

Tracking Antibiotic Resistance throughout Agroecosystems

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ABSTRACT

Widespread use of antibiotics in livestock production can result in the dissemination of bacteria carrying antibiotic resistance genes (ARGs) to the broader environment. Within agroecosystems, ARGs can pose a risk to livestock handlers, farmers, and ultimately consumers. The overall goals of this dissertation are to examine the presence of resistance (antibiotic, metal) in agricultural soils and evaluate the most critical potential points of best management control of antibiotic resistance spread along the agricultural production chain. The relative impacts of agricultural practices, manure management, native soil microbiota, and type of crop grown and harvested on the agricultural resistome are multi-dimensional and cannot be captured via a single analytical technique or by focusing on one specific point in the agricultural process. Culture-, molecular “indicator”-, and next-generation sequencing- based methods were employed to characterize antibiotic resistance via taxonomic and functional profiles on the broader manure, soil, and vegetable surface microbial communities through 16S rRNA amplicon sequencing and shotgun metagenomics. Although antibiotic concentrations dissipated in the soil after 28 days after amendment application, antibiotic resistance presence was recoverable throughout the entire 120d growing season in the compost and manure amendments, the amended soil, and on vegetable surfaces. The addition of organic fertilizers increased antibiotic resistance presence compared to background levels. Further, metals and metal resistance were also measured in the amended soils and were found to be at greater levels in the inorganically fertilized soils compared to the manures and compost amended soils. Analysis of the widespread agroecosystem microbial community composition and broader metagenome has characterized varying genera profiles in the soil and on the vegetable surfaces and specific ARG and mobile genetic element (plasmid) co-occurrences. These co-occurrences highlight which ARGs may be most critical for future antibiotic resistance dissemination research. It is imperative to employ multiple methods when measuring agricultural resistance, as one method alone may miss significant patterns and lead to different best management recommendations. Linking the livestock manure, soil, and vegetable surface-associated ARBs, ARGs, resistomes, and microbiomes will help identify critical control points for mitigation of agricultural dissemination of antibiotic resistance to the environment and food production.

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GENERAL AUDIENCE ABSTRACT

By 2050, it is estimated that antibiotic resistant infections will be the leading cause of death worldwide. It is important to consider human, animal, and environmental health when researching antibiotic resistance, which is known as a “One Health” approach. In this dissertation work, I focus on the environmental side of antibiotic resistance in our agricultural systems. Agriculture is a known source of antibiotic resistance due to its use of antibiotics in livestock as a treatment for illness, and in some instances, as a growth promoter. Over one growing season, I measured antibiotic resistance in an agricultural setting using many techniques. First, I analyzed the effects of inorganic (chemical) versus organic (manure and compost) fertilization on antibiotic resistance in the soil. I measured antibiotic resistance by growing antibiotic resistant bacteria, quantifying specific antibiotic resistant genes (ARGs) using DNA amplification, and quantifying all the ARGs in the soil using a next-generation sequencing (NGS) technique called shotgun metagenomics. I found that adding manure to the soil increases ARGs compared to background soil levels, and that composting is an effective management strategy in decreasing ARGs in the soil over time. Second, I analyzed the same effects of fertilization on metal resistance in the soil. I was able to use the same NGS dataset to measure metal resistance genes (MRGs). I found that adding inorganic chemical fertilizer increases MRGs in the agricultural soils compared to the organic (manure or compost) fertilizer. Additionally, I studied the microbes that live in the agricultural soils using another kind of NGS data specific for microbial identification. I found that although there were small differences between the microbial populations in the soil when fertilizers were added, they returned to similar composition over the growing season. Lastly, I measured antibiotic resistance and microbes throughout the entire agricultural system. I picked the point of fertilization (manure management), soil, and the lettuce surface to evaluate if antibiotic resistance spreads from the farm to the vegetable that ends up on a consumer’s plate. I found that at each point antibiotic resistance is present, but at different levels. Composting reduces ARGs compared to raw manure. Agricultural soils may act as a natural buffer to antibiotic resistance. Lettuce plants grown in compost fertilized soils have less ARGs than lettuce plants grown in manure. There are many agricultural management practices that effectively reduce antibiotic resistance and using all of them plus many measurement methods will ultimately help farmers and consumers reduce antibiotic resistance in our agricultural systems.

Dedication

I dedicate this dissertation to my father and mother.
I will be forever grateful for the sacrifices you made to support my academic journey.

“The aim of science is to discover and illuminate truth. And that, I take it, is the aim of literature, whether biography or history or fiction. It seems to me, then, that there can be no separate literature of science.” -Rachel Carson

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Table of Contents

List of Figures	ix
List of Tables	x
List of Abbreviations	x
Preface and Attributions	xi
Chapter 1. Introduction	1
1.1. Antibiotic Resistance in Agroecosystems.....	1
1.2 Methodological Strategies for Tracking Antibiotic Resistome	3
1.3 Resistance Mechanisms	6
1.4 Current Manure Best Management Practices	7
1.5 “Moo”ving Targets	7
References.....	8
Chapter 2. Research Goals and Specific Questions	12
2.1 Methods Comparison of the Agricultural Soil Resistome	12
2.2 Microbial Communities and Cross-resistance of metals and antibiotic resistance genes in Agricultural Soils.....	12
2.3 Agricultural Resistomes, Microbial Communities, and Co-Occurrences.....	12
Chapter 3. Cross-comparison of methods for quantifying antibiotic resistance in agricultural soils amended with dairy manure and compost	13
3.1 Graphical Abstract	13
3.2 Abstract	13
3.3 Introduction.....	14
3.4 Materials and Methods.....	18
3.4.1 <i>Study site and amendment description</i>	18
3.4.2 <i>Soil Collection and DNA Extraction</i>	19
3.4.3 <i>Culture analysis</i>	19
3.4.4 <i>Molecular analysis</i>	19
3.4.5 <i>Shotgun metagenomic sequencing analysis</i>	20
3.4.6 <i>Statistical analyses</i>	21
3.5 Results.....	22
3.5.1 <i>Trends in qPCR measures of AR targets</i>	22
3.5.3 <i>Annotation of class 1 integrons in metagenomic data</i>	26
3.5.4 <i>Comparison of culture-, molecular-, and metagenomic-based measures of resistance</i>	27
3.6 Discussion	29
3.6.1 <i>Detection of resistance markers in agricultural soils</i>	29
3.6.2 <i>Differences in detection of antibiotic resistance by associated drug class</i>	30
3.6.3 <i>Metagenomic diversity and subsequent limitations in agricultural soils</i>	31

