmacopeia (Rockville, MD, USA) reference standard. At least one internal standard was used for tetracyclines, the sulfonamide, and the macrolides, with labeled standards used when possible (Table 2.1). No internal standard was used for β-lactams, which were calibrated externally. Demeclocycline hydrochloride (DEM) at 92.4%, a European Pharmacopeia (Strasbourg, France) reference standard, was used as the

internal standard for the tetracyclines. Sulfadimethoxine-d6 (SUL-d6) at >99% was used as the internal standard for SUL, roxithromycin (ROX) at >90% was used as the internal standard for TYL, and nalidixic acid-d5-(ethyl-d5) (NAL) at >99% was used as the internal standard for TUL; all purchased from Sigma-Aldrich, St. Louis, MO, USA. High-performance liquid chromatography (HPLC) grade methanol (MeOH) and acetonitrile (ACN), formic acid (88%), oxalic acid (99^{+}%), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA) at 99^{+}%, and sodium phosphate dibasic heptahydrate (Certified ACS Crystaline) were purchased from Fisher Scientific (Fairlawn, NJ, USA). Anhydrous citric acid (99%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Organic-free water was obtained from a Picotech UV Plus system (Hydro Service and Supplies, Gaithersburg, MD, USA). EDTA-McIlvaine (pH 5) solution used for extraction was prepared by combining 500 mL of 0.1 M Citric Acid, 312 mL of 0.2 M disodium phosphate, and 30.25 g EDTA in a 1 L volumetric flask, with organic-free distilled water (DI) used to bring the solution to volume.

Individual stock solutions of reference standards and internal standards (IS) were prepared at a concentration of 100 µg/mL in MeOH and stored in amber glass screw-capped vials at −20 °C and replenished every 6 months. A mixed solution of reference standards for spiking was prepared fresh on the day of the experiment at a concentration of 5 or 7 µg/mL in MeOH. On the day of analysis, the mixed reference standard was prepared at 1 or 5 µg/mL in 50:50 ACN:DI, and a mixed solution containing the internal standards was prepared at a concentration of 10 µg/mL in MeOH.

2.3.2 Collection of Dairy Manure Samples

A blank dairy manure (BDM) sample was collected at the Beltsville Agricultural Research Center (Beltsville, MD USA) farm from dairy cows that were free of antibiotics and included in the extraction experiments. Dairy manure substrates from a farm located in New York, US that used solid/liquid separation, with the liquid manure sent to a lagoon and the separated solids subsequently heat treated using a bedding recovery unit (BRU), were collected and used to compare extraction efficiencies from different manure types (Oliver et al. 2021). Manure was collected at four points in the bedding recovery treatment process: (1) unprocessed pit manure (UPM), (2) separated liquid manure (SL), (3) separated solids (SS) from the top of a screw press, and (4) solids heat treated using the bedding recovery unit (BRU). After collection, all manure types were stored at 4 ℃ for no longer than two weeks before extraction.

2.3.3 Sample Preparation

Due to the varying sorptive properties of the different antibiotics extracted in this study (Table 1), the mass of extracted manure was based on the TS concentration (dry matter content) of each manure type, not the total wet mass, that has been used in other studies. The TS concentrations of the manure were determined using the Standard Methods for the Examination of Water and Wastewater (APHA, 2005), with the TS values of each manure substrate tested as shown in Table 2. In brief, this method dries 10 to 25 g of homogenized sample in tared ceramic crucibles at 105 ℃ overnight, accounting for the dissolved and settled solids masses. Previous studies have extracted antibiotics from 1 to 2.5 g raw manure manure (Arikan et al., 2006; Martinez-Carballo

et al., 2007; Zhao et al., 2010; Ho et al., 2012; Berendsen et al., 2013; Gao et al., 2016; Van den Meersche et al., 2016b) and from 0.1 to 5 g dried material (Jacobsen and Halling-Sorensen, 2006; McKinney et al., 2010; Zhou et al., 2012; Guo et al., 2016; Wallace and Aga, 2016). For this study, the wet mass of the manure fractions corresponded to a dry matter TS content of 0.25, which ranged from 2 to 0.77 g wet weight (2.00 g for BDM; 3.61 g for UPM; 4.20 g for SL; 0.770 g for SS; and 0.722 g for BRU manure substrates).

For the extraction, triplicate manure samples were weighed and, when required, antibiotic standard solutions were added to achieve a concentration of 350 µg/kg of each analyte, following the procedure used by Jansen et al. (2019). All samples were then vortexed for 30 sec and allowed to equilibrate in the dark at room temperature for one-hour prior to extraction. After equilibration, 10 mL of EDTA-McIlvaine buffer was added to each sample. The samples were vortexed for 10 sec, then sonicated in an ultrasonic bath (Elmasonic E 100 H, Singen, Germany) at room temperature for 15 min. Following sonication, the samples were placed on a rotary mixer (Lab-Line Orbit Shaker Model 3520, Melrose Park, IL, USA) and mixed at 50 rpm at room temperature for 15 min. After mixing, the samples were centrifuged at 3300× *g* for 20 min. The supernatant was decanted into a fresh 50 mL polypropylene centrifuge tube. The extraction process was then repeated with 10 mL MeOH. The extracts were either combined or kept separate as described in the various extraction iterations below in Section 3.6.

2.3.4 Sample Cleanup

Throughout the extraction trials, a Phenomenex 33 μm polymeric reverse phase 200 mg/6 mL SPE cartridge (STRATA™-X, Torrance, Canada) was used for sample cleanup. Before use, the cartridge was conditioned with 5 mL MeOH followed by 5 mL EDTA-McIlvaine buffer. The samples were continuously loaded onto the cartridges at a pressure of 10 psi (6 mL/min). After sample loading was complete, the cartridges were washed with 5 mL of DI water and dried by applying vacuum for 5 min. The residues retained on the column were eluted from the cartridge with 5 mL of MeOH into 15 mL polypropylene tubes and subsequently evaporated to <200 μL under a steady stream of N_2 (10 psi) at 45 °C (Caliper Life Sciences TurboVap LV, Charlotte, NC, USA). Residues were reconstituted to a final volume of 1 or 1.5 mL with 50:50 ACN:DI, vortexed briefly, filtered through a 0.45 μm polytetrafluoroethylene (PTFE) syringe filter, and then analyzed immediately or placed in a −20 °C freezer until analysis on the UPLC-MS/MS (Waters, Milford, MA, USA).

2.3.5 UPLC-MS/MS Conditions

The analysis of antibiotics in manure was conducted using a Waters Acquity H-Class Plus ultra-performance liquid chromatograph (UPLC) tandem Xevo TQ-S micro triple quadrupole MS (Waters Corp. Millford, MA, USA). Chromatographic separation was performed on a Kinetex C-18 (2.6 μm 100 Å, 2.1 mm × 100 mm) column (Phenomenex, Torrance, CA, USA) protected by a C18 guard column. The mobile phase composition was 1 mM oxalic acid and 0.1% formic acid in DI water (mobile phase A) and 0.1% formic acid in ACN (mobile phase B). The analytes were eluted at a flow rate of 0.4 mL/min under the following gradient: 0.0–0.5 min 95:5 (A:B); 0.5–

0.7 min linear increase to 35:65 (A:B); 0.7–2.0 min hold; 2.0–2.5 min linear increase to 13:87 (A:B); 2.5–4.0 min linear increase to 100% B; 4.0–6.0 min hold; then 6.0–7.0 min linear decrease to initial 95:5 (A:B); and 7.0–10.5 min hold. Detection was performed in positive electrospray ionization mode (ESI+) with multiple-reaction monitoring (MRM), where each compound was optimized for cone voltage and collision setting. The injection volume was 5 µL and column temperature was held at 40 °C. The MS/MS conditions were as follows: capillary voltage (0.5 kV), source temperature 150 °C, desolvation temperature 450 °C, cone gas flow 50 L/hr, and desolvation gas flow 1000 L/hr. Analyte retention times and mass transitions are presented in Table 2.1. Quantitation was conducted using TargetLynx software (Waters, Milford, MA, USA).

2.3.6 Method Trials

2.3.6.1 Comparative Analysis of Buffer vs. Solvent Extraction Efficacy

To determine the efficacy of an aqueous buffer compared to an organic solvent on the extraction of the antibiotics in this study, an experiment was conducted that used either EDTA-McIlvaine buffer or a 50:50 blend of MeOH:CAN. These solvents were tested using six control dairy manure samples that were each spiked with 350 µg of the 10 antibiotics/kg of manure. For one set of triplicate spiked samples 0.1 M EDTA-McIlvaine buffer (pH-4) (10 mL) was used for the extractant, and for the other set of triplicate spiked samples, 50:50 MeOH:CAN (10 mL) was used. The manure was extracted twice as outlined in our methods except that prior to further handling each extract was mixed with 10 mL of hexanes on the rotary mixer (20 rpm, 15 min), following a method used by Pan et al. (2011) to help remove interfering fats. After

centrifugation, the upper hexane layer was removed and discarded. To limit unwanted impurities, the EDTA-Mcllvaine extract was additionally cleaned up by passing it through SPE and the sorbed antibiotics were eluted off with 50:50 MeOH:CAN. Each extract was concentrated down to <200 µL using a stream of N_2 (45 °C) and reconstituted to a volume of 1 mL using 50:50 MeOH:CAN prior to analysis using UPLC-MS/MS. Recoveries were calculated based on the concentrations of antibiotics spiked into the manure.

2.3.6.2 Initial Two-Step Extraction with Buffer Followed by Methanol

This extraction trial employed the extraction solvents stepwise, with aqueous EDTA-McIlvaine used first, followed by MeOH. The use of a two-step protocol over a mixed extractant method was based on the preliminary data from above (Section 3.6.1). For the two-step method, 2 g of the BDM substrate was weighed out in quadruplicate, and three of the samples were spiked with 350 µg/mg antibiotics, with an additional sample carried through as a blank for post-extraction spiking and recovery calculations to account for matrix effects. All samples were placed in the dark for 90 min after spiking. The manure was first extracted using EDTA-McIlvaine (10 mL) following the mixing and centrifugation procedures detailed in Section 3.3. After the EDTA-McIlvaine buffer was decanted into a fresh 50 mL centrifuge tube, MeOH (10 mL) was added to the previously extracted manure pellet for the second extraction. The two extraction fractions were kept separate, and hexanes (10 mL) were added to each fraction, mixed on a rotary mixer (50 rpm, 15 min), and then centrifuged (3300× *g* for 20 min). The EDTA-McIlvaine fraction was further cleaned up using SPE (Section 3.4.), with MeOH as the elution solvent. These extracts were prepared for injection on

the UPLC-MS/MS, as described above in Section 2.3.4. The two extraction fractions were analyzed separately, and recoveries for each fraction were calculated based on the response of the post-spiked extract. Total recoveries were calculated by summing the recovery for the two extraction fractions.

2.3.6.3 Two-Step Extraction on Solid and Aqueous Manure Samples Based on Total Solids

This method was applied on manure collected from a commercial NY dairy farm utilizing a bedding recovery unit (BRU) for the separated solids. The manure samples were collected at each of the four points in the processing line. In lieu of drying samples prior to extraction, extraction masses were based on the TS content of each of the manure types (Table 2.2). Samples were weighed on a wet basis, according to the TS concentration to keep the solids content consistent across each sample type. The manure samples were weighed out in quadruplicate. Then, three samples of each manure type were spiked to a final antibiotic concentration of 350 µg/kg manure prior to extraction, and one sample was left as a blank for determining recoveries after post-extraction spiking. The four manure-types were extracted using the initial two-step extraction method discussed above in Section 2.3.6.2 (without the hexane cleanup step).

2.3.6.4 Optimizing the Two-Step Method to Combine Extracts for One Injection

A final extraction experiment was conducted to adapt the method employed in Section 2.3.6.3. above by optimizing it so that the EDTA-McIlvaine and MeOH fractions could be combined prior to analysis on UPLC-MS/MS. This then negates the need to have two extracts to analyze for each sample. In this experiment, two extract

combination methods were tested: Method A and Method B (Figure 2.4). In both Method A and Method B, the manure was extracted as outlined in Section 2.3.3., the extract fractions were then treated differently prior to being combined. In Method A, the two extraction fractions (10 mL of EDTA-McIlvaine and 10 mL of MeOH) were decanted into the same 50 mL centrifuge tube, the volume was brought up to 30 mL with DI water, centrifuged (20 min at 3300× g) to remove suspended matter, and decanted into 470 mL of DI water prior to sample cleanup using SPE (Figure 4A). In Method B, the 10 mL of EDTA-McIlvaine buffer extract was cleaned up using SPE and the 10 mL MeOH extract was blown down to a volume of 5 mL under N_2, and then it was combined with the SPE eluant prior to the final drying step. These combined extracts were prepared for injection on the UPLC-MS/MS as described above in UPLC-MS/MS section (Section 2.3.4.). The data were reported as averages of triplicate samples and recoveries were calculated based on triplicate blanks that were spiked post-extraction. Statistical comparisons of recoveries for each antibiotic were made between the five treatments using a one-way ANOVA followed by Tukey–Kramer's post hoc test in R (R Core Team. (2020) Vienna, Austria). The final, optimized method was selected based on the results from careful comparison of Method A and Method B (Figure 4) using replicate spikes of the blank manure.

2.3.7 Evaluation of Performance of Method Trials

Method performance for the method trials were used to establish data reliability based upon linearity of calibrants in the matrix, selectivity, recovery, and repeatability (Araujo et al. 2009). Matrix linearity was determined for each analyte by adding solutions of the antibiotics into the blank manure matrix at five concentrations from 10

ng/mL to 1 µg/mL. Least squares regression was conducted on the calibration lines constructed by plotting the ratio of the peak area of the analyte to the area of the internal standard (IS) against the ratio of the added concentration of analyte to the IS. A correlation coefficient greater than 0.99 was considered linear. Method limit of detection (LOD) (Equation (1)) and limit of quantitation (LOQ) (Equation (2)) were determined based on the standard deviation (SD) of the response of the lowest calibrant in the matrix-matched calibration curve and the slope of the curve (Araujo et al. 2009). Selectivity was conducted by comparing the chromatograms of the blank manure and spiked manure to ensure there was no interference in the blank sample at any of the antibiotic ion transitions. Recovery quantitation was based on the concentrations of analytes in the blank matrices fortified before and after extraction (Equation (3)). Repeatability was assessed by using the relative standard deviation (%RSD) of triplicate samples during one run (Equation (4)). Matrix effect (ME) was evaluated by comparing the slope of the calibration curve in the matrix extract to the slope in the curve prepared in solvent (Equation (5)) (Karaca et al. 2018).

$$LOD = \frac{3.3 \times SD}{slope}$$

$$LOQ = \frac{10 \times SD}{slope}$$

$$Recovery\ (\%) = \frac{Concentration\ in\ pre{-}spiked\ sample}{Concentration\ in\ post{-}spiked\ sample}\ x$$

$$\%RSD = \frac{SD\ of\ the\ mean}{mean} \times 100$$

$$ME\ (\%) = 1 - \frac{Slope\ of\ curve\ in\ extract}{Slope\ of\ curve\ in\ solvent} \times 100$$

2.4. Conclusion

This study and the final method presented builds on traditional extraction methodology and contributes three major concepts: (1) better understanding of solvent/buffer interaction based on manure matrix and antibiotic type, (2) an extraction process based on the sample TS, and (3) a uniform method for extraction of four antibiotics classes that can be used for the various forms of manure throughout the manure treatment and land fertilization processes. The most reliable method for extracting and analyzing the antibiotics evaluated in this study was a two-step extraction, with the final combined extracts diluted to an organic solvent concentration of 2% and then cleaned up using SPE (Method A). When multiple manure types were extracted, keeping the TS concentration of each manure constant resulted in consistent antibiotic extraction among the manure types. This method of determining the mass of manure to extract for similar recoveries can be used to evaluate antibiotic concentrations in manure as it moves through a treatment/land fertilization system. Furthermore, the assessment of antibiotic concentrations in multiple manure treatment effluent streams, with liquid and solid fractions, is vital to understanding antibiotic contamination risks between substrate type and final fertilizer applications using a mass balance approach.

Chapter 3: Antibiotic Resistance Partitioning in Dairy Manure Through a Continuous High Temperature Rotary Drum Bedding Recovery Unit

3.1 Introduction

Historically, antibiotics were administered in feed as growth promoters for livestock animals in the United States, while currently antibiotics are only given by veterinarians for medicinal care to livestock in the US. Any antibiotic administration can lead to antibiotic residues in manure due to incomplete metabolic catabolism and contribute to antibiotic resistance (AR) in the environment (Zhu et al. 2013; Youngquist et al. 2016; Guo et al. 2018). Manure application to fields as fertilizer can be a route of dissemination for antibiotics, antibiotic resistance gene (ARG) and resistance bacteria (ARB) into soils and adjacent systems (Huer et al. 2011; Youngquist et al. 2016). Policies and legislation, such as the US veterinary feed directive (CVM, 2019) and improvements to livestock housing and manure management practices, have made strides in reducing antibiotic use on-farms. Studies continue to infer connections between antibiotic administration on-farm, manure application, and increasing AR in the environment (Zhang et al. 2018a). The potential selective pressure of antibiotics and corresponding ARB can influence the local flora, with the ability of mobile genetic elements and genes to spur resistance within various taxonomic groups, including potential pathogens. While the exact contribution of dairy farms and other animal agricultural industries remain uncertain, exploration of the fate of antibiotics, ARGs, and ARB in these systems are necessary to understanding AR development.

There are a wide range of dairy manure management technologies designed to reduce manure odor, volume, and to optimize nutrient accessibility, yet these technologies are rarely designed or extensively assessed for AR mitigation (Newtrient 2019; Oliver et al. 2020). Solid liquid separation (SLS) is a technique that has been increasingly utilized within the dairy industry that generates two separate effluent streams, with the liquid fraction used as a fertilizer and the solid fraction available for reclamation as stall bedding or further processed via composting (Aga and Wallace 2016). Studies have demonstrated that SLS systems can have adverse impacts on ARG abundance (Tien et al. 2017; Wallace et al. 2018). Wallace et al. (2018) found that SLS concentrated ARGs, reporting 15% and 18% increases in *sul1* and *sul2*, respectively, as well as increases in several other ARGs surveyed, yet no increases in *tetW*. These previous studies also found corresponding increases in total tetracycline and sulfonamides concentrations post separation. While Tien et al. (2017) found mechanically dewatered manure to significantly reduce *ermB* (p-value <0.05), there were minimal reductions to the other seven ARGs studied compared to the raw manure. The vastly different substrate (liquid and solids) characteristics generated from the SLS process hosts various implication for antibiotics, ARGs, and ARBs. The physicochemical properties of each antibiotic dictate the partition and degradation potential of each compound within each manure fraction. Antibiotics, such as tetracyclines, macrolides, sulfonamides, and fluoroquinolones, have been shown to have greater sorption and corresponding persistence in solid organic material, such as dairy manure, while β-lactams are shown to be more hydrophilic and tend to partition

with more aqueous substrates (Wallace and Aga 2016; Li et al. 2017a; Gros et al. 2019).

Few studies have examined the combined effects of SLS with thermophilic rotary drum composting (>40 °C) in a continuous system. Composting has been suggested as an effective technique for reducing antibiotic residues and pathogens manure prior to land application or use as bedding (Youngquist et al. 2016; Speilmeyer et al. 2018; Zhang et al. 2018a). Previous work indicated that thermophilic composting has higher overall antibiotic degradation, alters bioavailability, and a lower viable pathogen risk for ARG and ARB dissemination compared to mesophilic temperatures or other non-heat-based treatments (Mitchell et al. 2015; Qian et al. 2016a; 2016b). While some composting studies have used conventional outdoor technologies, such as windrows and static piles (Rodriguez et al. 2012), it should be noted that composting effectiveness is variable based on maintenance of optimal conditions. Schueler et al. (2021) showed little to no reduction of tetracyclines in a 35-day on-farm compost windrow study despite previous literature reporting 85-99% removal (Masse et al. 2014). Composting parameters such as turnover rate, moisture and internal temperature can all influence microbial activities and compound degradation. Lab-based composting studies cite higher rates of antibiotic and ARG removal follows strict controlled condition that cannot be replicated in the field (Kim et al. 2012; Scheuler et al., 2021).

Rotary drum composting is seen to be a more efficient and promising technology that provides agitation, aeration, and consistent mixing to produce a more consistent and uniform product that also limits odor and leachate potential.

Additionally, this technology can reduce composting time to three weeks or less (Kalamdhad et al. 2009). Though previous studies investigating the impact of composting on antimicrobial resistance can provide some reference, composting results have been inconsistent due to differing conditions used in the field. In this study, a bedding recovery unit (BRU) system was studied, which provides a consistent product. There have been limited studies using BRUs for AR implications. Additionally, most previous studies are not based on the mass flow and manure characterization data from a full-scale continuous manure management system, with tracking of the solid and liquid components and understanding of the quantity of antibiotics, ARBs and ARGs that partition to each fraction. Our preliminary study, detailed in Oliver et al. (2021), was the first study to look at BRUs at dairy farms and AR implications and showed significant reductions (50%) of oxytetracycline in the treated SLS and 60 - 98% reductions across the four ARGs included in the study.

This study builds on Oliver et. al (2021) report on antibiotic residual and ARGs based on mass balance approach, as there have been limited previous studies analyzing AR parameters based on the mass flow and manure characterization data from full-scale continuous manure management systems. The objectives of this study were to 1) quantify the partitioning of antibiotics, ARGs and ARB throughout SLS based on a mass flow, 2) determine the impact of substrate characteristics on resistance factor partitioning, and 3) measure the impact of the high temperature rotary drum composting and retention time of solid separated manure on resistance reduction. The total mass and flow of manure processing throughout the SLS and BRU unit was determined. The antibiotic residual and bacterial analyses were then

correlated to total mass and concentrations in the separated solids, separated liquids, and BRU effluent, to create a mass balance of the antibiotics, ARBs, and ARGs through the entire manure management system on-farm. The study results can be used to better understand the transport and movement antibiotic resistance factor and corresponding risk assessment based on SLS, BRU utilization of the solid fraction, and field application after lagoon storage of the liquid fraction.

3.2 Material and Methods

3.2.1. Experimental Design:

3.2.1.1 Study Location:

The mass flow analysis of the manure was conducted on dairy farm managing ~625 lactating cows in the northeastern US. This farm operates a continuous solid-liquid separator screw press in tandem with a high temperature, rotary drum composter - bedding recovery unit (BRU) to manage daily manure loads and produce bedding material for the cows and/or fertilizer for field application. For this study, the 105,000 L manure influent pit was emptied and filled with manure transported from the farm's on-site hospital barn (manure most likely to contain antibiotic residuals). The influent pit is semi continuously mixed while simultaneously pumping the raw manure to a mounted solid liquid separation (SLS) screw press separator. Separated liquids (SL) were then pumped to an adjacent 80,000 L effluent pit before being piped to a nearby open-air lagoon for storage. The separated solids (SS) were collected and pumped directly into the continuous rotary drum composter completing the BRU system. The unit temperature monitoring system documented the average BRU inlet temperate at 48.6°C and outlet at 70.8°C.

3.2.1.2 Residence Time Tracing in the Full-Scale BRU System

The mass flow of the BRU system was determined using a tracer study to determine sampling frequency at appropriate intervals. Mass flow was calculated throughout the experiment to account for solids settling in the system influent pit, changes in the influent manure density, and the changes in total solids (TS) content during the treatment period. The final estimated residence time and mass flow rate of BRU was determined using corn kernels (2.27 kg) as the tracer. Half of the corn tracers were weighed and added with the hospital barn manure in the influent pit as it began to move through the system, with the other half of the tracer placed in the SS fraction of the hospital barn manure as the solids entered the rotary drum portion of the BRU system. This tracer served as a visual indicator of when the hospital barn manure samples arrived at the BRU unit, and the residence time for the manure traveling through the BRU system.