3.6.4 <i>Moving forward: Recommendations for AR detection in agricultural soils</i>	32
3.6.5 <i>Limitations</i>	33
3.7 <i>Conclusions</i>	34
3.8 <i>Acknowledgments & Funding</i>	35
References	36
Supplemental Information	42
Chapter 4. Examination of metal presence and metal resistance within inorganically and organically fertilized agricultural soil microbial communities	50
4.1 Abstract	50
4.1.1 <i>Importance</i>	51
4.2 <i>Introduction</i>	51
4.3 <i>Materials and Methods</i>	54
4.3.1 <i>Experimental Design</i>	54
4.3.2 <i>Soil Sampling and DNA Extraction</i>	55
4.3.3 <i>Metals Analysis</i>	55
4.3.4 <i>Microbial Community Analysis</i>	56
4.3.5 <i>Metagenomic Analysis</i>	57
4.3.6 <i>Statistical Analysis</i>	57
4.3.7 <i>Data Availability</i>	58
4.4 <i>Results</i>	59
4.4.1 <i>Metal Concentrations in Soils</i>	59
4.4.2 <i>Soil Microbial Community Composition over the Growing Season</i>	60
4.4.3 <i>Metals Resistance Genes in Soils</i>	63
4.4.4 <i>Cross-resistance of antibiotic- and metal resistance genes</i>	65
4.5 <i>Discussion</i>	66
4.5.1 <i>Metals Enrichment in agricultural soils</i>	66
4.5.2 <i>Treatment effect on soil microbial community</i>	67
4.5.3 <i>Heavy metals, MRGs and ARGs correlation in agricultural soils</i>	68
4.5.4 <i>Potential agricultural soil assembly and biases</i>	69
4.6 <i>Conclusions</i>	69
4.7 <i>Acknowledgments & Funding</i>	70
References	72
Supplemental Information	77
Chapter 5. Characterizing the antibiotic resistome and associated microbial communities at multiple points along the vegetable production chain	86
5.1 Abstract	86
5.2 <i>Introduction</i>	87
5.3 <i>Materials and Methods</i>	90

5.3.1 Study Overview	90
5.3.2 Field Study Design and Treatment Description.....	90
5.3.3 Amendment, Soil, and Vegetable Sample Collection	91
5.3.4 DNA Extraction.....	92
5.3.5 Shogun Metagenomic Sequencing	92
5.3.6 Antibiotic Resistome Analysis	93
5.3.7 ARG-MGE Co-Occurrence and Resistome Risk Analysis	93
5.3.8 Microbial Community Analysis.....	94
5.3.9 Statistical Analysis	94
5.4 Results.....	95
5.4.1 Comparing ARG Profiles Across Amendments, Soil, and Lettuce in the Vegetable Production Chain.....	95
5.4.2 Co-Occurrence of ARGs across the Vegetable Production Chain	98
5.4.3 Relative Resistome Risk Comparison.....	100
5.4.4 Taxonomic Composition Across the Vegetable Production Chain.....	100
5.4 Discussion	103
5.4.1 Synopsis of the Agricultural Resistome.....	103
5.4.2 Distinct Agricultural Microbial Communities	104
5.4.3 ARG-MGE Co-Occurrence highlights integral focus areas	105
5.4.4 Assembly Difference along the Agricultural Production Chain	106
5.4.5 Limitations	107
5.5 Conclusions.....	107
5.6 Acknowledgements & Funding	108
References.....	110
Supplemental Information	116
Chapter 6. Conclusions.....	125
Chapter 7. Recommendations & Future Work.....	126
References.....	128
Appendix A.....	129
Appendix B	129

List of Figures

Figure 1-1: Schematic of Antibiotic Resistance Spread in Agroecosystems.....	3
Figure 1-2. The Resistance Matrix and Associated Quantification Methods.	4
Figure 3-1. Treatment and time effects on the relative abundance (i.e., normalized to 16S rRNA genes) of genes quantified via qPCR in amended and un-amended soils over the 120-d study in Blacksburg, VA.	23
Figure 3-2 Total ARG relative abundance in soils.	25
Figure 3-3. Venn Diagram of total number of soil ARGs	26
Figure 3-4. A methodological decision-tree	33
Figure 4-1. Relative abundance of major phyla.....	62
Figure 4-2. Non-metric dimensional scaling (NMDS) plot using the Bray-Curtis dissimilarity distances to compare the bacterial microbiota profiles.	63
Figure 4-3. Total MRG relative abundance in soils.....	65
Figure 5-1. Non-metric multidimensional (NMDS) scaling of the Bray Curtis dissimilarity distances to compare agricultural ARG profiles.	96
Figure 5-2. Venn Diagram of total number of agricultural ARGs.....	96
Figure 5-3. Unique ARG-MGE co-occurrences	99
Figure 5-4 Agricultural microbial community alpha and beta diversity	102

List of Tables

Table 3-1. Summary table of antibiotic resistance studies in agroecosystems.....	16
Table 3-2. Correlation of antibiotic resistance quantification methods.....	28
Table 4-1. Summary statistics of MRGs characterized within the soils.	60
Table 4-2. Cross-resistant ARGs and MRGs agricultural soils.	66

List of Abbreviations

AR = Antibiotic Resistance
ARB = Antibiotic Resistant Bacteria
ARG = Antibiotic Resistance Gene
BDL = Below Detection Limit
BLQ = Below Limit of Quantification
LOD = Limit of Detection
LOQ = Limit of Quantification
MGE = Mobile Genetic Element
MLS = Macrolide-Lincosamide-Streptogramin
MRG= Metal Resistance Gene

Preface and Attributions

Chapter 3 was co-authored by Leigh-Anne Krometis, W. Cully Hession, and Amy Pruden, and has been published in the *Science of the Total Environment* (Wind et al., 2020).

Chapter 4 was co-authored by Leigh-Anne Krometis, W. Cully Hession, Brian Badgley and Amy Pruden. It is in preparation for publication to the journal of *Applied Environmental Microbiology*.

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Chapter 1. Introduction

1.1. Antibiotic Resistance in Agroecosystems

Antibiotic resistance is a serious human and animal health concern. Antibiotic resistant related infections are predicted to cause ten million deaths globally between 2014 and 2050, with an associated production loss to the world economy of one-hundred trillion USD (O’Neil, 2014). In 2019, there were 2.8 million reported resistant infections resulting in 35,000 deaths annually in the United States (CDC, 2019). In conjunction with these sobering statistics, the World Health Organization (WHO) strongly encourages the development of holistic action plans to preserve drug efficacy for usage in humans and animals (European CDC, 2015). There is increased recognition that the preservation of antibiotic efficacy requires not only examination of clinical protocols and nosocomial infections, but also the design of best practices specific to agroecosystems (Zhao et al., 2020). European and North American countries have introduced new policies and regulations to mitigate antibiotic resistance dissemination, including requirements for veterinary oversight, judicious antibiotic use, antibiotic sales surveillance, and animal-by-product (i.e. manure and slurry) management (UK HM Gov, 2015; FDA, 2018, WHO, 2017). However, as the need for food and agricultural products (e.g. crops, livestock, and livestock associated products) increases globally to meet the demands of a growing population, there is concern that environmental contamination and associated human illness may increase in nations that have not yet adopted these antibiotic resistance mitigation strategies and policies (Van Boeckel et al., 2015).

Although previous national-scale examinations of bans on antibiotic usage to promote livestock growth indicate significant and rapid decreases in the incidence of antibiotic resistant fecal bacteria among animals (Aarestrup et al., 2001; EC, 2005; FDA, 2013), it is important to note that these stringent regulations still do not eliminate the presence of resistant bacteria entirely. Continental-scale examinations of producer compliance and antibiotic resistance incidence in livestock following more recent widespread legislation in Europe and the United States have yet to be completed. Despite increasingly stringent North American and European policies, the global use

of antibiotics to support livestock agriculture is expected to rise 67% by 2030 to keep up with the growing demands of the global food chain (FAO, 2020).

Antibiotics are used for treatment, control (metaphylaxis), prevention (prophylaxis) and/or growth promotion in livestock (Capita and Alonso-Calleja, 2013). Agricultural industries accounted for at least 75% of all antibiotic usage in the US and EU combined in 2014 (OECD, 2016). Between 2009 and 2017, there was a 14% decrease in antimicrobial drugs approved for use in livestock in the US (FDA, 2017), which is likely due to the increase in regulation previously highlighted. In 2015, 9.7 million kg of antibiotics considered critical for human health were sold in the US to support livestock production (FDA, 2015; WHO, 2017). Encouragingly, antimicrobial drug sales for use in livestock production decreased 33% in the United States the following year (2016-2017) (FDA, 2017); however, it is unclear what immediate impact this will have given the long-term widespread use of livestock-derived manures and composts on agricultural lands.

Current and predicted trends in therapeutic usage of antibiotics coupled with the high excretion rates (40-90%) of antibiotic residuals in livestock urine and feces (Arikan et al., 2009; Kemper, 2008) suggest that agricultural soils fertilized with livestock manure amendments may serve as a reservoir for antibiotic resistance that can be disseminated into the environment more broadly. By 2050, 470 million tons of meat and 3 billion tons of grain products will be required to meet the food needs of the global population, which represents a 70% increase from 2009 production estimates (FAO, 2009). At least 1.66 billion ha of harvested agricultural land will be required to meet these production targets (FAO, 2012), with an increase in fertilizer likely required to assure sufficient yields. Therefore, as food production continues to increase to meet global demands, there is the potential for a concurrent reliance on livestock-derived organic fertilizer (i.e. manure, slurry, compost), rendering soil a significant environmental reservoir for antibiotic resistance. Given the complexity of agroecosystems, it is critical to consider multiple points of intervention to reduce antibiotic resistance, including herd health management, manure treatment and application practices, and crop harvesting and preparation (Figure 1-1).