The tracer study demonstrated immediate uptake of the raw manure to the solid-liquid separator once added to the influent pit. To monitor the BRU effluent, samples were taken every 30 min starting 12 hrs after the initial addition of the manure to the influent pit until 28 hrs after addition. The targeted BRU effluent was expected to have a retention time of ~ 26 hrs after manure addition, sampling included an additional 2 hrs to ensure no more kernels corresponding to BRU of interest were found. Six 5-gallon buckets of BRU solids were massed during each sampling time, with documentation of the accumulation time and the number of corn kernels found

within each bucket. The kernels were obtained by gently removing BRU solids from each bucket by hand and spreading them on a flat surface. The total mass and residence time of the of the BRU solids was determined using a calibrated hanging scale to weigh the BRU effluent (accounting for bucket tare weights) and timer used to document when samples were collected.

3.2.1.3 Mass Flow Experiment

Samples were collected over a 28-hour period from the initial addition of hospital barn manure into the inlet pit of the rotary drum system to the final 28-hour processing of the final bedding product. Two 3000-gallon truckloads of hospital barn manure were added to the influent pit, and raw manure samples were collected at the designated time point (t = 0 hrs). Sequential samples were collected immediately following the SLS processing of the system in 30-min intervals over a 2-hr period, as the SLS operated and emptied the pit. Based on the tracer study, there were 31 mass flow rate measurements taken over a 2-hr period, which encompassed the full emptying of the influent manure pit through the SLS system. The mass flow rate of the SL fraction was estimated by measuring the change in height of the manure from the influent raw manure pit prior to the SLS and the two receiving pits (SL and SS pits) using a measuring tape throughout the study period and factoring in the density of the SL, SS, and raw manure sources. The raw manure mass flow rate into the screw press was calculated by summing the solid and liquid fraction mass flow rates due the continuous addition of manure into the two receiving pits.

The mass flow rate for the solid fraction through the BRU was estimated by weighing the mass of solids collected at the exit of the BRU in a bucket over a 13-hr

duration. The processing of SS manure in the BRU occurred from 13 to 28 hrs. after the raw manure was loaded into the pit based on the tracer study. Starting 13 hours after initial manure addition, the BRU samples were collected every 2-4 hrs until the 28th hour to cover the complete mass flow of the manure from the initial addition of raw manure through BRU processing. The BRU effluent mass flow rate was based on an average of 177 measurements of the final BRU product taken over 18 hours based on the tracer protocol outline described in section 3.2.1.2. Both liquid and solid samples were quantified for antibiotics, bacteria, and corresponding ARGs.

3.2.2 Antibiotic Quantification

To measure antibiotic concentration and monitor antibiotic portioning within the manure, the method from Poindexter et al. (2022) was used for extraction and analysis. Due to the manure treatment system generating four unique substrates spanning influent manure to final BRU effluent bedding material, the extraction samples for antibiotic analysis were normalized by weight (grams) based on TS concentrations. Sample were analyzed in triplicates. In brief, the extraction method was a two-step method that utilized ultrasonic and mechanical mixing using two separate solvent extractions: 0.1 M EDTA-McIlvaine followed by methanol. The two extracts were combined and diluted to a solvent concentration less than 2%. The diluted extract was then concentrated using solid phase extraction through a C-18 cartridge. The cartridge was eluted with methanol, and the eluent was concentrated under a steady stream of N_2 gas. The final concentrate was reconstituted to a known volume using a 50% acetonitrile and 50% deionized water (DI) mix and stored in the

freezer at -20°C until liquid chromatography and tandem mass spectrometry (LC-MS-MS) analysis.

3.2.3 Sample DNA Isolation and qPCR:

Genomic DNA from the four manure substrates was isolated using 0.25 mg of the raw and separated liquid manure and 0.10 mg of the separated solid manure and BRU effluent samples using QIAamp DNA stool Kit (cat 51504, QIAGEN, Hilden, Germany) following the manufacturer's protocols. DNA extraction was performed in triplicate for each sample to account for extraction variable, efficiency, and heterogeneity of the samples. The quantity of the extracted DNA was determined and normalized based on Qubit 1.0 Fluorometer (Life Technologies, Grand Island, NY, USA) for DNA concentration and NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) for DNA sample quality analysis. DNA extract was diluted to 1 ng/uL in preparation for qPCR. All extracted DNA was stored at -20°C before qPCR analysis.

This study quantified the abundance of seven resistance genes, including macrolide (*ermB*), beta-lactam (*bla-2*), sulfonamides (*sul-1*), tetracycline *(tetX*, *tetM, tetW, tetQ*), as well as mobile genetic element (*intl1*) and 16S DNA for bacterial population normalization. Primer sequences of targeted genes listed in appendix A1 were cloned into the pGEM-T easy vector, purified using QIAprep Miniprep kit (cat# 27104, Qiagen, Hilden, Germany), and 16S illumina sequencing for gene verification. Verified plasmids were selected as standards for qPCR, quantified, and adjusted to 1 ng/uL. Serial dilutions of the plasmids were conducted to generate an internal standard curve. Data extracted from standard curves had r^2 values > 0.99. All the

qPCR analytics were performed using the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) following amplification of all genes: heat inactivation at 95°C for 5 2 min, followed 40 cycles of 95°C for 5 sec (denaturation), 65°C for 10 sec (annealing), and 72°C for 10 sec (elongation). 20 uL qPCR reactions included 10 uL Forget-Me-Not qPCR Master Mix Hi-ROX (cat#31046, Biotium, Fremont, CA, USA), 1 uL of forward primer, 1 uL of reverse primer, 5 uL of sample DNA, and 3 uL of dH_2O.

3.2.4 Bacterial Culturing for Fecal Indicators

Two fecal indicator bacteria (FIB) species, *Enterococcus* and *Escherchia co*li, were used to quantify ARBs. Culturing samples taken from the raw manure, SL, SS manure, were taken during the first 2 hrs after manure addition and BRU effluent at 16, 20 and 24 hrs after intial manure addition to capture the full range of the BRU treatment and processing of the system based on the material retention time. Raw manure and separated liquid manure samples were individually homogenized and 2 g were added to new 15 mL tubes, while 1 g of separated solid manure and BRU effluents were homogenized and weighed into new 15 mL tubes. Following the addition of 10 mL of phosphate buffer solution (PBS) (Fisher Scientific, Hampton, NH USA), the tubes were manually mixed via inversion. The samples were then 10-fold serial diluted three times (10^{-3}) in PBS. Aliquots of 100 uL of each dilution were plated in duplicates and enumerated via manually spreading on two different selective culture mediums. Gram positive *Enterococcus spp.* were evaluated on Slantez-Bartley (Fisher Scientific, Hampton, NH USA), and the gram-negative *E. coli spp.* were evaluated on *E. coli* Chromagar (Fisher Scientific, Hampton, NH USA). Three sets of

plate were made with one of the following additions on each plate: oxytetracycline (5 mg/L) and ampicillin (0.5 mg/ L). These concentrations were used to replicate environmental concentrations of antibiotic in manure based on previous literature (Wohde et al. 2016; Spielmeyer 2018). Tetracycline and ampicillin concentrations were selected to match potential resistance based on administration records verified by farm management. The *E. coli* plates were incubated at 37 °C for 24 h, while the *Enterococci* plates were incubated for 48 h (24 h at 37 °C and 24 h at 44 °C). After incubation and lactose fermentation on selective media, *E. coli* colonies (blue), *Enterococcus* colonies (red), and other bacteria (white) were distinguished on each respective media, and the specific bacterial colonies were counted.

3.2.5 Sample Characterization:

Samples were stored on ice and frozen until analysis for pH, TS, volatile solids (VS), and nutrient content in the University of Maryland's Bioenergy and Bioprocessing Technology Laboratory (College Park, MD, USA). The sample pH was measured using an Accumet Basic As 15 pH Meter. Solids pH was conducted using soil and waste pH standard method (EPA, Method 9045D). The TS and VS analyses were conducted using the Standard Methods (APHA, 2005). For TS analysis, 10 mL of aqueous sample (raw manure and separated liquid manure) were pipetted and 25 g of solid samples (separated solid manure and BRU effluent) were weighed into pre-weighed and pre-dried (at 550 °C) porcelain crucibles. The samples were then dried at 105 °C until a constant weight was maintained. For VS analyses, the crucibles were placed in a furnace oven at 550 °C until a constant weight was obtained. Nutrient analysis for ammonia, total Kjeldahl nitrogen (TKN) and total phosphorus (TP)

samples were analyzed on the Lachat autoanalyzer (Quikchem 8500, Hach Company, Loveland, CO, USA) using the QuickChem method 13-107-06-2-D for TKN and method 13-115-01-1-B for TP. Metals analysis was conducted for copper and zinc following the EPA method 3051A at Agrolab Inc, (Harrington, DE, USA).

3.2.6 Statistical Analysis:

One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons were applied to compare ARB, ARG, antibiotic concentrations in different substrates and sampling times. All *p*-values <0.05 were considered significant. Bartlett's test was be used to compare variance in measures for ARB, antibiotics, and ARGs between sample location and time. Pearson bivariate correlation analysis was used to determine correlations between antibiotics, ARB, ARGs, nutrient levels, and heavy metal concentrations.

3.3 Results and Discussion

3.3.1 Fate of Antibiotics in BRU Processing

Five antibiotics were detected in the influent raw manure out of suite of 13 antibiotics tested across four antibiotic classes (B-lactams, macrolides, sulfonamides and tetracycline). Three of the five antibiotics were the parent compounds tetracycline, tulathromycin, and penicillin G, which matched the farmers records for antibiotic administration. The metabolites 4-epitetracycline and benzylpenicilloic acid were also found at detectable levels within the manure, indicating epimerization and degradation of tetracycline and penicillin G. Average antibiotic concentrations are shown in Table 3.1, with tetracyclines recording the highest concentration in the raw manure at 9.78 ug/kg. The tulathromycin, penicillin G, and benzylpenicilloic acid

concentrations were substantially lower at 0.508, 0.706, and 0.436 ug/kg, respectively. Influent concentration of the antibiotics was within previously reported concentrations that range from ~ 0.01 – 91 mg kg dry weight (Wallace and Aga 2016; Speilmeyer et al. 2018). The SLS process reduced the B-lactams (penicillin G and benzylpenicilloic acid), as they were non-detectable in the solid fraction of the separated manure and had a significantly lower concentration in the SL manure samples at 0.376 and 0.186 ug/kg, respectively (*p*-value = 0.001). Tetracycline/4-epitetracycline and tulathromycin also had lower concentrations in the separated liquid manure fraction at 7.38 and 0.36 ug/kg, respectively. Moreover, the concentration of these antibiotics in the SL fraction was significantly higher than in the SS fraction (*p*-value = 0.0001). There was a significant difference between the SS and SL fractions concentrations for both the tetracyclines and tulathromycin (*p*-values = 0.0001). Yet there was no significant difference between the SS fraction of tetracyclines and tulathromycin concentrations before and after BRU processing. Conversely, tetracyclines (tetracycline and 4-epitetracyline) and tulathromycin concentrations were higher in the solid fraction at 23.59 and 1.95 ug/kg, respectively, compared to the separated liquids. The three antibiotics (tetracycline, 4-epitetrcycline and tulathromycin) that appeared in the separated solids had no significant differences in concentration between pre (SS) and post treatment (BRU) treatment, with a higher concentration found in the final BRU effluent at 29.35 (*p*-value = 0.272) and 2.04 (*p*-value = 0.058) ug/kg respectively. This contrasts with most studies that have found thermophilic composting of dairy or beef manure to be effective at removing common antibiotics, such as oxytetracycline, chlortetracycline, and tylosin (Arikan et al. 2009,

Mitchell et al. 2015, Youngquiest et al 2016, Zhang et al. 2019). It should be noted that most composting processing occurs over the course of weeks, or months, compared to the 13 to 26 h period for composting within the rotary drum BRU. Furthermore, after SLS, the solids fraction had a lower moisture content (62%), which has been shown to inhibit the transportation of dissolved nutrient required for microbial metabolism and antibiotic degradation (Zhang et al. 2019). Tetracyclines and macrolides, such as tulathromycin, are known to display strong sorptive properties within manure, which can pose challenges for extraction and detection, especially within manure solids (Kim et al., 2012; Wegst-Uhrich et al., 2014; Wallace and Aga, 2018). Similar occurrences were reported by Wallace and Aga (2018), where both chlortetracycline and epi-chlortetracycline concentrations increased following SLS. Wallace and Aga (2018) theorized that higher detection may be the result of the compounds binding affinity to organic materials and changes to organic material concentrations between substrates.

Table 3.1: Concentration of antibiotics detected in manure from the farm-scale experiment. Results reported as average and (± standard error). Concentrations are based on percent solids per 1 kg of wet manure not total mass.

Manure Type	Total Tetracyclines (Tetracycline & 4-Epitetracycline) (ug/kg)	Tulathromycin (ug/kg)	Penicillin-G (ug/kg)	Benzylpenicilloic Acid (ug/kg)
Un-processed (Raw)	9.78 (± 0.88)	0.508 (± 0.01)	0.706 (± 0.130)	0.436 (± 0.071)
Separated Liquid	7.35 (± 0.79)	0.364 (± 0.01)	0.376 (± 0.044)	0.186 (± 0.028)
Separated Solids	23.59 (± 1.9)	1.95 (± 0.03)	ND*	ND*
BRU effluent	29.35 (± 3.1)	2.04 (± 0.02)	ND*	ND*

* ND stands for not detected in the sample.
* (BRU) Bedding Recovery Unit

Using the concentration of the antibiotics (Table 3.1) and measured manure flow rates, the total mass flow rates were calculated for each antibiotic across the four substrates (Table 3.2). Of the total mass of manure processed (7730 kg/h), 95% of the mass (7320 kg/h) fractioned with the separated liquid fraction, which was sent to an open-air storage lagoon. While 5% of the manure fractioned with the solids (410 kg/h), which continued to the thermophilic rotary drum processing (Table 3.2). The antibiotics followed a similar pattern as the manure with 71% of the tetracyclines, 68% of tulathromycin, and 40-50% of the beta-lactams partitioning with the separated liquid fraction of the manure, while 13% of tetracycline, 14% of 4-epitetracycline, and 20% of tulathromycin antibiotics remained within the separated solids fraction and proceeded to the BRU inlet for processing. These results parallel the Oliver et al.

(2020) study, with 80 and >90% of the total mass of the two antibiotics (oxytetracycline and sulfadimethoxine) partitioning with the liquid fraction.

Table 3.2: Average mass flow rate of tetracycline, 4-epitetracycline, tulathromycin, penicillin G and benzyl-penicilloic acid in unprocessed manure, separated liquid, separated solid and BRU effluent. Mass flow rates were based on antibiotic concentrations in Table 1 multiplied by the calculated manure flow rate.

Manure Type	Manure Flow Rate (kg/hr)	Antibiotic Flow Rates (mg/h)			
		Total Tetracyclines (Tetracycline & 4-Epitetracycline)	Tulathromycin	Penicillin-G	Benzyl-penicilloic Acid
Unprocessed Manure	7730	75.63	3.93	5.46	3.37
Separated Liquid	7320	53.80	2.66	2.75	1.36
Separated Solid	410	9.67	0.80	ND	ND
BRU Effluent	375	11.00	0.77	ND	ND

ND: not detected in the sample

The storage of the manure in the influent pit and the SLS processing impacted the antibiotic mass flow and degradation, with reductions across all antibiotics ranging from 8 – 50%. This reduction in the mass of antibiotics may also be attributed to compounds settling in the bottom of the influent pit with the solids. Following SLS, the average amount of antibiotic mass not portioned to the liquid or solid fraction was 8%, 16%, 40%, 50% for tulathromycin, tetracyclines, benzylpenicilloic acid, and penicillin G, respectively. The high loss of Beta-lactams can be attributed to their highly unstable beta-ring, which can be easily hydrolyzed by both abiotic and biotic factors (Berendsen et al. 2015; Cha and Carlson 2018). These compounds are also known to possess strong hydrophilic physiochemical properties with a low Log K_{ow} value (1.83), which collectively supports the higher detection and mass flow within the separated liquid fraction and no detection within the separated solid

fraction. With 95% of the mass fractionating with the liquid separated portion, this data suggests that large quantities of the antibiotics proceed untreated and could accumulate within the storage lagoon in the current on-farm manure processing.

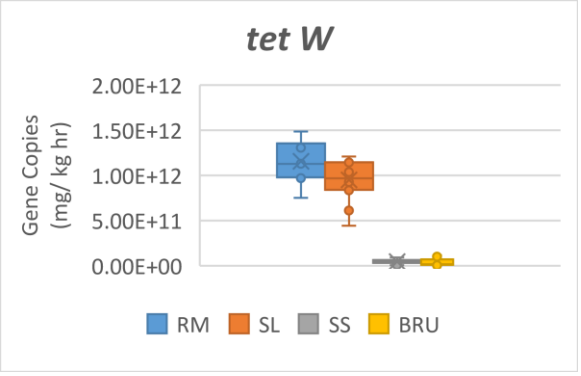
Of the 5% of the total manure mass that is contained in the separated solids, only 13% of the tetracycline mass and 20% of the tulathromycin mass proceeded to thermophilic processing with the solid fraction. Tetracyclines and macrolide chemical structure allows for adsorption to solids material via cation exchange, surface complexation, and cation bridging that can impact the fate and mobility of the compounds (Gothwal & Shashidhar 2015). Surface water and sediment studies have reported detectable concentration of tetracyclines, floroquinolones, and macrolides in water and sediment samples (Zhao et al. 2016). While there was a slight increase in total average mass flow rate for tetracyclines (9.68 to 11.00 mg/h) following BRU treatment, tulathromycin decreased from 0.80 to 0.77 mg/h, but these changes were not significant (p-value = 0.961) indicating that the rotary drum process had no significant effect on antibiotic degradation. Oliver et al. (2020) reported a similar increase in mass (20%) with sulfadimethoxine. Documenting the mass in the SS at 1409 mg then increasing to 1809 mg following BRU treatment, though their increase was determined to be significant. This slight increase in antibiotic concentration following BRU processing was likely the result of changes in organic matter concentrations, potential antibiotic releases, and increased extraction efficiency which directly impacts the mass flow analysis (Wallace and Aga, 2018). Antibiotic half-life can span a few hours to many days depending on the antibiotic class, physiochemical properties, and treatment conditions. Furthermore, composting studies can vary in

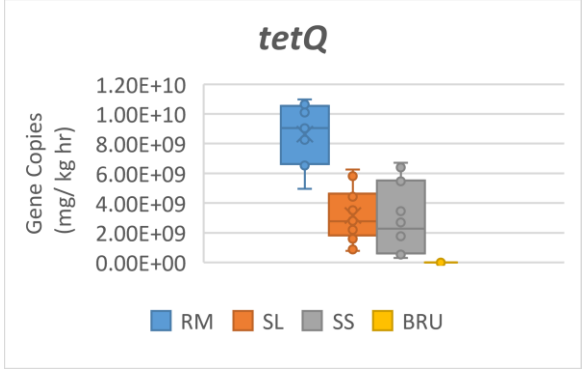
length and effectiveness in removing antibiotics. Arikan et al. (2007) conducted a lab-scale manure composting study, which reported 99% removal of extractable oxytetracycline after 35 days. Ho et al. (2013) reported >99% removal of nine antibiotics and one hormone in a 40-day broiler manure composting study. While Zhang et al. (2019) composting study reported > 89% removal for macrolides (ex.lincomycin, trimethoprim), <63% removal was documented for sulfonamides, tetracycline and fluoroquinolones included in the study over 171 days. The thermophilic processing that occurred in this study was withing reported ranges that have been documented to degrade antibiotics. While the processing time of <24 hours demonstrate that one day is not enough time to effectively degrade antibiotics under these conditions and reinforces the significance of the antibiotic stability and effect of half-life in relation to composting time.

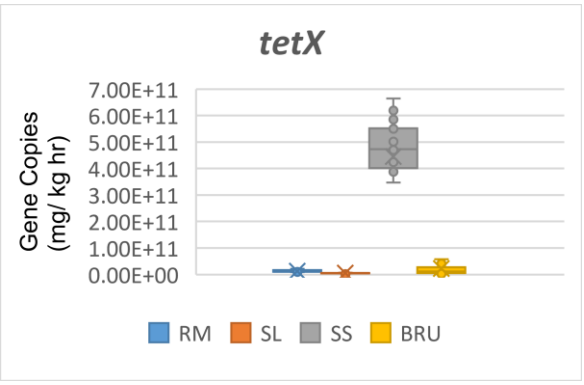
3.3.2 Fate of Antibiotic Resistance Genes (ARGs) and Gene Partitioning

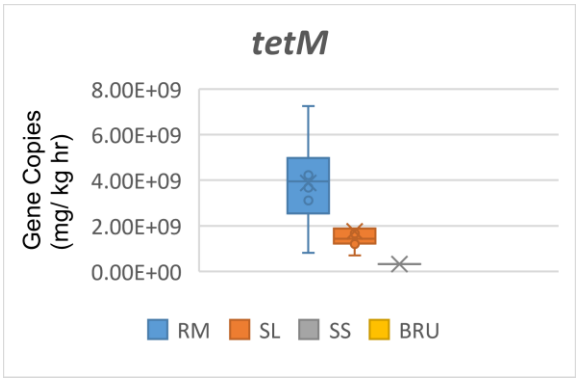
Seven of the eight ARGs were found in all the samples, except for *tetM* which was had minimal to no detection in the SS and BRU samples as shown in figure 3.1. *TetW* and *sul1* had the highest reported relative abundance (RA) of genes at 1.15 $x10^{12}$ and 1.72 x 10^{11} mg/ kg hr in the raw manure, followed by *tetX* and intl1 at 1.32$x10^{10}$ and 9.93$x10^{9}$ mg/ kg hr, and *tetQ*, *tetM, bla2* with lower abundances, *ermB* having the lowest relative abundance at 2.88$x10^{9}$ *g*/ manure. There was a significant reduction (*p*-value = 0.001) between *tetQ, tetW, tetM* and *bla2* when the raw manure and SL samples were compared. *TetX, intl1*, and *sul1* relative abundance were 4.45$x10^{11}$, 6.5$x10^{10}$ and 1.07$x10^{12}$ mg/ kg hr in the SS fraction, which were all significantly higher (*p*-value =0.001) than the raw manure and SL, which provides some indication

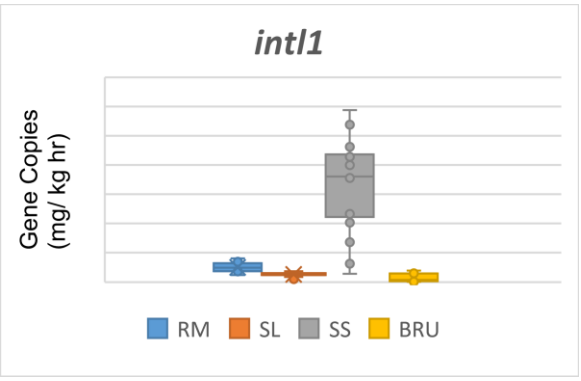
that the bacteria harboring these specific genes may have a higher affinity for binding to solid material. The *ermB* relative abundance remained consistent during BRU treatment ranging from $9.93x10^9$ – $3.54x10^9$ *g*/ manure, with both *ermB* and *tetW* remaining constant from pre- to post-BRU treatment. The *intl1*, *sul1*, *tetQ*, *tetX*, and *tetM* genes significantly decreased (95, 97, 99 and 95%, respectively; *p*-value < 0.0001), with each gene reduced by ~6-9 folds between the SS and final BRU product (figure 3.1).