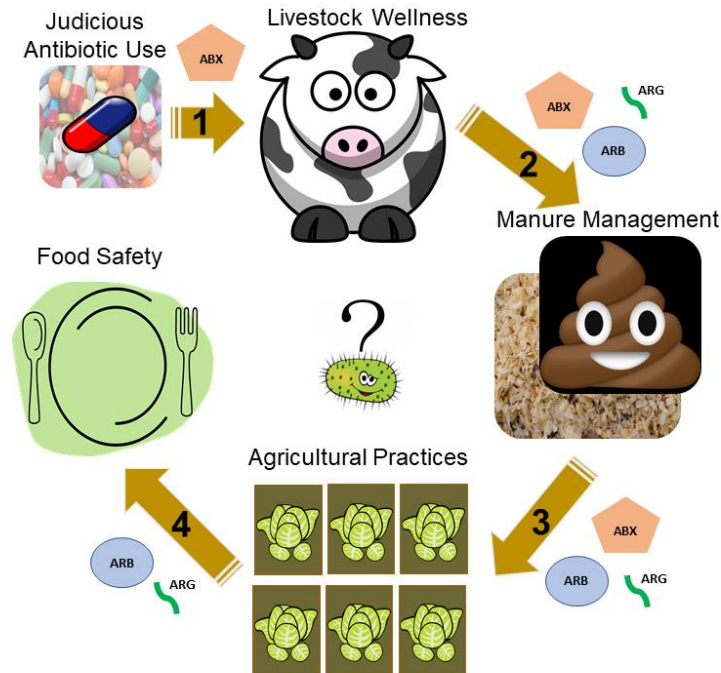


Figure 1-1: Schematic of Antibiotic Resistance Spread in Agroecosystems. Potential antibiotic resistance dissemination along the agricultural production chain by means of: (1) administration of antibiotics to livestock; (2) implementation of manure best management practices, including composting; (3) growing crops in agricultural fields amended with compost, and (4) vegetable harvesting and sanitation practices.

1.2 Methodological Strategies for Tracking the Antibiotic Resistome

The antibiotic resistome comprises all antibiotic resistance genes (ARGs) and precursor genes (cryptic embedded genes, genes that encode protein resistance etc.) found in bacteria, whether pathogenic or non-pathogenic (Wright, 2007). The natural diversity of the agricultural soil resistome is potentially expanded through the addition of manure-derived amendments (Chen et al., 2019; Fang et al., 2015; Udikovic-Kolic et al., 2014). When considering the potential for resistance in a given ecosystem, it is critical to focus not only on measures of resistant bacteria (ARB) or genes coding for resistance (ARGs) but also potential selection pressures, such as residual antibiotics and other contaminants like metals. Examination of the “resistance matrix” must consider not only the presence of ARB and ARGs but also antibiotic presence, autochthonous bacterial communities, and their associated functions through multiple detection methods (Figure 1-2). Characterizing antibiotic resistance for each resistant determinant (ARB, ARG, antibiotic) will allow for a more systematic and thorough approach to understanding how resistance moves within agroecosystems.

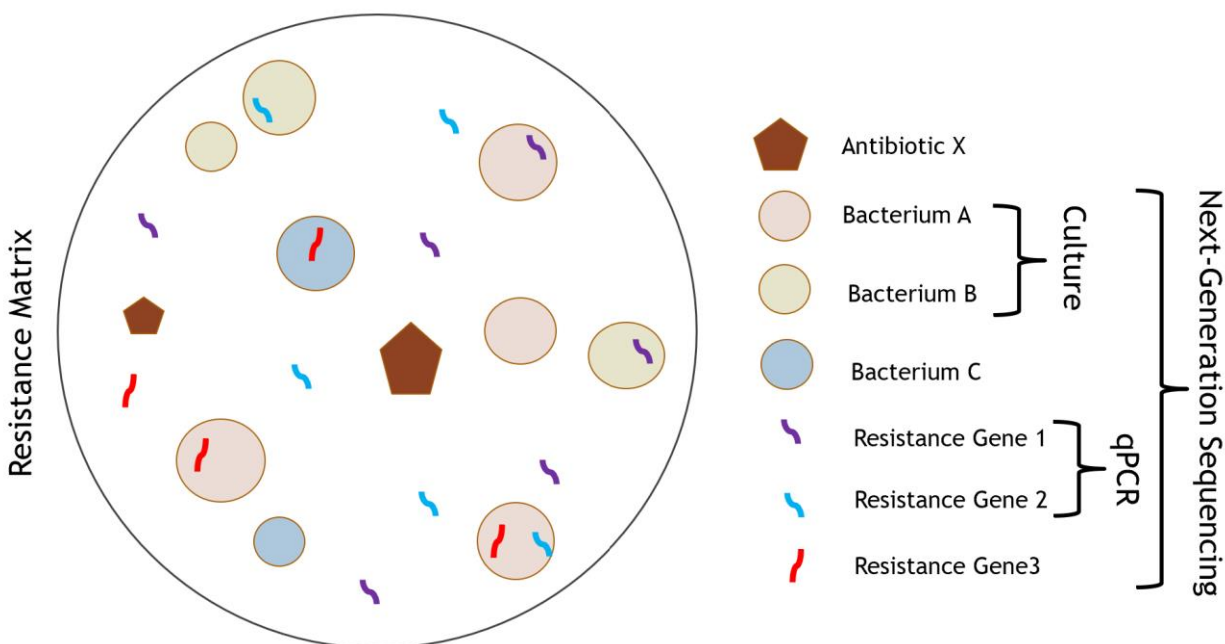


Figure 1-2. The Resistance Matrix and Associated Quantification Methods. The “resistance matrix” is comprised of potential antibiotic resistance sources and selection pressures the soil resistome may be affected by. These include antibiotic presence, antibiotic resistance gene presence, bacterial communities and associated functional characteristics. These resistance determinants (ARGs) can be either expressed or not expressed, in viable hosts or non-viable hosts, and in pathogenic or non-pathogenic bacteria. Antibiotic resistance can be measured through a multitude of methods including but not limited to culture, molecular (qPCR), and NGS techniques.