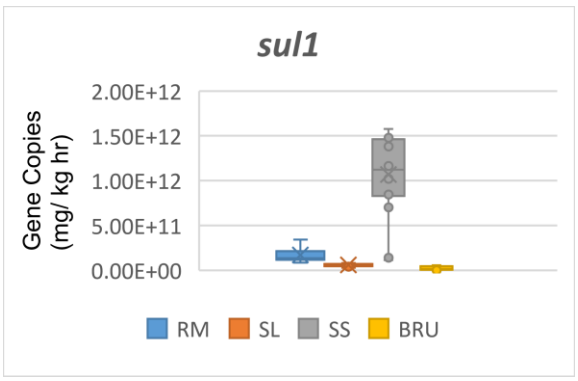


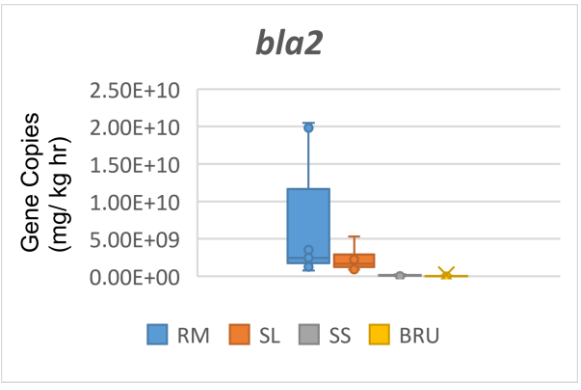












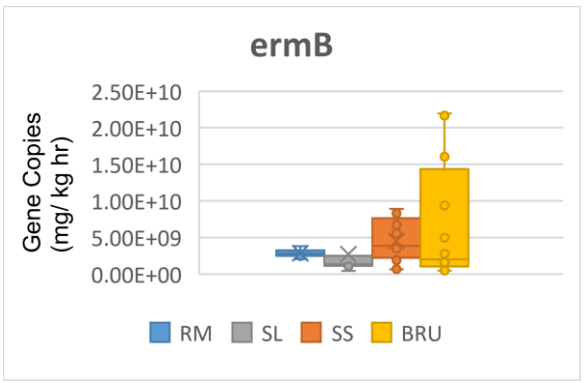


Figure 3.1 Average samples ARGs relative abundance normalized per gram manure based on mass flow (mg/kg hr) and standard deviation. Raw manure (RM), Separated liquid (SL), separated solids (SS) and BRU (blue, orange, grey and yellow respectively) each show distribution of samples and mean.

The fate of ARGs during manure composting is highly variable, as some ARGs can decrease while others are enriched dependent on the treatment, manure sources, and bacteria harboring the genes (Youngquist 2016; Zhang et al. 2018; Cheng et al. 2019; Cao et al. 2020). Cheng et al. (2019) completed a compost study on swine manure and found the tetracycline ribosome protection protein genes (*tetM*, *tetQ* and *tetW*), tetracycline efflux pump gene (*tetX*), and *sul1* increased in abundance during the compost piles transition for mesophilic stage to the thermophilic phase. The *tet* genes sustained abundance throughout the thermophilic phase, suggesting that these gene were present in thermophilic bacteria. This largely contrasts the results of this study, as most *tet* resistance genes decreased under thermophilic conditions within the BRU, except *tetW*. This highlights the high variability of ARG harboring bacteria, bacteria association with livestock, and the impact of composting.

Generally, thermophilic composting has been shown to eliminate the majority of bacteria found in animal manure and effectively reduce most ARG abundance (Bernal et al. 2009; Guo et al. 2018). As it is believed that most bacteria in animal manure are coliforms that are susceptible to higher temperatures or composting conditions (Leclercq et al. 2016). Qian et al. (2016b) compared thermophilic composting conditions and found normal and continuous conditions had greater reduction of 5/10 ARGs surveyed in cattle manure compared to incomplete thermophilic conditions. Under continuous thermophilic conditions, there were significantly greater reductions of *tetG, tetX, sul2, ermB,* and *aac(6')-ib-cr* compared to normal thermophilic conditions, with the genes composing 1.5-19.5% of the abundance in the normal thermophilic conditions at Day 40. In our study, sul1 was reduced by 97% from the

SS fraction through BRU processing, which were higher than *sul1* reductions (75%) reported by Oliver et al. (2020) using the same BRU system.

Sul1 is a prevalent gene found ubiquitously in the environment alongside the mobile genetic element *intl1*, with both genes often used as indicator genes (Subirats et al. 2018; Duan et al. 2019). *Intl1* is often used to track potential occurrence of horizontal gene transfer that allows transmission of resistance genes between bacteria. The relative abundance of *intl1* level between the raw manure and SL were similar at 9.93 x 10^9 and 5.25 x 10^9 mg/ kg hr, respectively. Following BRU, the abundance of *intl1* significantly decreased form 6.5 x 10^{10} to 3.54 x10^9 mg/ kg hr (p-value< 0.001). Previous studies have reported decreases in the *intl1* through composting (Duan et al. 2019; Ciu et al. 2020), while other studies reported increases (Ciu et al. 2020; Sardar et al. 2021). Thermophilic bacteria harboring this gene at this temperature may be less effective at mitigating dissemination risk, yet, in this study the carriers seemed to be vulnerable to thermophilic temperatures and gene reduction occurred during BRU processing. More metagenomic studies are needed to target and discover the key bacterial species needed for monitoring ARGs and mobile genetic elements.

3.3.3 Fecal Indicator Bacterial Analysis

ARB species that are most commonly associated with dairy manure include *Enterobacteriaceae, Campylobacter*, *Staphylococcus* and *Enterococcus*. Most studies also include *E.coli* as barometer of human risk through both water and manure systems (Oliver et al. 2020). Overall, *Enterococci* were shown to be more susceptible to both ampicillin and oxytetracycline concentrations, compared to *E. coli*, in this study, with lower bacterial counts across all substrates. Differences in gram-positive

and gram-negative cell wall structure may play a role; gram (+) bacteria solely possess a thick peptidoglycan layer, while gram (-) bacteria have a thin peptidoglycan layer in addition to outer lipid membrane. Initial raw manure bacterial counts for *E. coli* resistant to ampicillin and oxytetracycline were 4.08 x 10^5 and 3.15 x10^4 CFU/mL, respectively, compared to 1.47 x 10^4 and 8.75 x10^4 CFU/mL enterococci, respectively, as shown in Figure 3.2.

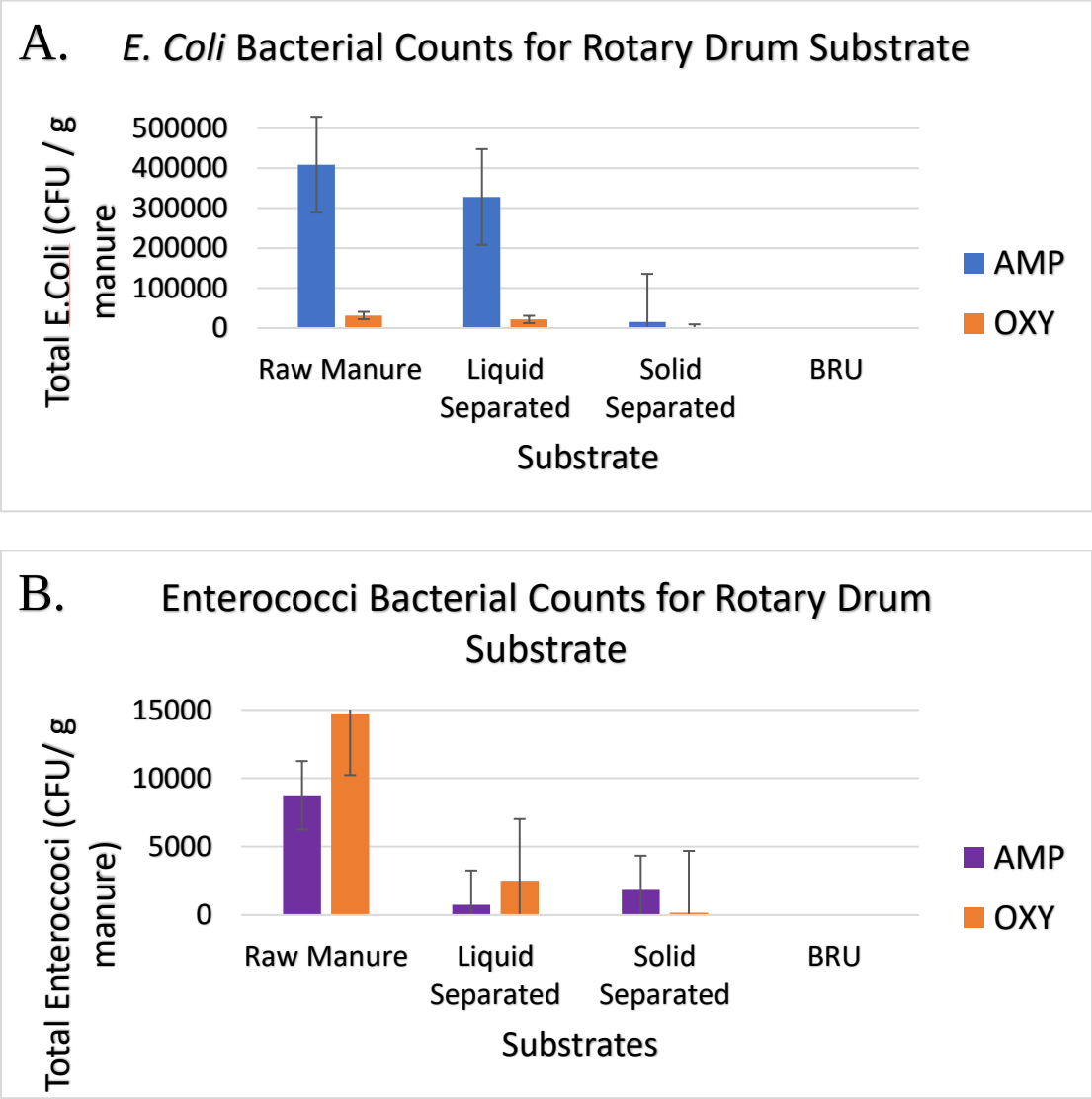


Figure 3.2: *E. coli* bacterial counts shown in (A), and *Enterococci* bacterial counts and standard error in raw manure, liquid separated, and solid separated samples grown on selective media shown in (B). Oxytetracycline plates are represented as "OXY" and ampicillin plates are represented as "AMP."

E. coli isolates associated with dairy manure have commonly been shown to exhibit tetracycline resistance (Oliver et al., 2020). Yet in this study, the bacterial counts on oxytetracycline plates were significantly lower than ampicillin plates (*p*-value=0.001). This trend may be due to oxytetracycline plates having a higher concentration (5 ug/mL) compared to ampicillin (0.5 ug/mL). Antibiotics, such as oxytetracycline, are usually found at higher concentrations in manure and other environmental samples, as they have greater structural stability compared to the ampicillin, which is one of the more easily degradable beta-lactams (Berendsen et al. 2015). In addition, higher rates of utilization in animal agriculture for oxytetracycline and other tetracyclines, their stability and longer half-life can lead to potentially longer exposure times and corresponding resistance (Li et al. 2017a).

After the raw manure was subjected to SLS processing, only ampicillin resistant *E. coli* were significantly reduced to 3.28×10^5 CFU/mL (*p*-value=0.007) in the SL fraction and 1.58×10^4 CFU/mL (*p*-value=0.001) in the SS fraction compared to raw manure at 4.08×10^5 CFU/mL. *E. coli* has been shown to attach to particulate matter that aids in the survival and transport within the environment (Soupir and Mostaghimi 2011; Liang et al. 2017). The propensity of *E. coli* to bind to particulate matter in combination with the more nutrient dense liquid fraction, which is more ideal for bacterial growth likely accounted for greater CFUs seen within the SL samples in contrast to the SS fraction in this study. Studies measuring *E. coli* from manure-amended fields have reported over 50% of the bacteria fractioning with the runoff compared to the soil concentration (Soupir and Mostaghimi 2011; Zwonitzer et al. 2016). No significant difference was found for either type of *Enterococci*

resistance for the raw manure, separated liquid, and separated solids at 1.47 x 10^4, 2.5x 10^3, and 1.66 x 10^2 CFUs (ampicillin) and 8.75 x10^4, 7.50 x10^2, and 1.83 x 10^3 CFUs (Oxytetracycline), respectively, as shown in Figure 3.2. Few studies have included analysis of gram (+) bacteria, such as *Campylobacter* and *Enterococci*, though tetracycline resistance has been documented (NARMS, 2018).

Thermophilic processing eliminated all FIB bacteria. No bacteria grew on any of the plates for samples taken from final BRU effluent during 13^{th}- 26^{th} hr. of sampling, including controls, indicating that BRU processing effectively eliminated viable bacteria (Figure 3.2). Studies with open air and thermophilic (55°C) composting have reported that erythromycin and tetracycline resistant bacteria decreased 4 to 7-fold, respectively, after 48 days (Wang et al. 2012; Wang et al. 2015). Sharma et al. (2009) monitored resistant *E. coli* in stockpiled windrowed cattle manure at >55°C and found variable reductions depending on stockpile location and achieved temperature, with the hottest compost pile temperatures (72°C) achieving the most efficient *E. coli* removal within 3 weeks. This study's removal of both *E. coli* and *Enterococci* within 13 – 24 hrs at 72°C is above the range of reported temperatures (55 -60°C) for the effective removal of pathogens. Additionally, conflicting studies suggest composting from 7 -14 days or just 2- 6 hrs. result in E. coli elimination (Turner 2002; Gong 2007, Sharma et al. 2009).

3.3.4 Nutrient and Heavy Metals Analysis

Along with nutrients, heavy metals aluminum, copper, iron, manganese, and zinc were detected within the manure. Copper (Cu), and zinc (Zn) has been shown to have positive correlation to antibiotic resistance (Han et al. 2002; Seiler and

Berendonk, 2012; Pal et al. 2017). Pearson correlation was used to determine potential relationships between the metals detected in the manure and ARGs. Metal concentrations were measured at 21.3 and 6.04 mg/L respectively for zinc and copper in the unprocessed manure and decreased after SLS to 17.5 and 4.9 mg/L within the liquid fraction, as shown in appendix Table A2. As seen with the other analytes, 77% of the metals mass partitioned with the liquid fraction, while only 7-8% of the metals partitioned with the solids. Zinc has been associated with both macrolide and aminoglycoside resistance. There was a significant increase in heavy metal concentrations in the separated solids fraction, with the BRU effluent having 34.8 mg/L for Zn and 10.06 mg/L for Cu. The increase in heavy metals can be attributed to water loss as result of the composting process, involving organic matter decomposition, water loss, and other processes (Chen et al., 2019; Dong et al., 2013). The high temperature BRU processing resulted in a slight decrease in Cu from 14.27 mg/L in the separated solids to 10.06 ppm in the final BRU product.

Only *tetW* and *tetQ* were found to have positive and significant correlation (*p*-value <0.05) to the copper (*p*-value =0.001, *p*-value =0.0134) and zinc (*p*-value =0.001, *p*-value =0.0168) concentrations (Table 3.3). The varied utilization of heavy metals in feed additives, organic or inorganic fertilizers, or pesticides in agriculture has resulted in increased metal contamination (Seiler et al. 2012; Pal et al. 2017). The environmental contamination associated with heavy metals can trigger cross resistance and co-resistance mechanisms against both metals and antibiotics (Seiler and Berendonk, 2012). *TetW* and tetQ both encode for ribosomal protection proteins (RPP) used to inhibit tetracycline disruptions of bacterial DNA synthesis. Liu et al.

(2019) reported that during vermicomposting of various animal manure Cu and Zn had a positive correlation to *tet* genes (*tetM, tetO, tetS*, and *tetW*), with R^2 between 0.46 – 0.87, with these R^2 values comparable to values found in this study for *tetQ* and *tetW* (0.61 – 0.92). Both Cu and Zn within antifouling paint have been found to increase marine bacterial abundance of metal and tetracycline resistance genes (Flach et al. 2017). Though *tetM* is also RPP gene, it showed no correlation to either metal, in addition to its non-detection in the SS and BRU samples demonstrates the diversity of bacteria harboring genes with similar mechanisms.

Table 3.3. Correlation analysis of ARGs and heavy metals mass flow based on concentrations found within the manure.

		16S	***ermb***	***intl1***	***sul1***	***tetQ***	***tetW***	***tetX***	***tetM***	***bla2***
Zinc	R^2	-0.4302	-0.4887	-0.3479	-0.3552	0.6054	0.8966	-0.4255	0.4004	0.3249
	p value	0.1095	0.0645	0.2039	0.1938	**0.0168***	**0.0001***	0.1138	0.5042	0.2373
Copper	R^2	-0.4081	-0.4854	-0.332	-0.3314	0.6216	0.9271	-0.4033	0.4252	0.3646
	p value	0.1311	0.0667	0.2266	0.2276	**0.0134***	**0.0001***	0.136	0.4755	0.1815

***Bolded values indicate statical significance (p-value <0.05)**

While other studies (Ji et al. 2012; Qui et al. 2021) have found Cu and Zn to have significant correlation to *sul1* and *intl1* and potentially triggering bacterial SOS responses and gene transfer, this analysis reported no significant interactions. It is essential to state that heavy metals have been documented to have correlation and not causation of AR. Additionally, the impact of environmental conditions which can affect metals selectively (toxicity) by determining their bioavailability has not been extensively studied regarding composting. The SLS fraction contained the highest concentration of Zn and Cu and being processed through thermophilic BRU composting could impact the heavy metals ionization state within the manure, thus,

affecting their ability to influence resistance, though these parameters where not examined in this study.

Initial nitrogen and phosphorus concentrations in the raw manure were 3524 mg N/L and 1483 mg P/L, respectfully, as seen the appendix Table A2. The majority of nutrients, specifically 73% of the nitrogen (2563 mg N/L) and 81% of the phosphorus (1198 mg P/L) were contained in the liquid fraction following SLS. The SS fractions nutrient concentration for phosphorus (35 mg P/L) was significantly lower than both the raw manure and SL fractions (p-value =0.001, p-value =0.004) reporting 98 and 97% decrease in the concentration. Partitioning of nitrogen and phosphorus in the aqueous fraction can be attributed to each nutrient being predominately in their dissolved forms, as ammonium (NH_4) and orthophosphate (PO_4) in manure slurry (Christensen et al. 2009; Hjorth et al. 2011). BRU processing had minimal impact on either nutrient in the separated solids, with concentrations of 110 N g/kg and 30 P g/kg in the final processed bedding material. The untreated liquid fraction is more nutrient dense and could be a more effective fertilizer than the SS and BRU, yet the final BRU can be applied in the stalls for bedding or used as fertilizer in the field.

3.4. Conclusions

The uniqueness of the BRU system allowed for the examination of antibiotic resistance partitioning across aqueous and solid substrates. Only the tetracyclines and macrolides were reported to continue with the SS fraction, likely due to their cation binding capabilities. Though studies have reported thermophilic composting to effectively reduce this antibiotic concentration. The rapid turnover (<24 hrs) of the

thermophilic rotary drum in this system did not generate significant degradation of the antibiotics compared to conventional composting. This BRU system was able to effectively eliminate the two fecal indicator pathogens surveyed in this study. However varied success in ARG reduction demonstrate the complexity and diversity of bacteria harboring ARGs. *Intl1, sul1, tetQ, tetX* and *tetM* were found to decrease after thermophilic composting, while *tetW, ermB* and *bla2* remained at constant concentrations pre- and post-treatment. Most importantly, the study demonstrated that most of the mass and antibiotic resistance factors portioned with the LS fraction. The combination of resistance bacteria, antibiotics, and numerous resistance genes alongside metals persistence are cause for the biggest concerns regarding resistance development. The lack of additional treatment of the liquid fraction that is likely applied to the field generates the greatest exposure risk. Furthermore, this study was able to execute and document a sample protocol that follows manure through this continuous system while demonstrating the variance of the fate of antibiotic resistance variables based on their distribution pattern.

Chapter 4: A Time Series Analysis of Antibiotic Resistance Response within Dairy Manure under Mesophilic and Thermophilic Anaerobic Digestion

4.1 Introduction

Antibiotics have been extensively used to treat human and animal illness since their initial discovery in the 1960s. Increase in livestock production over the past few decades have led to higher demand for innovations livestock health and more precise incorporation of antibiotics as treatment and prevention of infections (Widyasari-Mehta et al. 2016; Feng et al. 2017; Gurmessa et al. 2020). Though these compounds have proven to be effective for human and livestock well-being, 30-90% of these compounds can be excreted out the body through manure and urine unmetabolized, depending on the antibiotic class and animal metabolism. As much as 70% of the antibiotic oxytetracycline was documented to be unmetabolized in urine and feces (Lui et al. 2018). Those compounds that are metabolized to intermediates can be transported as biologically active metabolites that accumulate in the waste products, such as manure. Additionally, the selective pressures generated by antibiotic administration can led to the accumulation of antibiotic resistance genes (ARG) and antibiotic resistant bacteria (ARB). Manure application as agricultural field fertilizer increases both potential human and ecological risk, as manure is transported, resulting in exposure of these antibiotic resistance components within the environment (Masse et al. 2014; Spielmeyer et al. 2018; Xie et al. 2018). Past studies have provided evidence around the emerging antibiotic resistance contaminants within manure and

the connection to increases in bacterial pathogen resistance (Heuer et al. 2011: Pruden et al. 2013; Yang et al. 2014).

Prior to manure application and amendments in the environment, various manure handling practices and technologies are incorporated to help manage nutrients, odor, and storage. Anaerobic digestion (AD) is a form of manure management that has been incorporated globally from the individual farm scale to community scale managing manure from a few animals to large commercial management of the waste from thousands of animal livestock in one location. Anaerobic digestion technology uses anaerobic microbial metabolism to break down organic matter and produce biogas that can be used as renewable energy. The technology is often seen as win-win for its energy production and reduction of environmental risk. One specific aspect of environmental risk remediation is that studies have shown AD to be effective in the mitigation of antibiotic resistance. The conditions in the AD environment have shown to have effective antibiotic reduction ranging from 0 to 100%, depending on the class and types of antibiotics being surveyed and effectively decreased the relative abundance of ARGs and ARBs. Feng et al. (2017) showed 100% degradation by day 90 of sulfonamides and trimpephoprim, with 99% reduction of erythromycin during thermophilic AD (52°C) of swine manure. Complete ampicillin, florfenicol, and tylosin compound removal was recorded by Mitchell et al. (2013) in an AD study at 37°C. Zhang et al. (2018b) found AD to reduce *ermF, qnrA tetW* and four mobile genetic elements by 36.7 - 96.5% during swine manure co-digestion. While Wallace et al. (2018) found

advanced AD to significantly decrease *sul1* and *sulII* ARGs but largely did not impact the tetracycline resistance genes *tetO* and *tetW*.

One key operational parameter that numerous studies have credited for AD effectiveness is temperature. Typically, digesters are operated at mesophilic temperatures (30 - 38°C), but some systems are operated at higher thermophilic temperature ranges (50 - 65°C). Both mesophilic (30 - 38°C) and thermophilic (50 - 65°C) AD conditions have shown to have effective antibiotic resistance mitigation, but there are conflicting results for which temperature condition is the most impactful. Higher temperatures are believed to generate higher rates of biological activity, exhibit higher selective pressure against antibiotic resistant bacteria and subsequential resistance genes. Numerous studies comparing mesophilic to thermophilic conditions have found higher temperatures to generate greater reductions of the ARGs surveyed in those respective studies (Gosh et al. 2009; Diehl and LaPara et al. 2010; Ma et al. 2011; Sun et al. 2016). Sun et al. (2016) observed that thermophilic conditions reduced 8 of the 10 ARGs tested in contrast to mesophilic conditions only reducing 5 of the 10 ARGs in dairy manure digestion. While Wallace et al. (2018) found advanced AD to significantly decrease *sul1* and *sul2* ARGs but had little impact on the tetracycline resistance genes *tetO* and *tetW*. Sun et al. (2019) conducted a more recent study on solid state AD (SAD) compared to liquid state AD across mesophilic and thermophilic temperatures and reported thermophilic SAD to have higher levels of *tetG, tetW, sul1, ermQ, qnrA* and *aac(6)* reductions following the 60 days of digestion. Contrary to the previous studies, Zou et al. (2020b) found higher concentration of *tetO* genes under thermophilic conditions

and lower mesophilic temperature (< 30°C). A metagenomic study by Zhang et al. (2015) reported thermophilic AD conditions actually enriched three (*sul1*, *macB* and *aadA*) of the 13 ARGs examined and concluded that AD did not have any effect on the total overall abundance of ARGs.

As stated previously, antibiotic removal has had mixed results as well. With Arikan et al. (2008) documenting only a 75% reduction in chlortetracycline in a 33-day, 35°C AD experiment on medicated calf manure compared to the 89% reductions (Varel et al. 2012) and 89-91% reductions (Alverez et al. 2010) at similar mesophilic conditions digesting swine manure. It should also be noted that the same studies reporting AD effectiveness on some antibiotics (Mohring et al. 2009; Mitchell et al. 2013; Feng et al. 2017,) also independently documented AD has minimal to no effect on select sulfonamides (sulfathiazole, sulfamethazine, and sulfamethoxypyridazine) and macrolide (clarithromycin) and 3,5 dichlorophenol. Previous literature generally supports thermophilic conditions as more effective than mesophilic. Yet, these removal efficiency rates can be variable and are not only dependent on temperature but also retention time of incubation at these temperatures (Watkinson et al. 2007; Youngquist et al. 2016).

Though AD has been shown by the previously referenced studies to be effective treatment technology, it does not completely eliminate antibiotics, ARGs and ARBs. In some cases, the relative/absolute abundance of genes or antibiotic concentrations can increase as result of biomass transformation. Thus, AD systems and their corresponding effluent retain AMR dissemination potential. Few researchers have conducted studies to examine all three components of antibiotic resistance

(antibiotics, ARGs, ARBs) in a synchronized time series study to observe potential correlations between antibiotic degradation/transformation, bacterial succession, and ARG prevalence. Further insight into the intricacies of microbial interaction and environmental conditions are essential in determining how antibiotic resistance is spread and how bacteria respond to AD conditions. This study is set to further examine and compare the impact of high temperature and mesophilic digestion of antibiotic removal and reductions in ARGs and ARBs. The two main objectives are to: 1) Quantify the effects of temperature (mesophilic versus thermophilic AD) on antibiotic resistance factors, and 2) Determine the effect of temperature on the timeline of antibiotic resistance degradation through a temporal correlation analysis. Conducted as a batch digestion experiment, this study generated a time series analysis through a 43-day digestion of dairy manure, resulting in an antibiotic degradation curve on select antibiotics. The results examine temporal dynamics of both mesophilic and thermophilic AD conditions regarding antibiotic concentration in relation to ARBs and ARG interactions.

4.2. Material and Methods

4.2.1 Experimental Design:

A batch digestion experiment was conducted based on biochemical methane potential (BMP) experiments which were used to determine the energy production potential developed by Owen et al. (1979) in the University of Maryland Bioenergy and Bioprocessing Laboratory (College Park, Maryland, USA). In this batch study, mesophilic (35°C) and thermophilic (55°C) anaerobic digester conditions were examined to determine difference in antibiotic degradation and reductions in ARGs

and ARBs during dairy manure digestion. Dairy manure collected from a farm in upstate New York, US was spiked with a 10 mg/L antibiotic solution composed of oxytetracycline (OXY), ampicillin (AMP), and erythromycin (ERY) and evaluated at both temperatures. The batch assay was conducted over a 43-day retention period to develop time and temperature antibiotic degradation curves, with post-testing of antibiotic concentration. The experimental setup consisted of triplicate bottles that were destructively sampled at six time points (Day 0, 3, 9, 21, 36, and 43) to determine the effect of time on degradation, with six inoculum control bottles (triplicates controls at each temperature), and triplicates of non-spiked manure, resulting in 48 samples total.

Mesophilic and thermophilic inoculums were obtained from on-site laboratory-scale inoculum reactors. Mesophilic inoculum was generated using USDA Beltsville Agricultural Research Center (BARC) dairy manure (Beltsville, Maryland, USA). The thermophilic inoculum seed culture was obtained from a Virginia Tech University facility (Asburn, Virginia, USA). The batch destructive assay was conducted in 300 ml serum bottles filled with 10 mL of manure and 130 mL of inoculum and 10 mL of the 10 mg/L antibiotic solution for a total of 150 mL. The mass of substrate to inoculum ratio was 2:1 in each bottle based on volatile solids (VS). Prior to incubation, the headspace in each bottle was purged with 30% CO_2 and 70% N_2 to establish anaerobic conditions and sealed with a rubber septum. Each set of assay bottles (n=24 per temperature condition) was subjected to either 35°C or 55°C conditions in respective environmental chambers for 43 days. All assays, including the inoculum control and non-spiked manure, were performed in triplicate.

Biogas production and methane (CH_4) content of the produced biogas was measured daily during the first week of the experiment, approximately every other day the following week, and then bi-weekly for the remainder of the experiment. The frequency of the biogas measurements was based on the quantity of biogas produced. When biogas production is high, the biogas was purged (and measured) more frequently to prevent pressure build-up inside the serum bottles. Biogas production was measured via volume displacement using a 50-mL wetted glass gas tight graduated syringe with 2 mL gradations. The produced biogas was analyzed for CH_4 and carbon dioxide (CO_2) content by injecting 0.10 mL sample, using a luer-lock, gas tight syringe, into an Agilent HP 7890A GC (Agilent Technologies, Santa Clara, CA, USA), equipped with a thermal conductivity detector (TCD) using the following parameters: (1) injection temperature of 250 °C, (2) detector temperature of 250 °C, (3) oven temperature of 60 °C, and (3) a carrier gas flow rate of 8.6 mL He/min. The average CH_4 production in the control (inoculum only) was subtracted from the other treatments to account for CH_4 production attributed to the inoculum source, thus, the results presented are the total CH_4 production from the diary manure only.

4.2.2 Antibiotic Extraction and Quantification:

To measure antibiotic concentration and monitor antibiotic degradation during digestion, extraction and analysis procedures outlined in Poindexter et al. (2022) were conducted. The extraction samples for antibiotic analysis were normalized by weight (grams) based on total solids (TS) concentrations. Samples taken from each select day during digestion were analyzed in triplicates. In brief, the extraction method was a

two-step method that utilized ultrasonic and mechanical mixing using two separate solvent extractions: 0.1 M EDTA-McIlvaine followed by methanol. The two extracts were combined and diluted to a solvent concentration less than 2%. The diluted extract was then concentrated using solid phase extraction through a C-18 cartridge. The cartridge was eluted with methanol, and the eluent was concentrated under a steady stream of N_2 gas. The final concentrate was reconstituted to a known volume using a 50% acetonitrile and 50% deionized water (DI) mix and stored in the freezer at -20°C until liquid chromatography and tandem mass spectrometry (LC-MS-MS) analysis.

4.2.3 DNA Isolation and qPCR Resistance Gene Quantitation

Genomic DNA from the four manure substrates was isolated using ~ 0.25 mg of the digestate samples using QIAamp DNA stool Kit (cat 51504, QIAGEN, Hilden, Germany) following the manufacturer's protocols. DNA extraction was performed in triplicate for each sample to account for extraction variable, efficiency, and heterogeneity of the samples. The quantity of the extracted DNA was determined and normalized based on Qubit 1.0 Fluorometer (Life Technologies, Grand Island, New York, USA) for DNA concentration and NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) for DNA sample quality analysis. DNA extract was diluted to 1 ng/uL in preparation for qPCR. All extracted DNA was stored at -20°C before qPCR analysis.

This study quantified the abundance of seven resistance genes, including a macrolide (*ermB*), beta-lactam (*bla-2*), sulfonamides (*sul-1*), tetracycline *(tetX*, *tetM*, *tetW*, *tetQ*), mobile genetic element (*intl1*), and 16S DNA for bacterial population

normalization. Primer sequences of targeted genes are listed appendix Table A1 were cloned into the pGEM-T easy vector, purified using QIAprep Miniprep kit (cat# 27104, Qiagen, Hilden, Germany), and 16S illumina sequencing for gene verification. Verified plasmids were selected as standards for qPCR, quantified and adjusted to 1 ng/uL. Serial dilutions of the plasmids were conducted to generate an internal standard curve. Data extracted from standard curves had r^2 values > 0.99. All the qPCR analytics were performed using the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, California, USA) following amplification of all genes: heat inactivation at 95°C for 2 min, followed 40 cycles of 95°C for 5 sec (denaturation), 65°C for 10 sec (annealing), and 72°C for 10 sec (elongation). The 20 uL qPCR reactions included 10 uL Forget-Me-Not qPCR Master Mix Hi-ROX (cat#31046, Biotium, Fremont, Californa, USA), 1 uL of forward primer, 1 uL of reverse primer, 5 uL of sample DNA, and 3 uL of dH_2O.

4.2.4 Detection and Quantifiaction of Fecal Indicator Bacteria

For bacterial counts, two fecal indicator species *Enterococcus* and *Escherichia coli*, were used to quantify antibiotic resistant bacteria. The BMP bottles were pulled at designated time points (Day 0, 3, 9, 21, and 43) and each bottle sample was separately homogenized, and 2 g was placed into new 15 ml falcon tubes. Followed by the addition of 10 mL of phosphate buffer solution (Thermofisher Scientific, Waltham, MA USA), the tubes were manually mixed via inversion. The sample was then 10-fold serial diluted up to 10^{-3} in phosphate buffer solution (PBS). Aliquots of 0.1 mL of each dilution were plated and manually spread on two different selective culture mediums. Gram positive *Enterococcus spp.* was evaluated on Slantez-Bartley

media (Fisher Scientific, Hampton, NH USA) and the gram-negative *E. coli spp.* were evaluated on *E. coli* Chromagar (Fisher Scientific, Hampton, NH USA). Three types of plates were made for each fecal indicator bacteria, with each group of plating spiked with one antibiotic addition of the initial antibiotics (oxytetracycline, ampicillin or erythromycin) spiked at 3 mg/ L. The *E. coli* plates were incubated at 37°C for 24 hrs, while *Enterococcus* plates were incubated for a total 48 hrs (24 hrs at 37°C and 24 hrs for 44°C). After incubation and lactose fermentation on selective media, distinguishing *E. coli* colonies (blue), *Enterococcus* colonies (red), and other bacteria (white) on each respective media were used to count specific bacterial colonies. Isolates were picked from various samples, struck for isolation, and grown overnight and stored and frozen in a 50% glycerol solution at -80°C for later characterization. The highest dilution (10^{-1}) used that produced microbial counts between 1 -200 colonies was used to estimate the number of resistant bacteria in the sample.

4.2.5 Digestate Physicochemical Characterization:

Triplicate samples taken at each time point were analyzed for pH, total solids (TS), VS, and chemical oxygen demand (COD) within 24 hrs of collection, followed by volatile fatty acids (VFA) and nutrient analysis according to standard methods (APHA, 2005). Analyses were measured in each bottle before incubation and after bottles were pulled for degradation analysis The sample pH was measured using an Accumet Basic As 15 pH Meter. The TS and VS analyses were conducted using the Standard Methods (APHA, 2005). For TS analysis, 10 mL of aqueous sample (raw manure and separated liquid manure) were pipetted and 25 g of solid samples

(separated solid manure and BRU effluent) were weighed into pre-weighed and pre-dried (at 550 °C) porcelain crucibles. For TS analyses, the samples were dried at 105 °C until a constant weight was maintained. For VS analyses, the crucibles were placed in a furnace oven at 550 °C until a constant weight was obtained. Nutrient analysis for ammonia, total Kjeldahl nitrogen (TKN) and total phosphorus (TP) samples were analyzed on the Lachat autoanalyzer (Quikchem 8500, Hach Company, Loveland, CO, USA) using the QuickChem method 13-107-06-2-D for TKN and method 13-115-01-1-B for TP. The COD concentration was measured using a Hach DR 5000 spectrophotometer (Hach Company, Loveland, Colorado, USA). The samples for VFA analyses (butyrate, propionate, acetate, and valeric) were acidified with concentrated sulfuric acid to a pH below 2 and filtered to 0.22 µm before injection into a HP 7890A GC (Agilent Technologies, Santa Clara, California, USA), equipped with a flame ionization detector (FID) with the following parameters: 1) injection temperature of 250 ºC, 2) detector temperature of 300 ºC, 3) oven temperature of 100 ºC for 2 minutes and increased by 10 ºC/min for a total run time of 10 min, and 4) a carrier gas flow rate of 1.80 mL He/min. For quality control, 10% of the samples included duplicates, spikes, and blanks.

4.2.6 Statistical Analysis:

Statistical significance between results of thermophilic and mesophilic digestion was evaluated using a two-way analysis of variance (ANOVA) on all pre- and post-digestion reactors to determine antibiotic concentrations, ARG abundance, ARB counts, cumulative CH_4, biogas production, TS, VS, COD, VFA, and nutrients to determine statistical difference between reactors. Differences based on sample time

and temperature were considered significant for *p*-values < 0.05. Spearman correlation analysis was used to measure association of ARGs and digestate characteristics under mesophilic and thermophilic conditions.

4.3. Results and Discussion

4.3.1 Methane Production

Cumulative biogas and CH_4 production were used as a barometer for AD processing and performance in the absence and presence of the antibiotics. The thermophilic CH_4 production over the 43-day digestion was 160 CH_4 g/VS, which was significantly lower (p-value = 0.003) than mesophilic production at 235 CH_4 g/VS (Figure 4.1). Higher digester temperatures have generally been reported to increase CH_4 yields due to more extensive hydrolysis of the initial feedstock and higher levels of microbial processing (Varel et al. 2012; Yu et al. 2014) Though Varel et al. (2012) reported lower CH_4 production at 55°C compared to 38°C with swine manure containing chlortetracycline and monensin. A combination of antibiotic inhibition and temperature selectivity could contribute to lower bacterial diversity and metabolism, thus, impacting CH_4 synthesis.

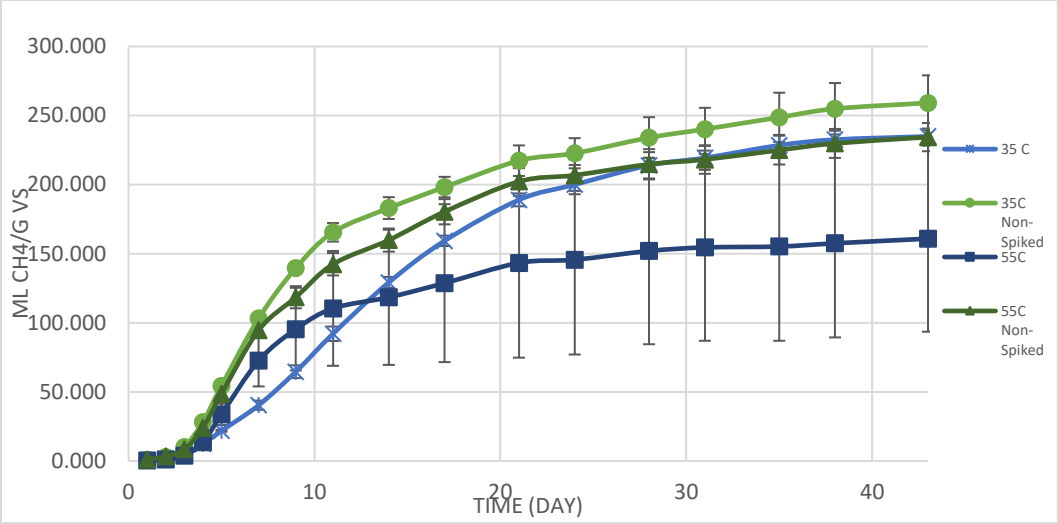


Figure 4.1. Average, cumulative and standard error of CH_4 production during the 43-day anaerobic digestion of dairy manure spiked and non-spiked with antibiotic solution at 35°C and 55°C.

Numerous studies have examined the impact of oxytetracycline on AD with these studies being primarily conducted under mesophilic conditions (28-42C) (Arikan et al. 2006; Ince et al. 2013; Yin et al. 2016; Andriamanohiarisoamanana et al., 2020; Chen et al. 2020). Studies have reported oxytetracycline to inhibit CH_4 production of digesters by 12 – 60%. A kinetic study of antibiotics (oxytetracycline and chlortetracycline) inhibition on diary manure digestion by Andriamanohiarisoamanana et al. (2020) found CH_4 yields to be between 10 – 26% lower than control without antibiotics, although cumulative CH_4 production was deemed not significantly different (p-value <0.05). While Yin et al. (2016) reported OXY concentration below 40 mg/kg TS actually increased CH_4 production, while concentration over this threshold value was inhibitory. Though the antibiotic concentration in this study is lower than reported inhibitory concentrations the combination of the various antibiotics (ampicillin and erythromycin) may have more influence. The presence of the antibiotic can have inhibitory effects on key microorganisms during digestion that can impact processing and overall CH_4 yields.

Lower concentrations of the antibiotics can lead to quicker removal, less inhibitory pressures and potentially add to metabolic hydrolysis of organic carbon sources.

In this study, both spiked conditions had lower total CH_4 yields (256 and 160 CH_4 g/VS) compared to the non-spiked controls (259 and 234 CH_4 g/VS) for mesophilic and thermophilic respectively, indicating the antibiotic solution did have an inhibitory effect (figure 5.1). Only the mesophilic control when compared to it spike conditions was deemed to be significantly different (p-value= 0.0013). Various studies have reported OXY to have inhibitory effects on biogas and CH_4 production, with a suggestive inhibitory concentration of 10 mg/L (Shi et al. 2011; Scheluer et al. 2021). Erthromycin on the other hand, showed increased CH_4 yields at higher concentrations in some cases. Wang et al. (2022) reported 13% higher CH_4 yields from fermentation dredges spiked with 500 mg/L ERY compared to the lower concentrations spiked at 100, 200 and 300 mg/L, which reported no inhibitory effects. Wang et al. (2022) credited this occurrence to greater relative abundance of acetogenic bacteria (*sedimentibacter*) and mixotrophic archaea (*methanosarcina*) and their potential syntrophic interactions. AMP is a broad-spectrum antibiotic that can be used against gram positive and gram-negative bacteria. AMP inhibitory impact on digesters is reported to primarily impact initial biogas production due to the antibiotic instantaneous hydrolyzation by both abiotic and biotic factors. Mitchell et al. (2013) independently spiked four antibiotics, including ampicillin, into cattle manure to examine their impact on digestion. Ampicillin was reported to have some inhibitory effects on biogas production within the first 30 days but was not significant compared to the control.

4.3.2 Fate of Antibiotics throughout digestion

The removal rates of the three examined antibiotics are displayed in appendix Table B1. The degradation curve for the spiked antibiotic concentrations throughout the 43-day digestion can be seen in figure 4.2. Previous AD studies have designated temperature to be a key parameter that can influence antibiotic degradation. In this study, overall degradation of the antibiotics was shown to be class and temperature dependent. Ampicillin was almost completely removed under both mesophilic and thermophilic conditions, while erythromycin and oxytetracycline appeared to be more temperature dependent. Both conditions were able to achieve > 30% removal across all digestion treatments.

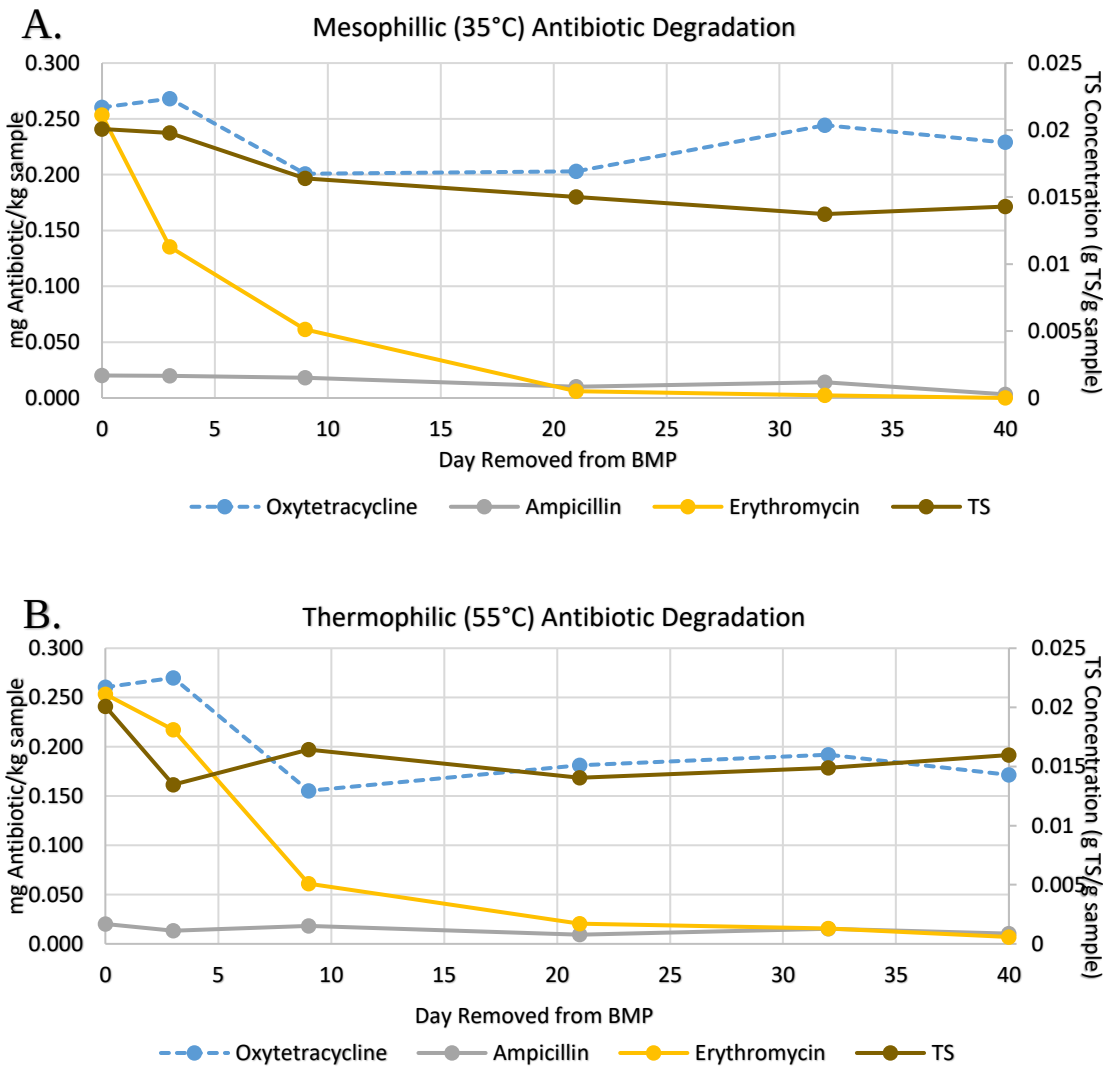


Figure 4.2. Quantified concentration of spiked antibiotics (oxytetracycline, ampicillin and erythromycin) throughout the 43-day digestion. Shown in A is the mesophilic conditions (35°C) and shown in B is thermophilic conditions (55°C), with changes in the total solids (TS) displayed for each condition.