Broad scale evaluations of the impacts of agricultural practices on amendment, soil, and crop associated antibiotic resistance are often limited as quantification focuses on only one part of the Resistance Matrix. Multiple analytical strategies, including culture-, molecular-, and next-generation sequencing (NGS)-based techniques are currently used to detect and quantify antibiotic resistance in agricultural samples (manure, compost, soil, vegetable surface etc.); however, it is critical to recognize that the information obtained from these different methods are not necessarily interchangeable (Figure 1-2). The direct recovery of resistant organisms via culture isolates specific microorganisms based on their known taxonomy and functions (i.e. bacteria vs fungi, selective vs non-selective growth media). However, laboratory culture can only quantify what is being targeted and fails to encompass the soil’s microbial diversity. The majority (~99%) of microorganisms cannot be cultured and must be quantified via molecular methods that recover nucleic acids (Vartoukian et al., 2010). Perhaps most well-known, the quantitative polymerase chain reaction (qPCR) has the ability to quantify specific resistance genes (e.g. nucleic acid sequences) (Pace, 1997). Reliance on qPCR is widespread within the field of microbial ecology as

it allows for relatively rapid targeted gene characterization from environmental samples; however, it does not identify the species of bacteria carrying these genes and does not verify whether the genes are expressed.

Next-generation sequencing (e.g. shotgun metagenomics, 16S rRNA amplicon sequencing) has emerged in the past decade as a strategy that allows for simultaneous characterization of all bacteria and their functional genes in a single sample. Shotgun metagenomics therefore provides a broad evaluation of resistance in sequenced soil bacterial communities without a priori selection of target genes via qPCR or target bacteria via culture (Chambers et al., 2015). However, at present shotgun metagenomics remains semi-quantitative, can be limited by low sequencing depth, and is expensive when compared to culture and qPCR techniques.

As each detection method highlights a different aspect of the “resistance matrix” (i.e. a living resistant microorganism, the presence of an ARG, the soil community abundance etc.), all methods must be used together to observe the entire scope of the agricultural resistome. Recent examinations of the resistome in agricultural soils pair multiple quantification methods to compare the relationships between soil resistomes and the microbial communities present (Qian et al., 2016; Su et al., 2014; Zhu et al., 2013). Examinations across methodological strategies can reveal broad trends, e.g. agricultural microbial communities, and their associated soil resistomes are altered when livestock-derived manure amendments are added to the soils as a source of organic fertilization. However, distinct differences in trends become apparent depending at which scale resistomes are being evaluated. Examinations of soils amended with compost vs raw manure in controlled benchtop scale greenhouse studies revealed persistent and distinct changes in the resistome between soil amendment types (Chen et al., 2019, Smith et al., 2019); field-scale evaluation of these same treatments indicated that resistome differences dissipated after a single growing system (Lopatto et al., 2019). The majority of past studies examine single points in the agroecosystem, e.g. manure application or livestock management. Ideally, the use of multiple quantification methods at multiple stages along the agricultural chain could be used to facilitate the evaluation of critical points of potential control of antibiotic resistance dissemination and human exposure (Figure 1-1). Examinations at the field scale can provide agricultural stakeholders

and policy makers the most accurate and reproducible insights for mitigating antibiotic resistances in agroecosystems.

1.3 Resistance Mechanisms

Resistance can occur through natural selection as bacteria with favorable natural genetic mutations transfer these genes vertically (Davies and Davies, 2010), or it can be acquired through the horizontal transfer (HGT) of resistance genes between cells (Aminov and Mackie, 2007; Heuer et al., 2011). HGT can occur through three possible genetic mechanisms: (1) transformation, (2), conjugation, and (3) transduction (Aminov and Mackie, 2007). Therefore, even non-pathogenic ARB can play a critical role in the spread of resistant infections because their corresponding ARGs may be transferred to human pathogens by plasmids, transposons, integrons, and mobile genetic elements (MGEs) (Gillings et al., 2015; Xie et al., 2018).

An emerging area of interest in the design of agricultural best management practices is the interaction of metals and antibiotics in agricultural soil reservoirs, as metal resistance genes (MRGs) and ARGs may be co-selected (Pal et al., 2017). Co-selection, in this case, is the ability of metal presence to drive and promote antibiotic resistance in the soils. Co-selection can occur through co-resistance or cross-resistance. Co-resistance occurs when MRGs and ARGs are co-located in the same cell (plasmid, mobile genetic element, transposon) (Pal et al., 2015). Cross-resistance describes when the same resistance mechanism (ie. efflux pump, enzymatic inactivation, cell permeability) confers resistance to both metals and antibiotics (Chapman, 2003). Metagenomics provides an efficient way to broadly evaluate the potential of co-selection within agricultural microbial communities through co-occurrence analysis (Luby et al., 2016; Pal et al., 2015; Yu et al., 2017). Addressing how co-selection in agricultural soils occurs via co- or cross-resistance is important as this mechanism allows for antibiotic resistance in the absence of antibiotics and will aid in developing best management practices.

1.4 Current Manure Best Management Practices

The use of manure-derived amendments in agricultural systems simultaneously improves soil quality while providing a means for onsite waste disposal and recycling (Risse et al., 2006). To preserve this agricultural practice while protecting the public health, best management practices must be designed intentionally to prevent further antibiotic resistance dissemination. A common management practice used globally to reduce overall pathogen content is composting. Composting is highly recommended by the USDA Food Safety Modernization Act (FSMA) to reduce potential foodborne pathogens in subsequently amended agricultural soils (FDA, 2015; Sharma et al., 2009; USDA Organic, 2012). Although previous work supports the use of composting to increase nutrient ratios and decrease pathogens, it is unclear whether this practice will completely negate any risk of antibiotic resistance spread. Temperature plays a significant role in ARG abundance following composting, and many studies emphasize that continuous thermophilic composting that maintains a core temperature of at least 55°C, is the best approach with regard to pathogen and ARG removal in compost (Qian et al., 2016; Ray et al., 2017; Keenum et al., in revision.). Although past efforts have observed significant decreases in ARGs in livestock manures during the composting process, ARBs and ARGs are often still detected in agricultural soils following compost amendment (Marti et al., 2013; Udikovic-Kolic et al., 2014; Wind et al., 2018). Given the additional cost and management required to compost, it is critical to determine whether this process should be recommended to reduce antibiotic resistance dissemination in agroecosystems, and to understand any residual risks.

1.5 “Moo”ving Targets

Identifying the most effective strategies to mitigate the spread of antibiotic resistance in agroecosystems requires a holistic approach that simultaneously examines the presence of resistant bacteria, incidence of specific genes of interest, shifts in microbial community structure, and patterns in the overall soil resistome. This requires the explicit consideration of data from culture-based, molecular, and next-generation sequencing techniques together to determine which stage(s) throughout the agricultural process (i.e. antibiotic administration to livestock, manure collection and treatment practices, amendment application, vegetable harvesting, vegetable washing) are

most critical in mitigating the spread of antibiotic resistance along the agricultural production chain.

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Chapter 2. Research Goals and Specific Questions

If ARGs do persist within agroecosystems, they could pose a risk to livestock handlers, farmers, and consumers. The goal of the proposed dissertation is to examine the presence and/or absence of antibiotic and metal resistance at the field scale in agricultural soils, and evaluate the most critical stages of resistance dissemination along the agricultural production chain. In order to realize this goal, this dissertation seeks to answer the following questions:

2.1 Methods Comparison of the Agricultural Soil Resistome

1. What nuances of antibiotic resistance are illuminated when examining amended soils across multiple analytical lenses (i.e., culture-, molecular-, and NGS-based methods)?
2. Does amendment type (i.e., inorganic versus organic fertilizer; prior animal antibiotic usage versus no antibiotic usage; composted manure versus raw manure) affect the overall agricultural soil resistome?

2.2 Microbial Communities and Cross-resistance of metals and antibiotic resistance genes in Agricultural Soils

1. Does amendment type (i.e., inorganic versus organic fertilizer; prior animal antibiotic usage versus no antibiotic usage; composted manure versus raw manure) alter metal, metal resistance genes, and microbial communities in agricultural soils?
2. Is there evidence of cross-resistance between metal and antibiotic resistance genes in agricultural soils?

2.3 Agricultural Resistomes, Microbial Communities, and Co-Occurrences

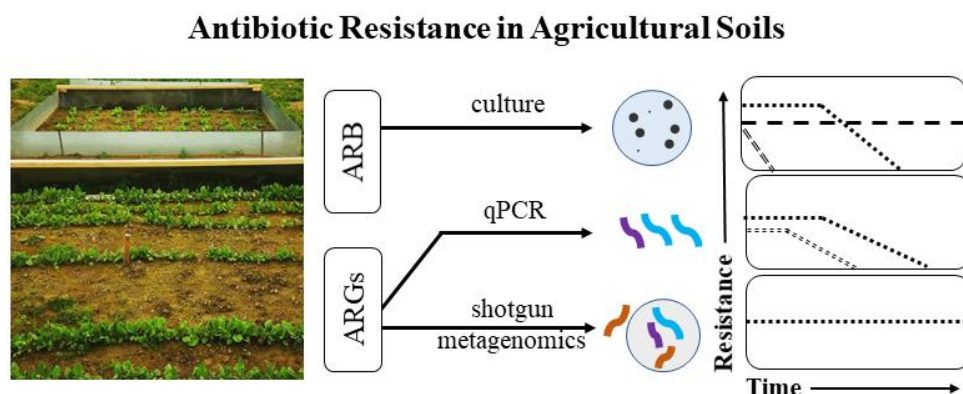
1. Do antibiotic resistomes and microbial communities shift along the agricultural production chain (i.e. amendment, soil, crop surface)?
2. Does co-occurrence of antibiotic resistance genes and mobile genetic elements shift along the agricultural production chain (i.e. amendment, soil, crop surface)?

Chapter 3. Cross-comparison of methods for quantifying antibiotic resistance in agricultural soils amended with dairy manure and compost

A research article in revision in *Science of the Total Environment*.

Wind, L.*, Krometis, L-A., Hession, W.C., and Pruden., A. 2020. Cross-comparison of quantification methods examining the similarity of agricultural soil resistomes amended with dairy manure and compost. *Science of the Total Environment*.

3.1 Graphical Abstract



3.2 Abstract

Agricultural soils are often amended with livestock manure, making them a key reservoir of antibiotic resistance genes (ARGs). Given that soils are among the most microbially-diverse environments on the planet; effective characterization and quantification of the effects of manure-derived amendments on soil resistomes is a major challenge. This study examined the effects of dairy manure-derived amendments on agricultural soils via two strategies: quantification of anthropogenic ARG markers via qPCR and shotgun metagenomic resistome profiling; and these strategies were compared to a previously published antibiotic resistant fecal coliform dataset. Soil samples were collected throughout a 120-day complete block field experiment to compare the effects of amendment type on antibiotic resistance. Results of all three measurements were

consistent with the hypothesis that the application of composted manure reduced antibiotic resistance in soil relative to the application of raw manure, although some differences were noted in comparing the patterns of the three measurements with time. Raw dairy manure-amended soils yielded high *sul1* and *tet(W)* relative abundances on Day 0 (following amendment application), but significantly decreased to background levels by Day 67 (harvest) and Day 120 (study completion). Shotgun metagenomics similarly detected a decrease in the relative abundance of sulfonamide and tetracycline-associated ARGs over time in the raw manure- and compost-amended soils; however, these levels were significantly lower than those estimated by qPCR. Interestingly, although patterns of sulfonamide and tetracycline resistance amongst culturable fecal coliforms echoed those observed via qPCR and metagenomics; erythromycin resistant coliforms were directly recovered by culture in amended soils, but corresponding ARGs were not detected by qPCR or metagenomics. This study supports both composting and time restrictions as means of reducing the potential for antibiotic resistance in manure to spread via soil application. Results suggest some differences in finer conclusions drawn depending on which antibiotic resistance monitoring target is selected