The initial ampicillin concentration 0.020 mg/kg was significantly lower (*p*-value= 0.0004) than the other two antibiotics despite all three compounds being spiked into the manure at the same concentration. Ampicillin is a beta lactam which has been documented to be easily hydrolyzed under acidic or mild alkaline conditions (Speilmeyer et al. 2018), resulting in minimal reporting of this class of antibiotics at detectable levels in manure or digestate (Speilmeyer et al. 2018). Ampicillin

degradation in this study was influenced by temperature, with 83% reduction at mesophilic conditions (0.003 mg/kg, *p*-value<0.0001) and only 48% reduction at thermophilic conditions (0.010 mg/kg, *p*-value = 0.0003) by the completion of the digestion. Showcasing that the mesophilic condition to have greater reduction compared to thermophilic (p-value= 0.0131). These results agree with Mitchell et al. (2013) who tested various concentrations of ampicillin (0.35 - 350 mg/L) and reported complete removal of the compound <10 days at 37°C.

Following the 43-day digestion, the macrolide erythromycin had 100 and 97 % removal under the mesophilic (*p*-value <0.0001) and thermophilic (p-value <0.0001) conditions, respectively (figure 4.2). The degradation patterns at the two temperatures nearly mirrored each other throughout this digestion study with no significant differences over the six sample periods, except Day 3 when a 46% reduction was reported under 35°C compared to only 14% reduction at 55°C. Differences in the Day 3 samples could be attributed to temperature or the abundance and efficiency of microorganisms actively metabolizing the compound.

Previous studies have observed *Firmicutes* to be a dominant bacterial group under thermophilic conditions, while *Proteobacteria* and *Bacteroidetes* have been found to be the dominant bacterial groups under mesophilic temperatures (Ma et al. 2011; Zhang et al. 2015). The mesophilic temperature in this study were slightly more effective and efficient condition at removing erythromycin. By Day 43, erythromycin was undetectable in the 35°C bottles in contrast to only 97% removal observed in the 55°C bottles. Wang et al. (2022) and Zhang et al. (2022) documented 80% removal of erythromycin in fermentation dredge after 30 days of AD and 90% removal under

31 days of anaerobic fermentation both at 37°C, accordingly. Feng et al. (2017) reported similar erythromycin degradation results in a comparative AD study digesting pig manure under psychrophilic (15°C) and thermophilic (52°C) conditions, with 89.9% (0.007 mg/kg) reduction by Day 8 and 100 % reduction by Day 40. Though it should be noted that Feng et al. (2017) used pig manure in comparison to dairy manure used in the current study and did not include mesophilic parameters. Regardless, both the current study and Feng et al. (2017) study's degradation patterning resemble first order degradation curve reported by Schleur et al. (2006) when monitoring erythromycin concentration during storage of pig manure in tanks. The higher level of reductions of erythromycin under mesophilic conditions may also contribute to the higher CH_4 production seen in previous section. Reduction of erythromycin not only eliminate it ability in inhibit bacteria but can also provide a carbon source for metabolic processing.

Total oxytetracycline concentrations include both the primary oxytetracycline compound and metabolite 4-epi oxytetracycline due to limitations in LC-MS quantification methodology confidence (baseline separation between primary and metabolite products). Thermophilic conditions resulted in significantly higher degradation of the oxytetracycline rates of 34 - 53% (p-value <0.001) during the 43-day digestion compared to 8 - 30% reduction under mesophilic conditions (p-value = 0.0011). Both the 35°C and 55°C conditions increased oxytetracycline concentrations by 8% and 6% respectively, between Day 0 and Day 3 (figure 4.2). This increase is likely due to the conversion of oxytetracycline to 4-epioxy, as epimerization can be the result of substrate pH changes before or after sample collection and extraction

procedures (Speilymeyer et al. 2018). Oxytetracycline reduction has been reported in previous dairy manure AD studies, ranging from 27 - 78%, with most studies operating at mesophilic conditions (Arikan et al. 2006; Ke et al. 2016; Turker et al. 2018) The greatest removal of oxytetracycline under both conditions occurred on Days 9 and 21 at ~43% for mesophilic conditions and 78% and 55% reduction under thermophilic conditions, respectively. This timing of greatest reduction in this study parallel the Turker et al. (2018) study report of sharp decrease in oxytetracycline concentration between Day 10 - 20 of the 30-day digestion of dairy manure treated at 37°C. Oxytetracycline removal may be attributed to NH_2 and OH groups on the compound that are key sites for bacterial metabolism during digestion (Yin et al. 2016), alongside other environmental and operational parameters of the AD system (Akyol et al. 2016). Though the thermophilic conditions in this current study had the greatest impact on oxytetracycline degradation, both conditions left residual compounds at 0.087 and 0.202 mg/ kg in the final digestate.

4.3.3 Fate of antibiotic resistant genes (ARGs) during anaerobic digestion

This study quantified eight ARGs in mesophilic and thermophilic conditions, as shown in figure 4.3. The manure pre-digestion had highest relative abundance (RA) concentrations of *tetW* and *sul1* at 1.98×10^7 and 2.83×10^6 gene copies/g digestate. Out of the eight genes detected, the 35°C conditions reduced the RA of five different ARGs over the course of the 43-day digestion, while the 55°C conditions reduced six of the eight genes surveyed. The 35°C reduced the RA of *ermB* (57%) *int1* (57%), *sul1* (40%), *tetX* (28%), *tetM* (31%) and *bla2* (94%), although significant reduction was only determined for *ermB, intl1*, and *bla2* (*p*-values = 0.0005, 0.0005,

and <0.0001, respectively). The 55°C conditions similarly reduced *ermB* (54%), *intl1* (54%), *sul1* (71%), *tetX* (82%), and *TetM* (100%) RA at *p*-values of 0.0005, 0.0005, 0.0404, 0.0309, and <0.0001, respectively, while also reducing *tetQ* (62%), which was not reduced in 35°C conditions; but was not deemed significant (p-value = 0.5152).

Figure 4.3. Gene copy values and standard error for antibiotic resistance genes (ARG) detected in diary manure under 35°C and 55°C anaerobic digestion conditions of diary manure. Samples were quantified in triplicates and gene copies are based on grams of digestate analyzed, with blue bars for 35°C and orange bars for 55°C digestion temperatures.

Previous studies have been inconsistent when reporting results of AD efficacy on ARG reduction, with some studies reporting thermophilic temperatures have

greater levels of gene reduction (Diehl & LaPara 2010; Sun et al. 2016; Xu et la. 2018), while others report mesophilic to be better at removal (Ma et al. 2011; Zhang et al. 2015; Huang et al. 2019). Thermophilic conditions in this study proved to move effective at reducing *tet* genes (*tetX*, *tetQ* and *tetM*), with >40% reductions and complete removal of *tetM* (100%) compared to mesophilic conditions which only reported 28-32% decrease in *tetX* and *tetM*. Xu et al. (2018) reported >80% reduction of *tetA*, *tetO*, *tetX*, *sul1*, and *sul2* under thermophilic AD of sewage sludge. Zhang et al. (2021a) compared lower temperature, mesophilic (25°C and 35°C), and thermophilic (55°C) AD and reported significant reductions and lower abundance of all the seven ARGs (*tetA*, *tetO*, *tetW*, *tetC*, *sul1*, *sul2*, and *intl1*), with thermophilic conditions removing 2 logs more ARGs compared to both of the lower temperature.

Other previous studies, such as Huang et al. (2019) analyzed four different AD temperature (25, 35, 37, and 55°C) and documented significant reduction of ARGs for all treatments, yet the 55°C conditions had higher total abundance of genes compared to the other treatments. Interestingly in our study, at the end of the digestion (Day 43) there were no significant differences between the RA of *ermB*, *sul1*, *intl1* and *tetW*. All genes in our study, except *tetW*, were reduced under both conditions, with reduction falling within previously reported ranges (40%< (Younguist et al. 2016; Aziz et al. 2022). This can be seen in Figure 4.3, where the RA values for *ermB* and *intl1* on Day 43 are $1.36x10^5$ and $1.70x10^6$ g / digestate for 35°C and $1.45x10^5$ and $5.01x10^5$ g/ digestate for 55°C. For most of the ARGs, apart from bla2, significant changes in the RA were documented within the first 9 days before appearing to stabilize. This initial period of significant reduction may likely

coincide with shock to the bacterial community as the bacterial communities adjust to each respective conditions during the selective pressure of both temperatures.

Conversely, both conditions also were documented to increase two ARGs, with the RA of *tetW* (109%) and *bla2* (159%) increasing from $1.98x10^7$ and 1.5x105 to $4.15x10^7$ and $3.9x10^5$ gene copies/g digestate accordingly at 55°C conditions. Enrichment of some genes is a common occurrence for both conditions (Ma et al. 2011; Huang et al. 2019). *TetW* was also enriched under 35°C conditions alongside *tetQ* (81% and 259%), with final abundances reported at $3.59x10^7$ and $2.79x10^5$ gene copies/g digestate.

The high utilization of tetracyclines and their ability to persist in the environment likely influenced higher levels of resistance and greater diversity of *tet* ARG harboring bacteria (Wallace et al. 2018). *TetW* encodes a ribosome protection protein that has typically been shown to decrease in abundance under both treatment conditions and has been reported to be significantly reduced under thermophilic conditions (Diehl and LaPara 2010; Ma et al. 2011; Zhang et al. 2015; Xu et al. 2018; Zhang et al. 2021a; Zang et al. 2021b). Ma et al. (2011) reported significant reduction of *tetW* under mesophilic conditions. Yet, both conditions in this study displayed an increase in RA, which could suggest that a different group of bacteria, not susceptible to either mesophilic or thermophilic digestion temperatures, may be harboring this particular gene compared to previous studies.

B-lactams are another class of antibiotics commonly used to treat dairy cows and likely drive resistance in cow microbiome (Whichman et al. 2014), alongside other livestock animals and humans. Previous studies have primarily focused on

extended spectrum B-lactamase genes (*bla-$_{ctx}$*) due to their high rates of transmission and resistance to various subclass of medically relevant B-lactams antibiotics (Rossolini et al 2008). The *bla2* gene is this study encode a beta lactamase enzyme that can inactivate antibiotics such as penicillin and cephalosporins (Chen et al. 2003). This gene is not typically included in ARG analyses but was shown to have polar response to the two conditions. The 94% reduction under 35°C starkly contrast the significant 159% enrichment under 55°C conditions (p-value = 0.0244). These results indicate that the bacteria harboring this particular gene favors thermophilic conditions or the selective nature of higher temperatures limit the diversity of bacteria and other microorganisms, thus selectively allowing other more adapted species to persist and fill the communal void.

As seen with previous studies, both conditions were effective in reducing the RA of the ARGs surveyed, with thermophilic conditions having greater reductions of some genes, while other genes had larger reductions under 35°C conditions indicating the treatment effectiveness is more dependent on the gene and the bacteria harboring the genes than the temperature itself. It is also important to highlight significant reduction of *intl1* under both conditions, as this gene is a mobile genetic element that is used as a barometer to assess potential transmission of genes via horizontal gene transfer. Furthermore, AD at any temperature should not be expected to completely eliminate ARGs, as there is such a diverse range of ARGs present, though digestion can be used to reduce the abundance in most cases yet serve as vessel of enrichment for a few ARGs. Advancement in technologies that can screen and better determine which bacteria species and groups are harboring the ARGs of most

importance/relevance would allow for better assessment of the effectiveness of AD and other mitigation strategies.

4.3.4 Fecal indicator Bacterial (FIB) Growth during digestion

Surveillance of putative pathogens, such as fecal indicator bacterial species (*E.coli* and *Enterococci*), that encompass both gram positive cocci and gram negative rods can be utilized to assess sanitary risk and monitor potential resistance to human and animals. Very little growth was recorded for any of the antibiotic plates over the 43-day digestion regardless of temperature. Severely limited growth of the FIB can be the result of the manure being frozen prior to its application within the experiment compared to results seen with a same fresh manure used in previous BRU study. Only *E.coli* on the erythromycin plates which were set at 3 mg/L were the only plates recorded to have any growth on Day 0, with $1.55x10^5$ CFU/mL at 35°C shown in Figure 4.4. By Day 3, there was some rebound in the FIB across the antibiotic plates. *E. coli* on the erythromycin plate was significantly reduced compared to Day 0 $1.55x10^5$ CFU/ mL to $7.5x10^3$ CFU/ mL (p-value < 0.0001) seen in figure 4.4. Growth was reported on all other plate expect for *E. coli* oxytetracycline plates in 35°C or 55°C conditions.

Figure 4.4. Bacterial colony forming units (CFUs) and standard error of *E.coli* and *Enterococci* resistance to Erythromycin spiked media (3 mg/L). CFU's documented at 5 times points for both 35C and 55C AD conditions throughout the 43-day digestion. Similair trend were shown or no growth reported in ampicillin and oxytetracycline plates (not shown).

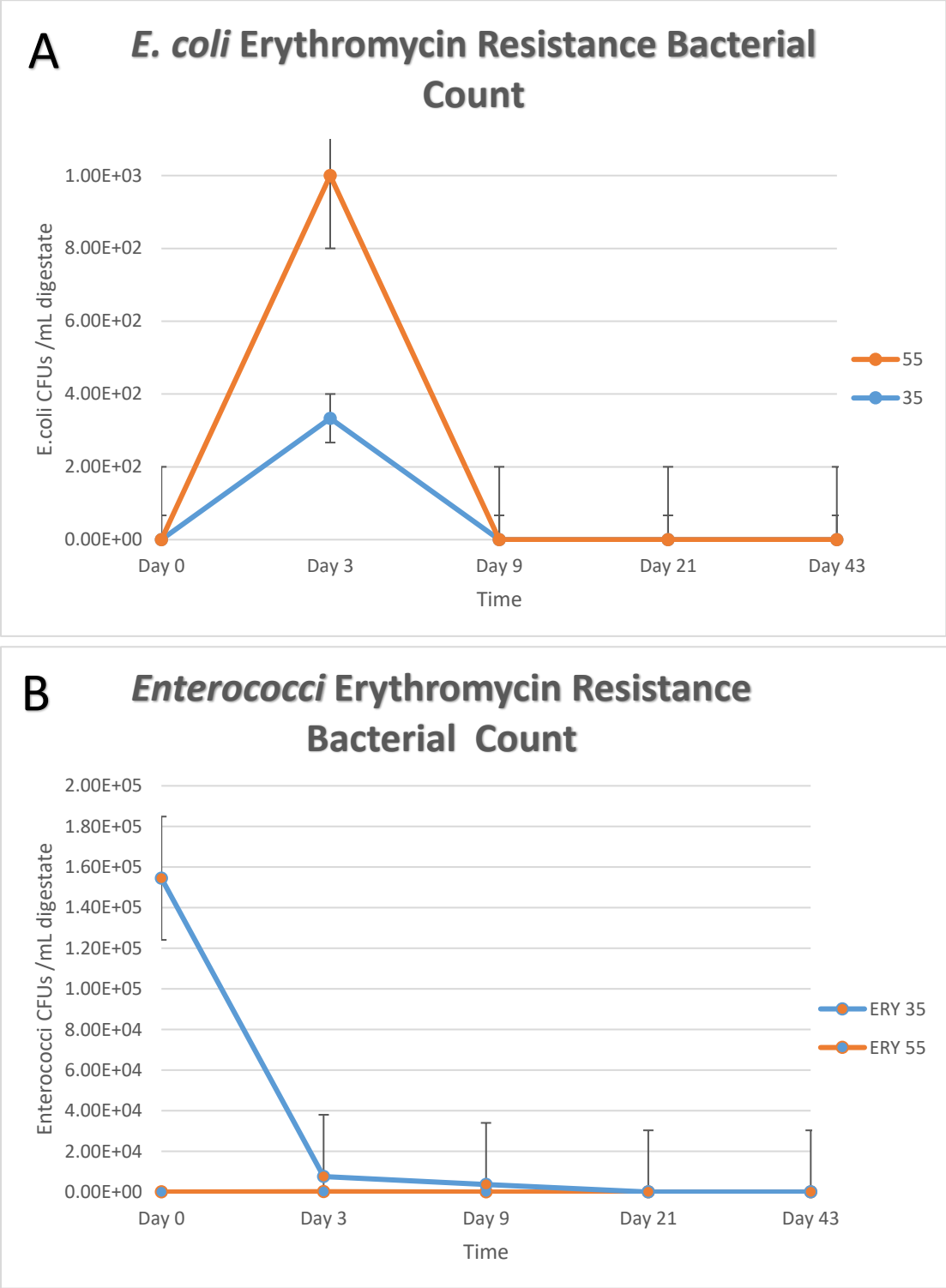


Figure 4.4. Bacterial colony forming units (CFUs) and standard error of *E.coli* and *Enterococci* resistance to Erythromycin spiked media (3 mg/L). CFU's documented at 5 times points for both 35C and 55C AD conditions throughout the 43-day digestion. Similair trend were shown or no growth reported in ampicillin and oxytetracycline plates (not shown).

E.coli isolates often found within the dairy manure have been shown to be susceptible to tetracyclines (Oliver et al. 2020), which may explain the no growth reported in the study. The tetracycline plates were set at 5 mg/L, as these antibiotics have high historical use and consistent detection within the environment. Under 55°C conditions, the ampicillin plate was found to not have any growth. On day 3 enterococci plates were found to have more CFU then the *E.coli* under erythromycin resistance at $7.5x10^3$ CFU mL compared to $6.67x10^2$ CFU mL. Enterococci prefer higher temperatures than *E.coli*, which could explain the higher CFUs. By Day 9, only *E.coli* on erythromycin plates from 35°C AD conditions were documented at $3.67x10^3$ CFU mL. By Day 20, no FIBs were reported for the duration of the study, including control plates.

As with antibiotics and ARGs, temperature is deemed essential to ARB reduction, often with higher efficiencies in thermophilic temperatures compared mesophilic (Varel et al. 2012; Beneragama et al. 2013; Tran et al. 2021) Though efficacy can be dependent on several parameters, Beneragama et al. (2013) found that mesophilic AD co-digestion of dairy manure and milk waste had>99% reduction of ARBs after 20 day, but a rebound of ARBs was also reported after 34 days. Tran et al. (2021) found that all six farms with AD operating temperatures ranging from 38 - 40°C significantly reduced *E.coli* (1.2 - 2.2 log 10 fold), yet, there was hardly any impact on gram positive bacteria. Whereas in this study, thermophilic conditions proved effective at eliminating the gram-positive enterococci. Moreover, Iwasakie et al. (2019) found thermophilic AD significantly reduced cefazolin, ampicillin and extend spectrum b-lactamase resistance bacteria, with survival rates of <2% following

digestion. However, this study also reported the thermophilic AD to be less effective at reducing non-coliform bacteria.

Studies typically screen for fecal indicator bacteria and other coliforms to assess risk, as digesters hold a unique position in their variety and abundance of bacteria and other microorganism (archaea) that remain constant. As stated above, both mesophilic and thermophilic conditions were capable of significantly reducing and/or eliminating these targeted organisms. Though non-coliform bacteria, which can be essential to digester function, may be key to examine as they: 1) might be ARG harboring bacteria, and 2) may be key organisms in gene transmission and prevalence both within the digester and effluent digestate exposed to the environment. Currently, only a fraction of the needed information is understood for microbial system dynamics and even less is understood when considering the development of antibiotic resistance. Non-pathogenic bacteria and other microorganism may hold value in understanding the gene movement and development of ARBs in the future.

4.3.5 Correlation analysis between AD characteristics and co-occurrence of ARGS

Physical and chemical characteristics have been shown to influence the fate of ARGs. Spearman correlation analysis was applied to evaluate correlation and trends between the AD characteristics of both mesophilic and thermophilic conditions, such as TS, VFAs, nutrients, COD, and pH, in relation to ARGs and any cooccurrence of resistance genes (Table 4.1). As stated, previously, *intl1* encodes for an integrase gene that has strong influence in the horizonal transference of genes between bacteria. This study reported *intl1* to have a strong correlation ($r^2 > 0.5$, p-value <0.05) to both *ermB*

and *sul1* under each condition. The 35°C conditions showed *intl1* and *ermB* to have r^2 = 1 and *p*-value <0.0001. Indicating that is very likely for *ermB* to be attached to *intl1*.

Table 4.1. Correlation analysis (r^2 value) between the relative abundance of antibiotic resistance genes and digestate characteristics for mesophilic and thermophilic condition (blue: p<0.05; green p<0.0001).

35°C	*intl1*	*ermB*	COD	TS	pH	tetX	N	P	*tetQ*	*tetM*	*sul1*	*tetW*	*bla2*	Acetic	Burtyric	Proponic	Valeric
intl1	1.000	1.000	0.591	0.392	0.664	0.177	-0.068	-0.268	-0.25	-0.095	0.681	-0.014	0.623	0.119	0.607	0.335	0.752
ermB	1.000	1.000	0.591	0.392	0.663	0.176	-0.067	-0.268	-0.249	-0.094	0.681	-0.014	0.623	0.119	0.608	0.336	0.753
COD	0.591	0.591	1.000	0.505	0.598	0.058	-0.322	-0.514	-0.161	-0.295	0.394	-0.216	0.500	0.857	0.292	0.471	0.669
TS	0.392	0.392	0.505	1.000	0.407	0.076	-0.255	-0.33	-0.025	0.263	0.263	-0.235	0.266	-0.563	-0.037	0.195	0.158
pH	0.663	0.663	0.598	0.407	1.000	-0.139	-0.337	-0.45	-0.457	-0.218	0.331	-0.489	0.370	0.714	0.425	0.086	0.694
tetX	0.177	0.177	0.058	0.077	-0.14	1.000	-0.284	-0.385	0.345	0.505	0.575	0.395	0.468	0.286	0.215	0.239	-0.061
N	-0.068	-0.068	-0.323	-0.255	-0.337	-0.284	1.000	0.781	0.363	-0.047	0.059	0.459	-0.21	-0.405	-0.038	-0.248	-0.12
P	-0.268	-0.268	-0.514	-0.33	-0.45	-0.385	0.781	1.000	0.162	0.005	-0.276	0.256	-0.295	-0.571	-0.21	-0.305	-0.385
tetQ	-0.25	-0.25	-0.161	-0.025	-0.457	0.345	0.363	0.162	1.000	0.389	0.115	0.5	-0.136	-0.429	-0.165	-0.343	-0.46
tetM	-0.095	-0.095	-0.295	0.263	-0.218	0.505	-0.047	0.005	0.389	1.000	0.119	0.114	0.193	-0.314	-0.006	0.412	-0.524
sul1	0.681	0.681	0.395	0.263	0.331	0.575	0.059	-0.276	0.115	0.119	1.000	0.345	0.684	0.071	0.442	0.014	0.441
tetW	-0.014	-0.014	-0.217	-0.235	-0.489	0.395	0.459	0.256	0.5	0.114	0.345	1.000	0.201	-0.452	0.094	-0.143	-0.193
bla2	0.623	0.623	0.501	0.266	0.370	0.468	-0.21	-0.295	-0.136	0.193	0.684	0.200	1.000	0.714	0.608	0.493	0.485
Acetic	0.119	0.119	0.857	-0.563	0.714	0.286	-0.405	-0.571	-0.429	-0.314	0.071	-0.452	0.714	1.000	0.742	0.095	0.714
Burtyric	0.608	0.608	0.292	-0.037	0.423	0.215	-0.038	-0.21	-0.165	-0.006	0.442	0.094	0.608	0.742	1.000	-0.07	0.920
Proponic	0.336	0.336	0.471	0.195	0.086	0.239	-0.248	-0.305	-0.343	0.412	0.014	-0.143	0.493	0.095	-0.07	1.000	0.110
Valeric	0.753	0.753	0.669	0.158	0.694	-0.061	-0.1204	-0.3851	-0.46	-0.524	0.441	-0.193	0.485	0.714	0.920	0.110	1.000