3.3 Introduction

Soils are of fundamental importance to food production and their potential to amplify the spread of antibiotic resistance (AR) is a critical area of investigation within agroecosystems. Given the historical and continued use of substantial quantities of antibiotics in feed to support livestock growth (Caniça et al., 2019; FAO, 2019; Landers et al., 2012; Van Boeckel et al., 2015), the expected recovery of antibiotic resistant bacteria (ARB) and associated antibiotic resistance genes (ARGs) in manure-amended agricultural soils presents an immediate human health concern. A growing body of research has begun to identify and discriminate both naturally-occurring and anthropogenic sources of resistance among various soil types (i.e., urban, forest, arctic, agricultural) and resistomes (Cytryn, 2013; D’Costa et al., 2011, 2007, 2006; Finley et al., 2013). The potential for manure-derived amendments to shift agricultural soil resistomes is of interest, particularly with respect to potential links between environmental and clinical AR in global food production systems (Ashbolt et al., 2013; Wright, 2010; Zhao et al., 2020).

Recent studies at microcosm, greenhouse, and field scales have demonstrated that manure amendments alter various measures of soil AR in agroecosystems (Table 3-1). In general, the application of manure or compost appears to result in an immediate increase in levels and diversity of ARGs in most soils compared to non-amended control soils (Chen et al., 2019a; Fang et al., 2015; Hu et al., 2016; Marti et al., 2013; Xie et al., 2018). However, there is evidence that this impact lessens over time, and soil resistomes (i.e., total ARG profiles) eventually return to background conditions (Chen et al., 2019a; Guo et al., 2018; Smith et al., 2019). Many agricultural soil studies suggest that implementing common best practices for manure management can aid in decreasing the potential for AR dissemination (Gou et al., 2018; Tien et al., 2017). Composting is an important manure management practice on farms that has well-established benefits for both soil health and pathogen reduction. However, the extent to which composting presents a barrier to the spread of AR is uncertain. Although composting techniques decrease ARGs compared to stockpiled raw manure (Gou et al., 2018a; Qian et al., 2016; Sharma et al., 2009), ARG proliferation may continue to occur within the soil when compost is used as an organic amendment (Xu et al., 2019).

The impact of manure amendment on AR in soil has been examined using a wide variety of analytical techniques and at multiple scales (Table 3-1). In addition to traditional culture-based techniques employing antibiotic-supplemented media, culture-independent methods, including quantitative polymerase chain reaction (qPCR) and next-generation sequencing (NGS), are increasingly used to quantify anthropogenic ARGs and discover novel ARGs and resistance indicators (e.g., integrons and mobile genetic elements (MGEs); (Chen et al., 2019a; Guo et al., 2018; Lau et al., 2017; Su et al., 2014). Given that the vast majority of soil microorganisms are not readily culturable in the laboratory, culture-independent methods can capture a wider spectrum of the microbial community and genetic diversity of environmental samples (Handelsman, 2004), although these methods are not without limitations. The use of qPCR to quantify specific ARGs of interest fails to identify the species of bacteria carrying these genes, cannot verify that the host organism is viable, and is dependent upon known target sequences. Shotgun metagenomic sequencing techniques are able to broadly evaluate the resistome, without *a priori* selection of target ARGs or bacteria (as required by qPCR and culture) but is regarded as semi-quantitative (Chambers et al., 2015; Handelsman, 2004; Luby et al., 2016b). As each technique captures

distinct dimensions of AR in soil and is characterized by different strengths and limitations, lack of comparability across studies is a major challenge to forming generalizable conclusions (Han et al., 2018; Sommer and Dantas, 2011).

Table 3-1. Summary of studies examining the impact of manure-derived amendments on antibiotic resistance in agricultural soils at multiple scales using varying quantitative methods.