55°C	*intl1*	*ermB*	COD	TS	pH	*tetX*	N	P	*tetQ*	*tetM*	*sul1*	*tetW*	*bla2*	Acetic	Burtyric	Proponic	Valeric
intl1	1.000	0.594	0.491	0.211	0.497	0.663	-0.24	-0.378	0.47	0.317	0.591	0.005	-0.226		0.395	-0.265	0.551
ermB	0.594	1	0.283	0.117	0.544	0.723	0.078	0.045	0.338	0.476	0.872	0.198	-0.619	0.109	0.406	0.013	0.764
COD	0.491	0.283	1	-0.039	0.253	0.241	-0.378	-0.536	0.39	0.5	0.33	-0.248	-0.249	0.5921	0.198	0.051	0.408
TS	0.211	0.117	-0.039	1	-0.054	0.253	0.32	0.111	0.152	0.084	0.091	0.163	0.288	-0.48	0.084	-0.106	0.325
pH	0.497	0.544	0.253	-0.053	1	0.462	-0.307	-0.375	0.395	0.476	0.45	-0.282	-0.585	-0.402	-0.125	-0.1665	0.25
tetX	0.663	0.723	0.242	0.253	0.462	1	0.18	0.032	0.706	0.429	0.806	0.089	-0.056	-0.068	0.404	-0.062	0.665
N	-0.24	0.0785	-0.378	0.32	-0.307	0.18	1	0.868	0.105	-0.943	0.175	0.569	0.294	0.041	-0.533	0.011	0.08
P	-0.378	0.045	-0.536	0.111	-0.375	0.032	0.868	1	-0.05	-0.829	0.174	0.674	0.172	0.046	-0.192	0.14	0.059
tetQ	0.47	0.338	0.39	0.152	0.395	0.706	0.105	-0.05	1	0.619	0.485	-0.133	0.076	-0.132	-0.005	-0.513	0.334
tetM	0.317	0.476	0.5	0.084	0.476	0.429	-0.943	-0.829	0.619	1	0.357	-0.19	-0.381	1	0.071	-0.257	0.395
sul1	0.591	0.872	0.33	0.091	0.45	0.806	0.175	0.173	0.485	0.357	1	0.229	-0.402	0.41	0.388	0.132	0.7
tetW	0.005	0.198	-0.248	0.163	-0.282	0.089	0.569	0.674	-0.133	-0.19	0.229	1	0.151	0.041	0.234	0.229	0.235
bla2	-0.226	-0.619	-0.249	0.288	-0.585	-0.056	0.294	0.172	0.076	-0.381	-0.402	0.1517	1	-0.378	0.189	-0.037	-0.271
Acetic		0.109	0.592	-0.48	-0.402	-0.068	0.041	0.0456	-0.132	1	0.41	0.041	-0.378	1	0.4	0.45	0.483
Burtyric	0.395	0.406	0.198	0.084	-0.125	0.404	-0.533	-0.192	-0.005	0.071	0.388	0.234	0.189	0.4	1	0.41	0.948
Proponic	-0.265	0.013	0.051	-0.106	-0.166	-0.062	0.011	0.141	-0.513	-0.257	0.132	0.229	-0.037	0.45	0.41	1	0.137
Valeric	0.551	0.764	0.408	0.325	0.25	0.665	0.08	0.059	0.334	0.395	0.7	0.235	-0.271	0.483	0.948	0.137	1

Numerous studies have reported similar strong correlations in manure and wastewater sludge studies (Liu. et al. 2012; Sun et al. 2016; Zhou et al. 2021a). *Intl1*

can carry other mobile elements, like transposons, which also help to facilitate gene movements. This analysis suggests potential clustering of the *ermB* and *sul1* genes on the *intl1* gene cassette that is also supported in the previous ARG analyses that displayed similar abundance reduction patterns. *Intl1* and *ermB* also showed positive correlation to pH ($r^2 = 0.59$, *p*-value = 0.0009) and COD ($r^2 = 0.66$, *p*-value = 0.001) under mesophilic conditions. During digestion the pH can decrease as a result of production of acidic molecules, such as VFAs. A pH change in the post-digestion samples at Day 0 from 8.22 to around ~7.6 for the remainder of the digestion period is documented in Table 4.1 This may indicate that this slight pH change is enough to impact the bacteria harboring this specific gene cassette. Furthermore, COD measurements report on the amount of organic material available for bacterial metabolism and generally decreases throughout AD processing. Collectively pH and COD correlation to these two genes may suggest that these bacteria are likely more active/ abundant during the early stages of AD, potentially derived from the cattle rumen/manure and have limited capacity during the AD treatment.

Nutrient levels and wastewater/waste quality have been shown to have positive correlations to ARG abundance. Cheng et al. (2016)'s case study on the behavior of antibiotics and ARGs in the eco-agricultural system found *tetM* to be significantly correlated to COD measurements and chlorophyll. McKinney et al. (2010) found similar results in an investigation of ARG in livestock lagoons, reporting *tetO, tetW* and total *tet* genes were positively correlated with COD, total nitrogen, ammonia, nitrate, and phosphates levels, which are all indicators of poor water quality. Numerous *tet* genes were detected and sustained throughout both

digestion conditions, yet there was not a correlation between *tet* genes in mesophilic conditions; under thermophilic conditions only *tetX* was found to be positively correlated to *tetQ* (r^2=0.7, p-value = <0.0001) and *sul1* (r^2=0.8, p-value = <0.0001) (Table4.1). This further highlighting the difference of bacterial community between each condition and corresponding gene profiles. *TetW* was found to have moderate positive correlation to total nitrogen (r^2=0.56, *p*-value = 0.003) and phosphorus (r^2=0.67, *p*-value = 0.0002) concentrations only under thermophilic conditions. Nutrient levels did fluctuate but overall, tended to decrease throughout the digestion process especially when implemented as a batch system. *TetW* harboring bacteria may be directly or indirectly benefiting from the nutrient availability that can also be seen in the enrichment of the *tetW* RA in Figure 4.3 above.

VFA production can also be used to gauge AD performance, as these compounds are intermediates produced during the second and third phase of AD (acidogenesis and acetogenesis). Of the four VFAs measured in this study (acetic acid, propionic acid, butyric acid and valeric acid), only valeric was found to have any correlation to ARGs. Under mesophilic conditions valeric acid was shown to have positive correlation to *intl1*, *ermB* (r=0.75, *p*-value = <0.0001). While at thermophilic conditions valeric displayed positive correlation to *ermB* (r^2=0.76, p-value = <0.0001), *sul1*(r^2=0.7, p-value = 0.0001), and *tetX* (r^2 = 0.66, p-value = 0.0004). The accumulation of VFAs can contribute to pH drop and microbial inhibition (Wainaina et al. 2019). Valeric acid may not be much a contributing factor in this study. As the general trend, ARGs and valeric acid concentrations decreased throughout the digestion. Bacterial groups, such as *Synergistetes* and *Clostridium*,

have been documented to be responsible for the fermentation in the hydrolysis step and the production of short chain VFAs (Guo et al. 2010; Lukitawesa et al. 2019; Wainaina et al. 2019). Correlation of these ARGs to valeric acid may be indicator that these bacterial classes may be the harboring these specific resistance genes. This correlational analysis further showcases the vast difference in ARGs and digestate characteristics and the complexity of each unique microbial system and processing.

5.4 Conclusion

Both mesophilic and thermophilic AD conditions demonstrated to be valuable options for manure treatment and AMR mitigation. Though higher temperatures have been credited to be a key parameter that can be manipulated to increase AD capability to reduce bacterial loads and corresponding antibiotic resistance. Variables regarding compound stability in tandem with microbial community and substrate physiochemical characteristics are even more essential to proper mitigation. As antibiotic degradation was variable throughout the 43-day digestion; degradation of the antibiotics was deemed to be more compound/ class specific rather than based on temperature conditions. As erythromycin had complete removal under the lower mesophilic conditions yet on almost complete removal at higher temperature. While oxytetracycline was shown to be in have the inverse response. The reduction of the ARGs was shown to be just as variable, as certain ARG abundances were lower in one condition but higher in another. While commonly surveyed genes such as *intl1, ermB, sul1 tetM* and *teX* were found have partial to significant reduction under either condition. The ARG *tetW* was found to increase >81% under both conditions suggest the possibility of an endemic species to digester harboring the gene. Even though

common indicator species like *E. coli* and *Enterococci* were completely eliminated by Day 9. Future work examining non-pathogenic bacterial species may be important in understanding the role digester play in mitigating or contributing to antibiotic resistance.

Chapter 5: Improving Public Engagement with Complex Scientific Issues Exemplified by Antimicrobial Resistance

As submitted to Journal of Extension

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ABSTRACT:

Effective communication to the public about complex scientific issues, such as antimicrobial resistance (AMR), is a priority for preserving the long-term health of humans, animals, and the environment. This paper surveys current efforts to evaluate and improve scientific communication and scientific knowledge of complex issues to develop an evidence-based outreach communication model for scientific topics. We propose a methodology for influencing audience behaviors. The proposed methodology incorporates five rhetorical elements to improve audience amenability to scientific messaging: 1) narrative structure; 2) normalization of the subject using human scaling; 3) non-agentive language; 4) trusted experts for message delivery; and 5) future simulation.

Keywords: health communication, public understanding of science and technology, risk communication, science communication: theory and models

5.1 Introduction

The ability to speak or write about a scientific topic in a manner that is trusted and compelling is known as "scientific discourse." An unfortunate reality of the highly globalized, connected, and digital world in which we live today, is the overwhelming quantity of information sources available, many of which are not evidence based. Ongoing debates around polarizing subjects, such as genetically modified organisms (GMOs) or climate change, highlight an important point about the social and political dynamics surrounding science communication: the way new technologies or scientific breakthroughs are communicated in social settings is at least as important as the scientific content that is being conveyed when lay audiences interpret new technologies or make decisions about public funding for science (Scheufele 2013). These mainstream topics have already transcended the realm of simple data presentation and entered the complexities of socio- scientific issues that encompass conflicting principles of ethics, cultural perspectives, and economic theories or human livelihoods. Complexities and conflict have placed these topics at the forefront of many news and media stories, further increasing the public face of the debate.

An emerging example of this is antimicrobial resistance (AMR), as the threat of AMR is evident to much of the scientific community. Regulatory agencies and policymakers have increasingly become concerned, as evidenced by the expansion of AMR-centric agencies and funding for research to address the spread of AMR. However, growing scientific concern has not effectively translated to public awareness. A primary example of this phenomenon is the public interest in antibiotic residuals within meat and dairy products. Seemingly, scientific concern with AMR in the food

chain has been received by the public indistinctly from other food contaminants like hormones and pesticides. Thus, the public consciousness has yet to conceive a clear link between their use of antibiotics and the growing scientific and medical concerns with AMR. As further illustration, research has found that individuals with little knowledge of AMR are less likely to seek out new information and may even willfully remain uninformed (Meerza *et al.,* 2019).

As seen with other complex scientific topics, the dearth of public knowledge and engagement has led to confusion and misunderstanding, exacerbated by the public perception of the media's authority. Knowledge deficit and distrust of authority can, in turn, promote messages of blame (Davis *et al.,* 2018). The result is the public is stranded in a storm of fact, media coverage, and mistaken information that encourages extreme opinions or complete negation of the topic. In this environment, AMR makes a useful example of a scientific issue that requires an understanding of both natural adaptation and the role of anthropogenic practices, that if presented in an isolated framework could instill grave mistrust and misconceptions, and potentially cause audiences to resist a message that seems too threatening (Meerza *et al.,* 2019).

Scientific communication on controversial and complex topics must incorporate two major components: 1) connection to scientific content and 2) social significance (Eastwood *et al.,* 2012). While the central scientific principles of AMR are well understood and widely accessible, AMR communications to the public are typically presented in a problem–solution format. However, such strategies tend to convolute information and confound certainty, leading to misinformation and controversy around an issue.

In this paper the authors will detail a process for creating effective messaging about complex scientific topics using AMR to illustrate a model of communication to meet these challenges. Based on evidence-supported practices for improving science communication and public reception. Select science communication research is summarized and systematically utilized to design a communication strategy that encourages public engagement in the example issue (AMR) and to measurably change public responses and influence relevant behaviors. Drawing upon established methods of communication, a proposed media campaign is designed to showcase and formulate elements of presentation that prove most effective in public assurance. A practical extension of extant science communication literature is used to frame an educational effort and then shape an output, ultimately yielding a model by which educators can develop an education campaign targeting one or more specific audiences.

5.2 Message Design

Drawing upon Swope's (1998) methodology, we isolate several features of science communication with the literature that have been shown to quantitatively be efficacious in spurring change. We then incorporate these features to develop a hypothetical media campaign that can then be evaluated in a sample population. The following elements were deemed of highest importance to our output: 1) narrative structure; 2) normalization of the subject using metaphors and human scaling; 3) non-agentive language; 4) message delivery by a trusted expert; and 5) future simulation.

5.2.1 Narrative Structure

Information framing has become a crucial aspect of scientific communication, as the logic model of data presentation has constantly underperformed within the public. Framing extends beyond the simplification of complex issues but includes the utilization of more active and lively rhetoric to emphasize certain aspects of the topic at hand. Dahlstrom (2014) makes the case that narrative formats are best for communicating with lay audiences; storytelling is inherently easier to comprehend, is more engaging and persuasive. The narrative structure links and explains events in a sequence of cause and effects, over a specific time frame that impacts a character. Down (2014) acknowledges the potential confrontation between the incorporation of new information into long-held beliefs and the familiar comfort of an existing worldview. Consequently, the use of narratives in science communication is a tool to help overcome the resistance by an audience to the information that challenges an existing paradigm. Dahlstrom (2014) elaborates that narratives are intrinsically persuasive because they "provide their users with a format of comprehension to simulate possible realities, which would serve to better predict cause-and-effect relationships". Winterbottom et al. (2008) made similar claims based upon a systematic review of the observed persuasiveness of narrative information on individual decision making, finding that scientific information presented through narratives influenced decision making more than the provision of statistical results, thereby illustrating that narratives generate interest in a topic and a willingness to engage with new ideas better than information gathering and processing. Narratives

are a default form of communication and allows the individual to infer general truths required for a desired outcome.

5.2.2 Normalization of the Subject Using Metaphors and Human Scaling

Scientists and science-literature are cautioned away from insisting that data should speak for itself. As research into more effective scientific communication has discovered, the untrained human mind is not well developed to grasp complexities outside of the realm of what can be directly experienced (National Academies of Sciences, 2017). The difficulty of defining and communicating antimicrobial resistance (AMR) as a socioscientific issue relates in many ways to the complexity of microbial systems. AMR is a naturally occurring process within microbial communities that is largely being expedited through various anthropogenetic behaviors. Yet extant AMR communication is missing an engaging format for how the issue arises and persist. The inherent hazard of this oversight can be seen in similar environmental issues like climate change and global warming, where failure to convey basic ideas of weather vs. climate have led to misinterpretation or "filling in the gaps" by the public.

Metaphors are an efficient and pervasive form of everyday communication which can be used within or alongside narratives to help people understand complex topics (Thibodeau et al. 2019). Given the difficulty of explaining AMR outside of its terminal impact, use of metaphors at the human scale could be used to describe AMR within the frame of reference of the intended audience. We recognize that there are limitations to any simplification; for instance, the viewer may over-extrapolate or misinterpret the information. Yet, the metaphorical portrayal of the complex nature of

microbial life in a context that matches the audience's scalar worldview brings microorganisms to an existing public framework, which could more easily spark audience interest and support decision making related to AMR. For example, during the 2018 presidential campaign and ensuing presidency, Trump consistently used the metaphor of "draining the swamp". A swamp is generally perceived as hot, messy, unchanging, and treacherous environment to provide a visual of the perceived corruption and stagnation of the American political system; with Trump claims to seeding change by draining its water. Gibbs and Colston 2012 psychology "Interpreting Figurative Meaning" found metaphors to be no more difficult to process then literal language. Metaphors are critical to human conceptualizations, using familiar domains to formulate unfamiliar, abstract, and complex concepts (Lakeoff and Johnson 1980; Thibodeau et al. 2019).

5.2.3 Non-Agentive Language

Despite the multitude of perspectives surrounding how AMR develops and spreads, all stakeholders are pursuing the same outcome: the health, safety, and economic well-being of society. Concomitant with thinking that tends toward in-group social commitments, Fausey and Boroditsky (2010) showed that agentive language—that which highlights a causal factor—tends to make people more willing to assign blame. Drawing together the connections of Fausey and Boroditsky with those of Kahan (2010), we assume that highlighting individual responsibility in public health issues using agentive language would increase the sense of interpersonal blame among audience members. This, in turn, could be expected to further polarize social groups by leading to thinking that reinforces social resistance to a given message and

mitigates a sense of individualized guilt. Thus, non-agentive language—that which presents an issue without assigning blame to any societal sector—would likely be more effective in such contexts. Similarly, Swope (1998) has shown that when dealing with potentially polarizing subjects, a message must be carefully selected to fit a given audience, or it runs the risk of increasing the sense of listener culpability and triggering fight-or-flight reactions instead of meaningful change.

5.2.4. Message Delivery by a Trusted Expert

Research has shown that the more a speaker is perceived to be of an "in-group" identity (such as a conservative speaker to conservative audiences), the more receptive an audience is to their message, regardless of the message itself. Thus, if the speaker delivering an anecdote is deemed trustworthy, their anecdote will more readily be deemed trustworthy, as well. Kahan (*et al.* 2012) showed that scientific literacy is not predictive of receptiveness to scientific data; instead, scientific literacy was positively correlated with the tendency to *resist* evidence-based findings if they appear to represent the views of an "out-group" mode of thought. People are social animals, as Kahan (2010) notes, and are much more likely to be motivated to maintain group connections than to accept abstract principles that threaten extant social networks.

5.2.5. Future Simulation

The use of narratives as tools to introduce AMR and its potential implications presents an opportunity to engage the public more closely through experiential, active learning. Similar to Choose Your Own Adventure (CYOA) books, which were an anomaly in the world of fictional literature upon their initial introduction in the 1970s.

CYOA books require the reader to make decisions at critical points throughout their literary journey rather than providing a single, linear storyline. Some choices lead to successful ends in the reader's adventure, others to the reader's unfortunate demise. This unique approach to storytelling, whereby the reader shapes the outcome of the story and faces the consequences of individual decisions, could arguably be considered a form of simulation-based or experiential learning. Simulation is simply an artificial representation of a real-world process to achieve educational goals through experiential learning (Flanagan *et al.* 2004). For instance, concerns about patient safety during the training of medical students, coupled with limited availability of patients with varying health conditions that students can learn to assess and treat, have contributed to the development of simulation centers and clinical skills laboratories in medical education (Al-Elq 2007; Scalese *et al.* 2008).

For learning to be considered "experiential," it must contain the following elements:

1. Reflection, critical analysis, and synthesis.
2. Opportunities for learners to take initiative, make decisions, and be accountable for the results.
3. Opportunities for learners to engage intellectually, creatively, emotionally, socially, or physically.
4. The possibility to learn from natural consequences, mistakes, and successes.

Using this approach (i.e., incorporating the basic elements of experiential learning into a low-stakes model of imaginative simulation that translates to real-

world outcomes) to learning about a scientific topic can easily be an iteration of CYOA books. Such an approach provides learners with a simplified model of a system for which the learner can make decisions that impact the outcomes of a problem or situation without being distracted by irrelevant information or potential risks associated with those decisions. Ironically, such a "low stakes" simulation methodology that provides a safe and straightforward way for a user to consider and weigh various courses of action can have positive, real-world impacts on "high stakes" issues (Ziv *et al.* 2005), such as the mitigation of AMR. The key features of a future simulation are that it thematizes openness, indeterminacy, virtuality, and the idea that every "now" contains a multitude of possible continuations; and it goes beyond this, by staging the fact that the future is a space of yet-unrealized potentiality.

5.3. Message Design and Delivery Flowchart

To aid in the development of an educational output that utilizes these principles, a decision-making flowchart is offered (Figure 5.1). While all the communication methods discussed are included in the flowchart, it is recognized that not all components of this decision-making tool may be applicable to every science communication endeavor. For instance, a topic that is not controversial or polarizing may not require employing a trusted expert to deliver the message successfully. Likewise, a topic with which an intended audience is already familiar may not require an introduction using human scaling, allowing the user to progress to the second step in the process: explaining the relevance of the topic to the intended audience using normalization and metaphors.

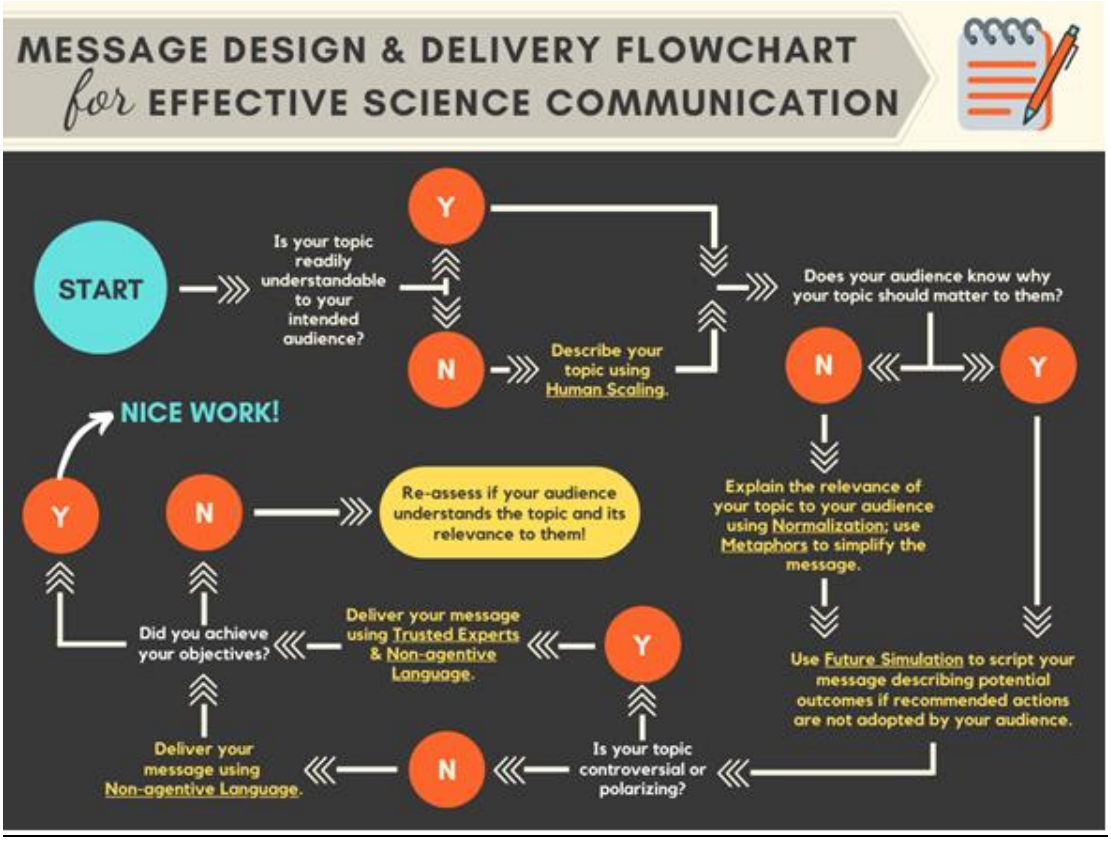


Figure 5.1: Decision-making flowchart for development of a science communication plan based upon relevant science communication principles.

5.3.1 Application

We applied the evidence-based principles of science communication reviewed herein to construct a hypothetical media output tailored to disseminating the basic principles of AMR to multiple public sectors. (Figure 5.2)


"We all have a role."
Storyboard 1/4
A man sits in bumper-to-bumper traffic, glancing at his watch with a look of annoyance and impatience, visibly stressed about making it to work on time.
He glances at the HOV lane's fast-moving traffic and sighs ruefully. But then his expression changes to one of satisfaction. He has an idea!
Cut to the same man, now with a satisfied smile as he drives in the fast lane with a mannequin in the passenger seat.
A few days later, two neighbors are watching as the man happily loads the dummy into his car. Their expressions slowly turn from confused to impressed.
Cut to an HOV lane full of cars with dummies in the passenger seat, including one the man's neighbors.
Zoom out farther and farther to reveal that the cars are, in fact, bacteria in a petri dish. On screen: "We've all looking for an advantage."


"We all have a role."
Storyboard 2/4
VO: Bacteria grow and change just like we do, sometimes learning new ways to gain an advantage - like resisting the effects of antibiotics.
VO: A single bacterium - or cell - can reproduce very quickly to form a colony of bacteria having the same characteristics as the single bacterium.
VO: With bees, a single sting may hurt, but not cause great harm. Likewise, a single bacterium may not harm us if our immune system can fight back against it.
VO: But our behaviors affect them. When we use antibiotics, bacteria look for an advantage, a way to survive a medicine used to kill them. When they succeed, we say they've become resistant to that drug.
VO: Bacteria that survive when exposed to an antibiotic continue to reproduce, passing on their new survival method to their offspring. Like a colony of killer bees, colonies of antibiotic resistant bacteria can be very dangerous.
VO: Bacteria that can cause disease and are resistant to one or more antibiotics can be very dangerous. Some common bacterial diseases are becoming hard to treat.

Figure 5.2: Media Model for Communicating Principles of AMR to Public

Narrative Structure

Communicating the complex topic of AMR through a narrative structure (or "storytelling") --rather than with an analytical explanation that includes scientific jargon--is a key feature of the proposed communication model presented. As such,

this structure for delivering the proposed content is interwoven throughout the remaining features of the model.

Normalization of the Subject Using Metaphors and Human Scaling

As outlined previously, human scaling provides a framework for communicating complex topics like AMR through everyday scenarios, allowing for greater audience relatability and potentially developing a better grasp of the subject at hand. In this case, we propose to utilize human scaling by building an analogy between microbial adaptation and a common human experience: the morning commute to work. Our media output begins by illustrating a parallel between AMR development and deceptive utilization of high-occupancy-vehicle lanes.

Once the audience is introduced to a new scientific concept, it is important that the audience recognize the relevance of the issue at both the individual and broader population/ societal level. Thus, we incorporate imagery that illustrates the risk associated with AMR through narratives metaphors to communicate this nuance, while employing a context that guards against potential alarmist public interpretations. We proposed the image of conceptualizing a bee and a beehive to showcase the micro and macroscopic impacts of smaller organism to describe the risks associated with disease-causing bacteria.

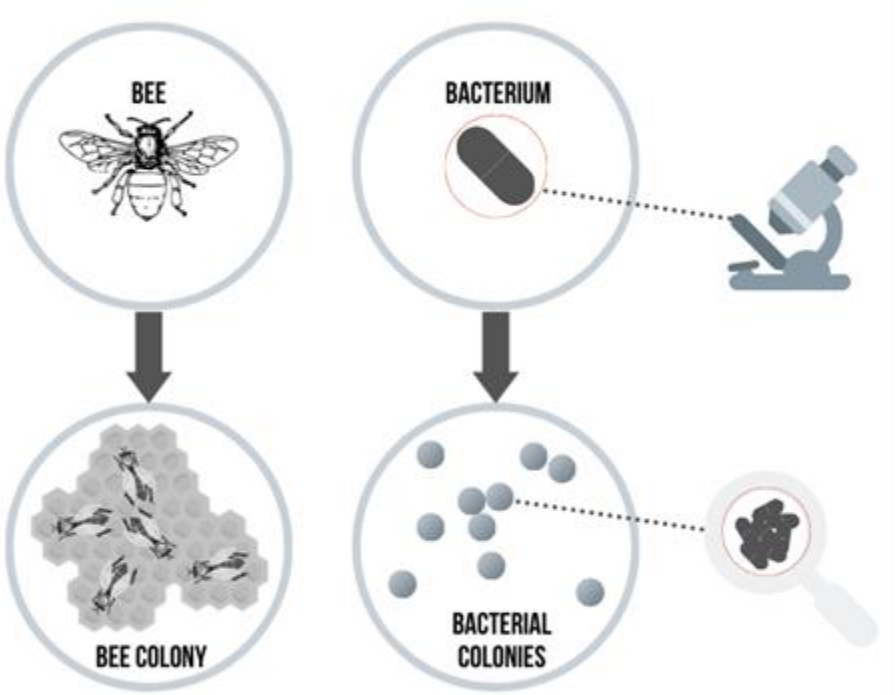


Figure 5.3. Metaphorical depiction of bacterial colonies as groups of individual bacterial cells, similar to the colonization of honeybees to form a hive.

Future Simulation and Message Delivery by a Trusted Expert

Many basic principles of science communication are grounded in data collected while studying contentious issues such as abortion, vaccination, and climate change. Previously cited studies supported that effective scientific communication about public health issues does not depend upon insisting repeatedly on the objective truths of data. Instead, we hypothesize that presenting future simulations within a narrative, communicated via a trusted expert, serves to demonstrate to different stakeholders because such data can and should matter to them.

Such as employing a mother figure to presenting the learner (e.g., other mothers) with a description of a human or animal infection for which the appropriate medical treatment must be selected. If the infection is caused by a virus and the learner selects an antibiotic for treatment, the feedback received may include an explanation of how

antibiotics do not impact the proliferation of viruses and may cause harm by contributing to the development of antimicrobial resistance.

Use of Non-agentive Language

AMR is a public health concern that can lead to antibiotic inefficacy. Several causes may contribute to AMR, including any use of antibiotics clinically and agriculturally, and the naturally occurring reservoir of resistant bacteria and resistance genes within the environment (Clardy *et al.* 2009; Cycoń *et al.* 2019). The perception of antibiotics as "the" tool for infection prevention and treatment have both been well cited as a driving factor of AMR. However, communication on AMR must avoid simplifications that blame specific sectors (ex. antibiotic use in animal agriculture) which would tend to immobilize those same sectors as potential partners in solving the problem. In parallel to the Center for Disease Control and Prevention One Health Initiative the overall model uses the tag "we all have a role" to illustrate the shared responsibility for the causes, and solutions, to this complex issue.

5.4. Conclusion

The last twenty years have seen increased intensity in debates surrounding global warming, vaccination, resource allocation, and disease. Clearly, we are at a crucial point of reflection regarding our understanding and acceptance of our role as a species within the built and greater natural environments. However, effective scientific communication necessitates more than the simple presentation of data; it requires the active integration of communication principles to convey complex issues such as AMR. The reviewed literature and hypothetical communication model proposed within this paper highlights elements of narratives that can be applied to

scientific communication. Narratives can be used to not only address the public's understanding of AMR but also provides a model to facilitate a broader application of these communication strategies. Thus, this proposed methodology brings together various elements of effective communication to reach a wide range of viewers on a more personal level. This approach may also provide a foundation to reform current models of scientific communication campaigns toward more integrative approaches of disseminating scientific information to the public.

Chapter 6: Conclusion

6.1 Intellectual Merit

Within the US regulation around antibiotic administration is still in its infancy especially in regard to utilization with animal agriculture. The 2018 Veterinary Feed Directive (CVM, 2019) has been the most recent and one of the first pieces of legislation within the US to begin regulating antibiotic administration for livestock animals. As antibiotics pollution increases and research continues to better enhance our understanding of the impacts of parallel resistance in livestock animal and antibiotic mobilization through the environment can contribute to human health. Standardization and improvement to antibiotic detection are essential. The research outlined in Chapter 2 presents a novel multi residual multi class method that can be applied for numerous forms of manure with < 30 % standard deviation all the antibiotics except for ceftiofur. The extent of animal manure antibiotic contribution to the environment is largely unknown. This method can be useful in quantifying the non-point sources of contamination and provide a uniform method of analysis. Furthermore, the method was also successful in detecting B-lactams like penicillin. Though this class has high utilization within animal agriculture and is medically relevant to human health, the instability of these compound makes them a challenge to HLPC-MS detection. This is one of the first methods to report consistent penicillin recover (> 50%) across the four manure substrates examined.

The BRU treatment technology SLS fraction capabilities allowed for the examination of antibiotic resistance factors and their variability based on substrate characteristics. The partitioning of the chelating antibiotics such as tetracycline and

tulathromycin further support the preexisting literature that find these compounds to have longer half-lives and higher concentration with sediments and soils. Interestingly the ARG analysis is one of few that provided a lens to indicate how bacteria and resistance partitions. *TetX, sul1* and *intl1* were all found to increase in RA in the separated solids that demonstrate these bacteria may be the few that have greater binding efficacy to solids that infers a shift of the bacterial community between substrates. The BRU technology was deemed to not be very effective at removing the antibiotics yet did report reduction to 6 of the 7 ARGS and sterilization of the fecal indicator species such as *E. coli* and *Enterococci*. This also highlights the importance that retention time plays in composts effectiveness in community succession and antibiotic degradation. Though the system setup was shown to transport most of the mass and the antibiotic resistance factors to the storage lagoon that doesn't have secondary treatment. It must be noted that this system was not promoted to effectively treating antibiotic resistance but is focused on in-house production of quality bedding material.

The comparative analysis between mesophilic and thermophilic AD was found to have variable results that compare to most studies that credit temperatures as key variable in resistance reduction. Oxytetracycline has been shown in the literature to more effectively degrade under thermophilic temperatures which held true in this study yet other compounds like erythromycin were found to have quicker and more efficient degradation at the lower temperature. In agreement with previous studies when comparing thermophilic ARG to mesophilic ARG RA both were found to significantly reduce *intl1, ermB, sul1*, and *tetM;* thermophilic RA was found to be the

marginally lower. Thermophilic conditions were found to have significantly lower (*p-value* = 0.0002) 16S at the end of the digestion. The simultaneous processing of digestion steps and the continuous temporal shifts of the microbial community that may correspond to ARG rebound make digesters hard to examine when evaluated regarding antibiotic resistance mitigation. Though the overall bacterial community may be lower certain bacterial species harboring various ARGs may be filling the void. This study suggests the need to intentionally examine the digester community and their participation its methane production but also resistance dissemination. Research beyond pathogenic bacteria would provide more holistic and accurate assessment of the role digester can play in both mitigation and the development of antibiotic resistance.

Effective messaging of research is key to increasing public knowledge and awareness of various issues and can be pivotal in behavioral and legislative actions. With AMR projected to be the leading cause of mortality by 2050 (Bloom et al. 2018; Dadgostar et al. 2019) the public fundamental understanding of this phenomenon is required to help combat this projection. Effective communication has to be facilitated between researcher, policymakers, doctors, farmer, and the general public. Chapter 6 proposes a media model and review of literature emphasizing the use of rhetorical elements within storytelling that highlights key features such as who is presenting the information and how that influences audience reception. This model attempts to bring the complex dynamics of microbial systems to more digestible scale by providing metaphorical examples to cultivate a message the targeted audience can receive. This

more integrated approach may help to reform current methods and challenges of science communication.

6.2 Future Work

Future planning for this research includes the completion of the thermal hydrolysis study as outlined in appendix C, including analysis for antibiotic concentrations, resistance gene quantification, and bacterial community sequencing. Together these metrics will be able to showcase the influence of thermal hydrolysis and AD on both diary manure and wastewater biosolids pertaining to antibiotic resistance mitigation and microbial community dynamics. Once completed, this study will be submitted for publication.

Appendices

Appendix A: Supplementary Information for Antibiotic Resistance Partitioning in Dairy Manure Processed Through a Continuous High Temperature Rotary Drum Bedding Recovery Unit

Table A1. Primers and PCR Conditions

Gene	***Primer/Probe***	***Sequence (5'→3')***	***Exp. Amplicon size bp***	***Annealing °C used***	***Reference***
ermB	ermB-F	GGATTCTACAAGCGTACCTTGGA	92	54	Bockelmann et al. 2009
	ermB-R	GCTGGCAGCTTAAGCAATTGCT			
intI1	intI1-F	GCCTTGATGTTACCCGAGAG	196	54	Barraud et al. 2010
	intI1-R	GATCGGTCGAATGCGTGT			
tetM	tetM-F	GGTTTCTCTTGGATACTTAAATCAATCR	88	54	Peak et al. 2007
	tetM-R	CCAACCATAYAATCCTTGTTCRC			
tetX	tetX-F	GCAAGCGCCCATTACCCATAA	97	54	McKinney et al. 2018
	tetX-R	AAGGCATCCACCAACCCACT			
tetW	tetW-F1	GAGAGCCTGCTATATGCCAGC	168	54	Wang et al. 2016
	tetW-R1	GGGCGTATCCACAATGTTAAC			
tetQ	tetQ-F4	AGAATCTGCTGTTTGCCAGTG	169	54	Qian et al. 2016
	tetQ-R4	CGGAGTGTCAATGATATTGCA			
Sul1	sul1-F5	CGGCGTGGGCTACCTGAACG	433	54	Qian et al. 2016
	sul1-R5	GCCGATCGCGTGAAGTTCCG			
β-lac2	bla2-F1	GCTACAAACCTGATACGCGT	200	54	Wichmann et al. 2014
	bla2-R1	CGAACAAATTTCCGCCAGCG			
16S	Eub 338F	5'-ACT CCT ACG GGA GGC AGC-3'	195	54	Daims et al. 1999
	533R	5'-TTA CCG CGG CTG CTG GCA C-3'			

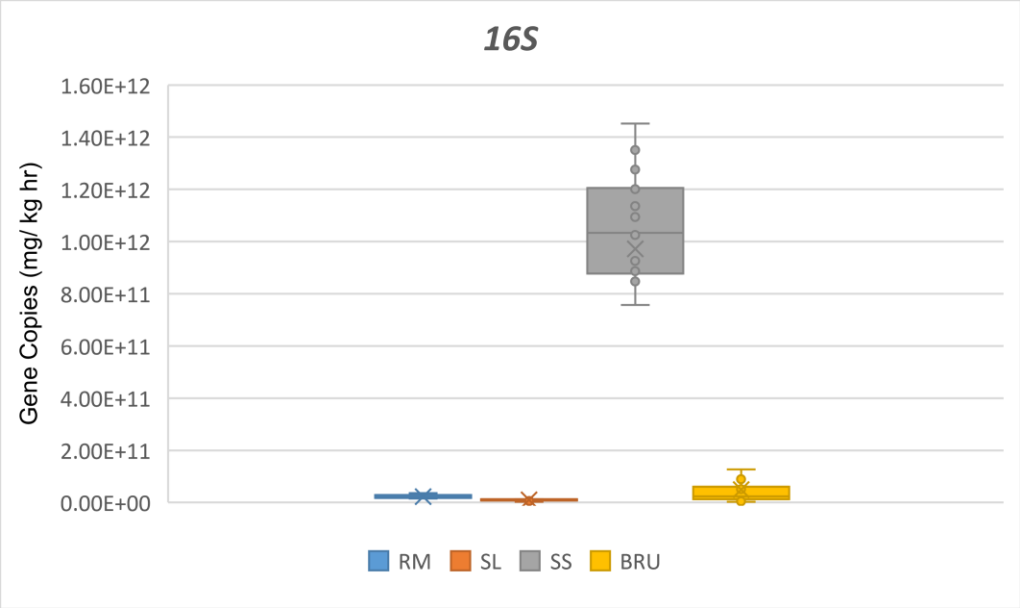


Figure A1. Average samples 16S relative abundance normalized per gram manure based on mass flow (mg/kg hr). Raw manure (RM), Separated liquid (SL), separated solids (SS) and BRU (blue, orange, grey and yellow respectively) each show distribution of samples.

Table A2. Nutrient and heavy metal concentrations detected within the manure and averaged mass flow of metals in manure and bedding recovery Unit (BRU). Results reported as averages and (± standard error).

Sample	Nitrogen (mg/L)	Phosphorus (mg/L)	Zinc (ppm)	Zinc Mass (mg/ kg hr)	Copper (ppm)	Copper Mass (mg/ kg hr)
Raw Manure	3524 (±176)	1483(±162.02)	21(± 7.48)	165 (±57.82)	6 (±1.35)	47 (±10.45)
Separated Liquid	2563 (±310)	1198 (±25.05)	18 (±1.22)	128 (±8.99)	5 (±0.51)	36 (±3.75)
Separated Solid	115 (±8.18)	35 (±2.36)	29 (±0.61)	12 (±0.25)	14 (±8.47)	6 (±3.47)
BRU	110 (±8.98	30 (±6.34)	35 (±2.19)	13 (±0.82)	10 (±1.71)	4 (±0.64)

Appendix B. Supplementary Information for Time Series Analysis of Antibiotic Resistance within Diary Manure under Mesophilic and Thermophilic Anaerobic Digestion

Table B1. Mesophilic (35°C) AD antibiotic removal rates during 43-day digestion

REMOVAL RATES (%)

	Total Tetracycline	Ampicillin	Erythromycin
DAY 3	+8	2	47
DAY 9	44	10	76
DAY 21	43	50	98
DAY 36	13	30	99
DAY 43	23	84	100

Table B2. Thermophilic (55°C) AD antibiotic removal rates during 43 day-digestion

REMOVAL RATES (%)

	Total Tetracycline	Ampicillin	Erythromycin
DAY 3	+7	35	14
DAY 9	78	9	76
DAY 21	58	54	92
DAY 36	52	25	94
DAY 43	67	49	97

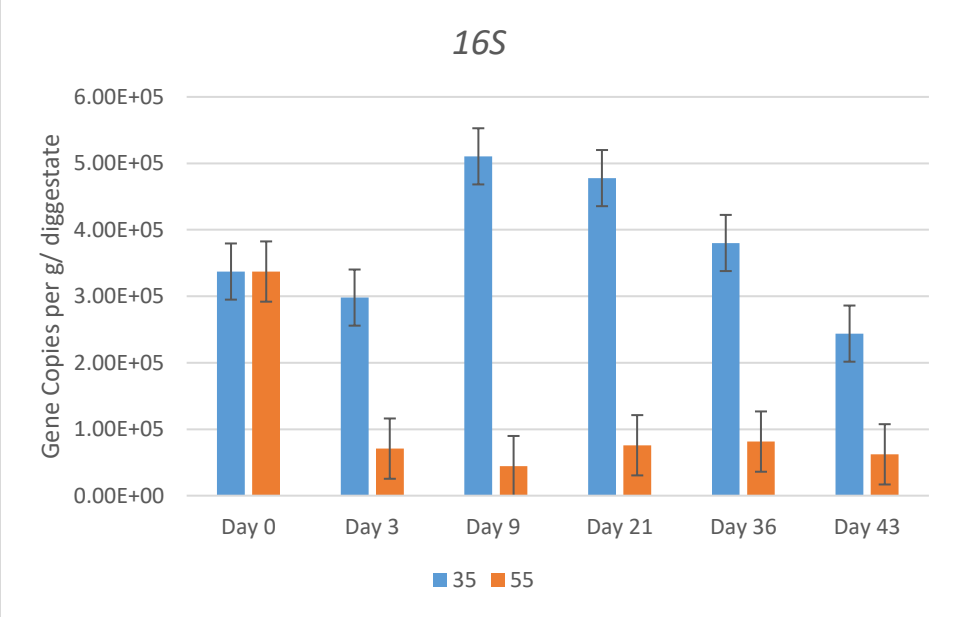


Figure B1. Gene copy values for *16S* detected in diary manure under 35°C and 55°C anaerobic digestion conditions of diary manure. Samples were quantified in triplicates and gene copies are based on grams of digestate analyzed, with blue bars for 35°C and orange bars for 55°C digestion temperatures.

Table B3: Pre and post BMP pH with % reductions in Total Solids (TS), Volatile Solids (VS), and Chemical Oxygen Demand (COD)

Sample	**pH**				**% Reductions**					
	35°C		55°C		35°C	55°C	35°C	55°C	35°C	55°C
	Pre-BMP	Post BMP	Pre-BMP	Post BMP	TS		COD		VS	
Day 0	7.87 ± 0.01	8.22 ± 0.01	7.87 ± 0.01	8.22 ± 0.01	N/A	N/A	N/A	N/A	N/A	N/A
Day 3	7.82 ± 0.03	7.97 ± 0.02	7.89 ± 0.02	7.59 ± 0.02	2%	33%	9%	11%	3%	37%
Day 9	7.88± 0.03	7.49 ± 0.01	7.96 ± 0.04	7.62 ± 0.01	18%	18%	4%	-1%	20%	22%
Day 21	7.92 ± 0	7.54 ± 0.09	7.83 ± 0.02	7.73 ± 0.05	25%	30%	77%	84%	27%	25%
Day 32	7.90 ± 0.03	7.17 ± 0.01	7.81 ± 0.01	7.47 ± 0.01	32%	26%	64%	60%	26%	21%
Day 43	8.01 ± 0.01	7.48 ± 0.06	7.89 ± 0.02	7.71 ± 0.02	29%	20%	53%	56%	32%	24%

Appendix C. Comparative Analysis of Thermal Hydrolysis Pretreatment and Anerobic Digestion Influence on Antibiotic resistance and Microbial Community Response in Dairy Manure and Wastewater Biosolids

C.1 Introduction

Intensive utilization of antibiotics to treat human and livestock animal's ailments has resulted in release of antibiotics into water and soil environments. Manure and biological waste movement and transport of antibiotics and antibiotic resistance genes (ARG) allows for greater dissemination and exposure risk potential that could have societal and environmental consequences. Emerging antibiotic pollutants in the environment in tandem with accruing resistance has quickly become an impending issue across the globe. With higher medical costs, longer periods of treatment, and increasing mortality for humans and animals, the world is re-evaluating health management (Lomazzi et al. 2019; Mutua et al. 2020). Key linkages between anthropogenic behaviors regarding antibiotic utilization and ecosystems can be examined at agroecosystem and wastewater treatment plant-scale (WWTP), as both sites are deemed potential hotspots for antibiotic resistance (AR) development and dissemination (Singer A.C. et al. 2016; Manaia et al. 2018; Graham et al. 2019). Subtherapeutic concentrations of antibiotic retreatment and high bacterial loads within manure and wastewater, even after treatment, have subsequent impacts on the surrounding microbial landscapes. Wastewater sludge, specifically, has been suggested to be a large reservoir of ARGs as they are an aggregate of influent streams

from a wide array of locations. Biological waste treatment technologies at these sites have been implemented to help reduce contamination risks by aiding in the removal of contaminants, reducing bacterial loads, and generating safer and more effective biosolids for land applications.

As more contaminants have been identified, including antibiotics and their downstream impact on resistance, there has been a growing demand for higher quality biosolids and more extensive inactivation/removal of pathogens, which has spurred more interest in alternative treatment methodologies (Ma et al. 2011). Thermal hydrolysis pretreatment (THP) involves the application of intense pressure (1-9 bars) and heat at or above autoclave temperatures (120 - 180°C) via steam injection for a designated amount of time. The combination of the pressure and heat followed by pressure release lyses the material within the sludge, leading to the destruction of cell walls and exposure of the cytoplasmic content. This process increases substrate availability and dewatering, which expedites the initial hydrolytic step of anaerobic digestion (AD) leading to reduced solid retention time and increased methanogen growth and corresponding methane (CH_4) production (Xue et al. 2015; Tong et al. 2019). Manure and other biological waste materials with high carbohydrate and protein content are highly favorable for enhanced hydrolysis processes through THP to enhance the release of organic matter and induce degradation (Xue et al. 2015; Barber 2016; Chen et al. 2018; Tong et al. 2019).

The ability of THP to destroy cell walls and release readily degradable biological components may translate to effective removal of antimicrobial resistance factors. Mesophilic anaerobic digestion (MAD) and thermophilic anaerobic digestion

(TAD) have been examined in previous studies to reduce most antibiotics, yet had mixed effects on ARG subtypes, as some studies have shown TAD to have greater removal efficiencies of various *tet* genes other studies have found MAD was more effective at reducing other ARGs. The additional pretreatment of the biosolids and manure through THP is suggested to assist in the mitigation of these antibiotic resistance parameters even further as discussed below. Ma et al. (2011) conducted a study on the impact of various sludge digestion conditions on sulfonamide, macrolide, and tetracycline resistance genes and integron 1, and confirmed DNA susceptibility to hydrolysis destruction through THP. Their findings show that all nine target genes (sulI, *sulII, ermB, ermF, tetO, tetW, tetC, tetG,* and *tetX*) and *intl1* were significantly reduced 1.59 – 2.60 log units and 2.46 log units, respectively, following THP. Later studies compared THP to other pretreatment techniques and supported the initial findings by Ma et al. (2011), displaying THP efficacy in ARG reduction (Pei et al. 2016; Tong et al. 2017, Tong et al. 2018, Sun et al. 2019). The temperature and lysis process that occurs within the THP process is also shown to increase antibiotics susceptible to high temperatures (Li et al. 2017b; Zhang and Li 2018; Sun et al. 2019). A study conducted by Zhang and Li (2018) study on the variation of antibiotics in sludge pretreatment and AD reported THP to reduce the total concentration of tetracycline, oxytetracycline, chlortetracycline, and azithromycin by 60 – 90%. Zhang et al. (2018) pretreatment comparison study found THP to reduce tetracycline, oxytetracycline, chlortetracycline, and azaamide by 78- 90%, with most of the AR reduction in these studies occurring after subsequent digestion regardless of mesophilic and thermophilic conditions in the AD after THP due to the bacterial

succession and activity credited for reduction. Cai et al. (2020) examined the impact of THP on intra and extracellular ARGs and reported lysis or dramatically decreased intracellular ARGs (p-value < 0.05) but the treatment appeared to lead to more extracellular ARG. Likewise antibiotics within the fluoroquinolone class have been found to increase in concentration after THP, demonstrating this technique is not effective on all antibiotics.

Since THP is a more advanced treatment, most studies have been primarily focused on wastewater sludge where this system is most likely to be utilized. Fewer studies have used this system for other complex substrates, such as dairy manure, or compared processing of both substrates. Dairy manure is more homogeneous than wastewater but presents a different composition of constituents, such as higher presence of humic substances and straw material that contain high levels of cellulose and lignin. Additionally, previous studies that have emulated lab-scale THP have used autoclave ovens that gradually heat and have lower levels of heat permeability through the substrate (Sun et al. 2022). The pilot THP unit utilized in this study replicates the conditions and processing of the full scale Cambi unit incorporated at WWTP that uses steam injecting and flash lysis.

Both substrates (dairy manure and WWTP sludge) are primary transporters of antibiotic resistance factors, yet no studies have compared this technologies' efficacy on these two different substrates, and specifically documenting substrate differences in ARG reductions/rebound, antibiotic degradation, and CH_4 yield in subsequent AD experiments. This study conducted a comparative analysis of THP impact on antibiotic resistance in both wastewater sludge and dairy manure. This study was

designed to: 1) Quantify antibiotic degradation and ARG abundance in a time series analysis of thermal hydrolysis pretreatment of dairy manure and wastewater sludge followed by thermophilic and mesophilic AD, 2) Quantify thermal hydrolysis efficacy on antibiotic resistance between the manure and wastewater sludge substrates, and 3) determine differences and shifts in bacterial community and ARG rebound during mesophilic digestion. These findings can help to distinguish the validity of THP as effective AD pretreatment for AR mitigation within various substrate. Also highlighting bacterial community response and significance regarding treatment effectiveness and the prevalence of ARGs.

C.2. Material and Methods

C.2.1 Sample Collection

Raw dairy manure slurry was collected in a five-gallon bucket from at the Beltsville Agricultural Research Center (Beltsville, MD USA) farm from dairy cows with limited to no use of antibiotics. For biosolids, the wastewater sludge settled from the primary tank at Little Patuxent Wastewater Treatment Plant (Laurel, MD USA) (prior to any biosolids lime or heat treatments) was collected in five-gallon buckets. Once collected, the samples were refrigerated at 4°C until use in the experiment.

C.2.2 Thermal Hydrolysis Pretreatment

Each sample was divided into four buckets each containing approximately 3 L. Two buckets were spiked with 1 L of 100 mg/L antibiotic solution containing oxytetracycline, erythromycin and ampicillin. After spiking, the antibiotic solution was mixed, and all samples allowed to settle for ~ 24 hr at room temperature prior to THP to allow the antibiotics to integrate into the sample.

Each mixture generated eight samples that were spiked and non-spiked with and without THP samples as shown in Table 5.1 for analysis. After ~24 hr, four samples from each substrate were spiked or non-spiked and taken to Loudoun Water Broad Run Water Reclamation Facility (Ashburn, VA USA) for treatment using a thermal hydrolysis pilot scale unit (CAMBI, Norway). The system can run a max of 5 L of sample per run, though this specific system generally processes 3 L per run. Substrates fed into the system are usually around 16% total solids (TS) of biosolids into the THP, resulting in an effluent of approximately 12% TS. The TS for both substrates were below 16%, and thus no adjustment was required. Once the substrate was fed into the THP, the sample was steam injected until it reached the desired temperature and pressure of 160°C at 6 bars PSI. Once these conditions were met, the system was allowed to run for 30 mins and then pressure was released for flash lysis. These conditions were within the parameters to produce Class A biosolids (US, EPA 1994). After each run was completed, the system was then thoroughly washed out with water and prepped for the next samples.

Table C1. Samples total solids (TS), volatile solids (VS) content and measurements for the biochemical methane potential (BMP) loading.

Sample	***TS (g TS/g wet mass)***	***VS wet (g VS/g)***	***Sample used for BMP (g)***	***Inoculum used for BMP (g)***
Dairy manure spiked w/ THP	0.081	0.083	12.514	148
Dairy manure non-spiked w/ THP	0.147	0.128	11.52	148
Dairy manure spiked no THP	0.106	0.094	10.464	148
Dairy Manure non-spiked no THP	0.094	0.088	7.929	148
Biosolids spiked w/ THP	0.02	0.018	53.795	148
Biosolids non-spiked w/ THP	0.02	0.01	51.123	148
Biosolids spiked no THP	0.025	0.023	44.14	148
Biosolids non-spiked no THP	0.025	0.028	40.02	148
Inoculum	0.02	0.013	0	148

C.2.3 Experimental Design

A batch digestion experiment was conducted based on biochemical methane potential (BMP) experiments to determine the energy production potential developed by Owen et al. (1979) in the Bioenergy and Bioprocessing Laboratory (College Park, MD, USA). In this batch study, mesophilic (35°C) anaerobic digester conditions were examined to determine differences in antibiotic degradation and reduction in ARGs in dairy manure and biosolids pretreated with THP. The batch assay was conducted over a 30-day retention period to develop time and temperature antibiotic degradation curves, with post-testing of antibiotic concentration. The experimental setup consisted of six bottles for each sample totaling 48 bottles with one bottle destructively sampled every 10 days, generating a 4-time points series (Day 0, 10, 20, and 30) over the 30

days to determine the effect of time on degradation. The non-THP samples were used as controls alongside six inoculum control bottles, resulting in 54 samples in total.

Mesophilic inoculums were obtained from on-site laboratory-scale inoculum reactors. Mesophilic inoculum was generated using USDA Beltsville Agricultural Research Center (BARC) dairy manure. The batch destructive assay was conducted in 300 ml serum bottles filled with a calculated amount of sample based on TS and corresponding inoculum until ~200 mL. The mass of substrate to inoculum ratio was 2:1 in each bottle based on VS measurements (Table 5.1). Prior to incubation, the headspace in each bottle was purged with 30% CO_2 and 70% N_2 to establish anaerobic conditions and sealed with a rubber septum. The assay bottles (n=6 per sample) were simultaneously subjected to 35°C condition in respective environmental chambers for 30 days. All assays, including the inoculum control and non-spiked manure, were performed in triplicate. Biogas production and CH_4 content of the produced biogas were measured daily during the first week of the experiment, approximately every other day the following week, and then bi-weekly for the remainder of the experiment. The frequency of the biogas measurements was based on the quantity of biogas produced. When biogas production was high, the biogas was purged (and measured) more frequently to prevent pressure build-up inside the serum bottles. Biogas production was measured via volume displacement using a 50-mL wetted glass gas tight graduated syringe with 2 mL gradations. The produced biogas was analyzed for CH_4, and carbon dioxide (CO_2) content by injecting 0.10 mL sample, using a luer-lock, gas tight syringe, into an Agilent HP 7890A GC (Agilent Technologies, Santa Clara, CA, USA), equipped with a thermal conductivity detector

(TCD) using the following parameters: (1) injection temperature of 250 °C, (2) detector temperature of 250°C, (3) oven temperature of 60 °C, and (3) a carrier gas flow rate of 8.6 mL He/min. The average CH_4 production in the control (inoculum only) was subtracted from the other treatments to account for CH_4 production attributed to the inoculum source, thus the results presented are the total CH_4 production from the dairy manure or WWTP sludge only. Biogas, CH_4 and CO_2 will be monitored over the 30-day digestion period.

C.2.4 Antibiotic Detection and Quantification

To measure antibiotic concentration and monitor antibiotic degradation pre and post THP and throughout mesophilic digestion, the extraction and analysis procedures outlined in Poindexter et al. (2022) were conducted. The extraction samples for antibiotic analysis were normalized by weight (grams) based on TS concentrations. Samples taken from each selected day during digestion were analyzed in triplicates. In brief, the extraction method was a two-step method that utilized ultrasonic and mechanical mixing using two separate solvent extractions, 0.1 M EDTA-McIlvaine followed by methanol. The two extracts were combined and diluted to a solvent concentration less than 2%. The diluted extract was then concentrated using solid phase extraction through a C-18 cartridge. The cartridge was eluted with methanol, and the eluent was concentrated under a steady stream of N_2 gas. The final concentrate was reconstituted to a known volume using a 50% acetonitrile and 50% deionized water (DI) mix and stored in the freezer at -20°C until liquid chromatography and tandem mass spectrometry (LC-MS-MS) analysis.

C.2.5 DNA Isolation And qPCR Resistance Gene Quantification

Genomic DNA from the four manure substrates was isolated using ~ 0.25 mg of the digestate samples using QIAamp DNA stool Kit (cat 51504, QIAGEN, Hilden, Germany) following the manufacturer's protocols. DNA extraction was performed in triplicate for each sample to account for extraction variable, efficiency, and heterogeneity of the samples. The quantity of the extracted DNA was determined and normalized based on Qubit 1.0 Fluorometer (Life Technologies, Grand Island, NY, USA) for DNA concentration and NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) for DNA sample quality analysis. DNA extract was diluted to 1 ng/uL in preparation for qPCR. All extracted DNA was stored at -20°C before qPCR analysis.

This study quantified the abundance of seven resistance genes including macrolide (*ermB*), beta-lactam (*bla-2*), sulfonamides (*sul-1*), tetracycline *(tetX*, *tetM*, *tetW*, *tetQ*), mobile genetic element (*intl1*) and 16S DNA for bacterial population normalization. Primer sequences of targeted genes listed Appendix A were cloned into the pGEM-T easy vector, purified using QIAprep Miniprep kit (cat# 27104, Qiagen, Hilden, Germany), and 16S illumina sequencing was used for gene verification. Verified plasmids were selected as standards for qPCR, quantified and adjusted to 1 ng/uL. Serial dilutions of the plasmids were conducted to generate an internal standard curve. Data extracted from standard curves had r^2 values > 0.99. All the qPCR analytics were performed using the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) following amplification of all genes:

heat inactivation at 95°C for 2 min, followed 40 cycles of 95°C for 5 sec (denaturation), 65°C for 10 sec (annealing), and 72°C for 10 sec (elongation). 20 uL qPCR reactions included 10 uL Forget-Me-Not qPCR Master Mix Hi-ROX (cat#31046, Biotium, Fremont, CA), 1 uL of forward primer, 1 uL of reverse primer, 5 uL of sample DNA, and 3 uL of dH_2O.

C.2.6. Microbial Community Analysis

All samples were frozen at -80°C until ready for analysis. The DNA was extracted from thawed samples using ~0.25 mg for the QIAamp DNA stool Kit (cat 51504, QIAGEN, Hilden, Germany) following the manufacturer's protocols. Samples lysed using the FastPrep-24 Instrument (MP Biomedical, Solon OH, USA) at 4.5m/s for 2 mins. Following extraction DNA was quantified using Qubit 2.0 Fluorometer (Life Technologies, Carlsbad CA USA). Extracts were then diluted to 6 ng/uL for amplification and sequencing. The V3- V4 regions of the 16S r DNA were amplified using 515F and 806F adapters. Amplified products were then cleaned and prepared for Illumina sequencing using 16S Metagenomic sequencing library preparation protocol (need catalog #). The 16S amplicon samples were then cleaned up using a AMPure XP beads (Beckman Coulter, Pasadena, CA, USA). The 8-nucleotide indexes and Illumina sequences were attached using Nextera XT Index Kit (Illumina, San Diego, CA USA) using N7 and S5 primers. Indexed samples were then re-cleaned using AMPureXP beads (Beckman Coulter, Pasadena, CA, USA).

Two uL of each indexed and cleaned sample were pooled together for 16S targeting. The pooled samples were quantified with Qubit 2.0 Fluorometer (Life

Technologies, Carlsbad CA USA). Pooled samples were then denatured and prepped for Miseq loading. Finally, samples were sent to the University of Maryland Baltimore, Genome Institute for sequencing using Miseq Sequencer (Illumina, San Diego, CA USA).

C.2.7. Substrate Characterization:

Samples were analyzed in triplicates at each time point for pH, TS, VS, and chemical oxygen demand (COD) within 24 hrs of collection, followed by volatile fatty acids (VFA) and nutrient analysis according to standard methods (APHA, 2005). The sample pH was measured using an Accumet Basic As 15 pH Meter. The TS and VS analyses were conducted using the Standard Methods (APHA, 2005). For TS analysis, 10 mL of aqueous sample (dairy manure and WWTP sludge) were weighed into pre-weighed and pre-dried (at 550 °C) porcelain crucibles. For TS analyses, the samples were dried at 105 °C until a constant weight was maintained. For VS analyses, the crucibles were placed in a furnace oven at 550°C until a constant weight was obtained. Nutrient analysis for ammonia, total Kjeldahl nitrogen (TKN) and total phosphorus (TP) samples were analyzed on the Lachat autoanalyzer (Quikchem 8500, Hach Company, Loveland, CO, USA) using the QuickChem method 13-107-06-2-D for TKN and method 13-115-01-1-B for TP. The COD concentration was measured using a Hach DR 5000 spectrophotometer (Hach Company, Loveland, CO, USA). The VFA (butyrate, propionate, acetate, and valeric) samples were acidified with concentrated sulfuric acid to a pH below 2 and filtered to 0.22 µm before injection into a HP 7890A GC (Agilent Technologies, Santa Clara, CA, USA), equipped with a

flame ionization detector (FID) with the following parameters: 1) injection temperature of 250 ºC, 2) detector temperature of 300 ºC, 3) oven temperature of 100 ºC for 2 minutes and increased by 10 ºC/min for a total run time of 10 min, and 4) a carrier gas flow rate of 1.80 mL He/min. For quality control, 10% of the samples included duplicates, spikes, and blanks.

C.2.8. Statistical Analysis

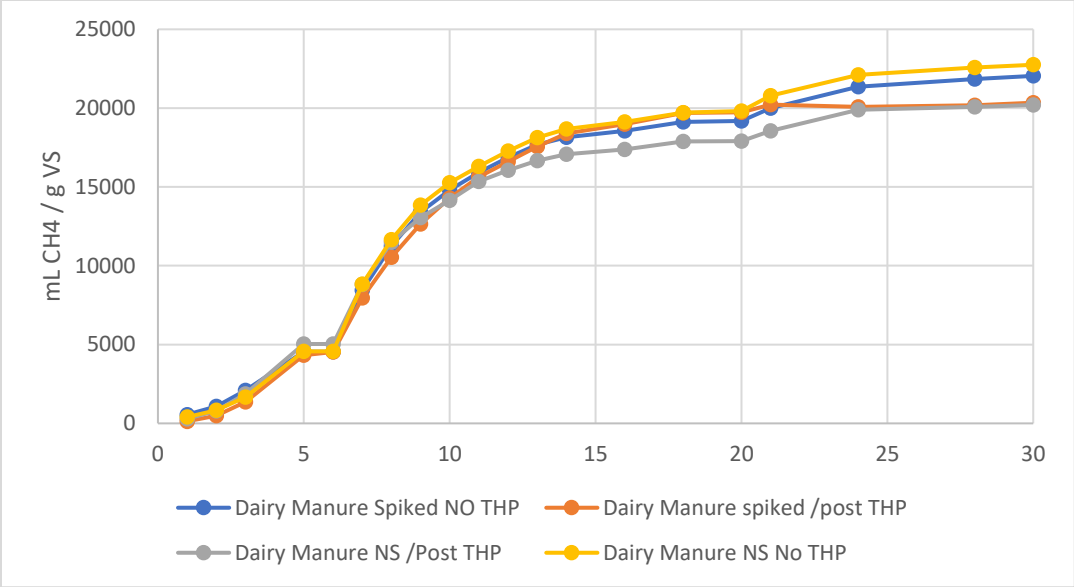
Statistical significance between substrates and pre- and post-THP and during mesophilic digestion were evaluated. A one-way analysis of variance (ANOVA) was conducted on all pre- and post-THP samples and digestion reactors to determine significant differences in antibiotic concentration, ARG abundance, cumulative CH_4, biogas production, TS, VS, COD, VFA, and nutrients. Temporal differences in bacterial community succession and diversity were examined for each sample. Differences based on sample time and substrate were considered to be significant for p-values < 0.05. Spearman correlation analysis was used to measure association of ARGs and digestate characteristics under mesophilic conditions.

C.3 Results and Discussion

C.3.1 Cumulative Methane Production

The average and cumulative CH_4 production found the biosolids to produce significantly higher (p-value <0.0001) CH_4 then the dairy manure samples. The biosolids CH_4 production ranged from ~4,4353 to 6,7310 ml CH_4 / g VS with the spiked post THP biosolids producing the highest yield throughout the 30-day study as

seen in Figure C1. While the dairy manure CH_4 production ranged between ~2,000 to 2,300 ml CH_4 / g VS. This agrees with the previous studies as biosolids are aggregate samples from numerous inputs resulting and nutrient rich and heterogenous substrate compared dairy manure that is more homogenous and has been pre-processed in the cow's rumen (Rafique et al. 2010; Passo et al. 2017; Wang et al. 2018; McVoitte and Clark, 2019).



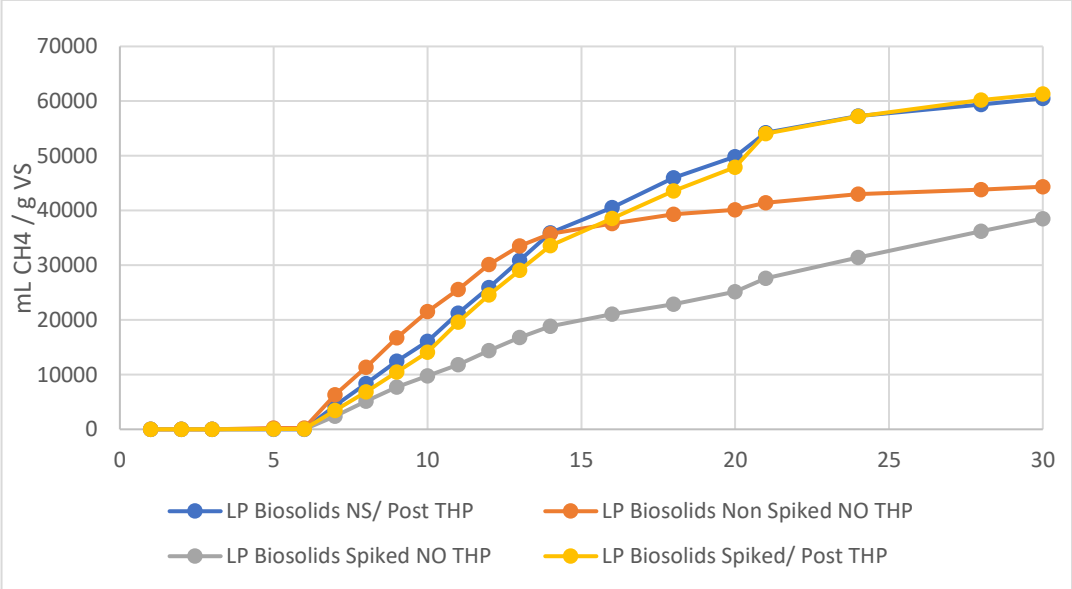


Figure C1. Average and cumulative CH4 production and standard error of dairy manure spiked and non-spiked with antibiotics and pretreated with thermal hydrolysis (THP) (A), spiked and non-spiked Little Patuxent (LP) wastewater biosolids CH4 production when spiked and no spiked with antibiotics ns pretreated with thermal hydrolysis.

THP processing had varied effects in the CH_4 production that was found to be substrate dependent. The non THP treated samples for the reported higher CH_4 yields at 2,2046 and 2,751 mL CH_4 / g VS for the spiked and non-spiked dairy manure respectively (Figure C1). The lower overall CH_4 production for the THP treated samples could be the of the formation of recalcitrant compounds formed under

elevated temperatures from hemicellulose and lignin solubilization (Rafique et al. 2010) The compounds can aggregate together and potentially be toxic ad inhibitory to AD microbial community functionality (McVoitte and Clark, 2019).This study found both the spiked and non-spiked untreated manure higher cumulative CH*4* to be marginally significant (p-value = 0.0320 and p-value = 0.0428) compared to the THP treated manure samples regardless of antibiotic spike. These results contrast Passos et al. 2017 study on thermochemical pretreatment of dairy manure AD found the pretreatment to not significantly impact CH4 yields compared to the positive controls at 0.29 and 0.33 L CH_4 g/ VS.

Yet, THP had the opposite effect on the biosolids with the pretreated CH_4 producing significantly higher yields of CH_4 regardless of the samples being spike or non-spiked with antibiotics (p-value <0.0001). An initial lag was documented in the study for all the biosolids samples with little to no CH_4 production until ~day 6. The phenomenon observed in this study is likely the results of some recalcitrant compounds in the much more complex biosolids that may take longer to convert to more accessible metabolite or may exhibit inhibitory effects. Regardless, THP processing significantly increased CH_4 yields and appeared to negate any potential impacts of the antibiotic spikes as the non-spiked and spiked THP samples had comparable (p-value = 0.4908) cumulative CH_4 production at 60,497 and 61,310 mL CH_4 / g VS accordingly. THP processing allows for sewage sterilization, and the reduction of organic micropollutants, ARGs, and ARB (Wang et al. 2019; Shin et al. 2022). Studies have shown THP to increase sewage sludge biogas production by 20 – 55% (Keep et al. 2010; Perez-Elvira et al. 2010 and Donoso-Bravo et al. 2011). THP

is set to help expedite AD's rate limiting step of hydrolysis with its lysis process, which explains why the non THP treated biosolids samples reported the lowest yields at 44,353 and 38,522 mL CH_4 / g VS for non-spiked and spiked samples (Figure C1). The spiked biosolids with no THP were also reported to have significantly lower (p-value < 0.001) CH_4 compared to the non-spiked demonstrating that the antibiotic solution may have inhibited some the microbial activity. Antibiotic such as tetracycline were reported to have lethal effects to AD microbial population at 8.5 mg/ L that led to inhibition of substrate utilization and biogas generation (Cetecioglu et al. 2013) while Ni et al. 2020 study suggested the macrolide roxithromycin to in negatively impact CH_4 production by hindering acidogenesis and methanogenesis in activated sludge digestion. Though this study samples were spiked with different antibiotics compared to the two previous studies just referenced, the antibiotics incorporated were of the same antibiotic class which could be indictor of residual class wide effects the methane production pattern of both sets of samples show how this the treatment has adverse effect depending on the substrate thus the value of this treatment in regard to CH_4 production is likely going to be industry dependent as well.

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