Scale	Country	Quantification Method Used			Specific Resistant Culture and qPCR Targets	Reference
		Culture	qPCR	NGS		
Microcosm	USA		X	X ^a	<i>sul1</i> , <i>tetW</i>	(Chen et al., 2019)
	Australia		X ^c	X ^b	144 ARGs (HT-qPCR)	(Gou et al., 2018b)
	China		X ^c	X ^b	285 ARGs, 10 MGEs (HT-qPCR)	(Han et al., 2018)
	Australia	X	X ^c	X ^b	<i>intI1</i> , <i>tnpA</i> (qPCR), 84 ARGs (HT-qPCR)	(Hu et al., 2016)
	China		X ^c	X ^b	144 ARGs (HT-qPCR)	(Xu et al., 2019)
			X		<i>sul1</i> , <i>sul2</i> , <i>intI1</i> , <i>tetAP</i> , <i>tetG</i> , <i>tetC</i> , <i>tetL</i> , <i>tetA</i> , <i>tetX</i> , <i>tetM</i> , <i>tetO</i> , <i>tetBP</i> , <i>tetW</i>	(Wang et al., 2015)
Greenhouse	USA			X ^a		(Smith et al., 2019)
	China			X ^a		(Fang et al., 2015)
	China		X		<i>sul1</i> , <i>sul2</i> , <i>tetA</i> , <i>tetG</i> , <i>tetM</i> , <i>tetO</i> , <i>tetW</i> , <i>tetX</i> , <i>qnrS</i> , <i>intI1</i>	(Zeng et al., 2019)
	USA			X ^a		(Giron et al., 2019)
Field	Germany		X		<i>sul1</i> , <i>sul2</i>	(Jechalke et al., 2013)
	Canada	X	X		Coliforms and <i>E. coli</i> resistant to 17 antibiotics; 46 ARGs (PCR)	(Marti et al., 2013)
	USA		X	X ^b	<i>erm(B)</i> , <i>erm(C)</i> , <i>sul1</i> , <i>str(B)</i> , <i>intI1</i> , <i>IncW repA</i>	(Lopatto et al., 2019)
	Canada	X	X		<i>sul1</i> , <i>aad(A)</i> , <i>str(A)</i> , <i>str(B)</i> , <i>erm(B)</i> , <i>erm(F)</i> , <i>bla_{OXA-20}</i> , <i>PSE</i> , <i>intI1</i> , <i>IncW repA</i> , <i>rrnS</i>	(Tien et al., 2017)
	USA	X	X		<i>enterococci</i> resistant to tylosin; <i>ermB</i> , <i>ermC</i> and <i>ermF</i> , <i>msrA</i>	(Luby et al., 2016a)
	USA	X	X	X ^{ab}	Bacteria resistant to cephalothin; <i>bla_{CEP-01}</i> , <i>bla_{CEP-02}</i> , <i>bla_{CEP-03}</i> , <i>bla_{CEP-04}</i> , <i>bla_{CEP-05}</i> , <i>bla_{CEP-07}</i> , <i>bla_{CEP-07}</i>	(Udikovic-Kolic et al., 2014)
	China		X ^c	X ^b	213 ARGs (HT-qPCR)	(Xie et al., 2018)
	USA	X			Coliforms resistant to ceftazidime, clindamycin, erythromycin, sulfamethoxazole, tetracycline	(Wind et al., 2018)
	China		X	X ^a	<i>tetA</i> , <i>tetG</i> , <i>tetM</i> , <i>tetO</i> , <i>tetQ</i> , <i>tetW</i> , <i>sul1</i> , <i>sul2</i>	(Guo et al., 2018)

^a metagenomics

^b 16S rRNA gene amplicon sequencing

^c high-throughput qPCR

A limited number of prior field-scale studies concurrently employed or compared culturable, molecular, and NGS techniques to study AR in agricultural soils (Guo et al., 2018; Udikovic-Kolic et al., 2014). Guo et al. (2018) paired qPCR and metagenomics to analyze ARG abundances after long-term swine-derived manure applications to soil in Jiaxing, China. Some sulfonamide and tetracycline ARGs were significantly higher as measured by qPCR in the soils that were amended with swine manure, and this trend was confirmed with metagenomic data. However, multidrug, fluoroquinolone, and bacitracin associated ARGs accounted for up to 86% of the ARGs profiled via metagenomics, which were not targeted via qPCR (Guo et al., 2018), emphasizing the utility examining the broader context of the resistome as a whole in order to identify potential ecological drivers of observed patterns.

Identifying the most effective strategies to mitigate the spread of AR in the agricultural environment requires a holistic approach that simultaneously examines the incidence of specific bacteria and genes of interest, along with patterns in the overall soil resistome. The overarching aim of this study was to discern the effects of application of different organic and inorganic amendments on AR in agricultural soils, while simultaneously comparing multiple AR methodological detection strategies. To this end, a 120-d field experiment examined patterns of AR detection in plots subject to five different amendment conditions: dairy manure collected from cows treated with pirlimycin or cephalixin at therapeutic levels and mixed together, compost derived from the same manure, control compost derived from cows not treated with antibiotics, inorganic fertilizer control, or no amendment. Samples were analyzed via qPCR targeting *sul1*, *tet(W)*, *ermB* and *intI1* as key markers of anthropogenic sources of AR, and a subset of samples via shotgun metagenomics to profile the resistome as a whole. Results from these analyses were compared to previously published findings detailing patterns of AR indicated by culture-recovered fecal coliforms within the agricultural soils (Wind et al., 2018). It was hypothesized that the relative abundance of ARGs in soils amended with organic fertilizer (manure or compost) would have greater relative abundance of ARGs compared to be greater than that observed in control and inorganically fertilized soils, and that composting, as a known best management practice, would reduce the relative abundance of ARGs detected in raw manure amended soils. We hypothesized that measures of specific target ARGs, while not equivalent, would be correlated across analytical methods (e.g. culture-based, qPCR, NGS). Together, these insights will support the design of

effective monitoring approaches to assess the potential for on-farm manure management practices to mitigate the spread of resistance in agroecosystems.

3.4 Materials and Methods

3.4.1 Study site and amendment description

Samples were collected at the Virginia Tech Urban Horticulture Center (UHC) in Blacksburg, VA, USA. Study details and discussion of patterns of culturable antibiotic resistant fecal bacteria in soil, release of fecal indicators and ARGs in surface runoff during natural storm events, and recovery of ARGs on the vegetables harvested have been previously reported (Fogler et al., 2019; Jacobs et al., 2019; Wind et al., 2018).

A complete block design, with plots grouped by treatment given typical wind patterns at the study site, compared the effects of organic and inorganic soil amendments on ARG recovery and resistome profiles. Five soil amendments were examined: (1) no amendment control (n=3); (2) inorganic chemical fertilizer control (n=3); (3) control compost (n=3); (4) compost from animals treated with antibiotics (n=3); and (5) stockpiled raw manure from animals treated with antibiotics (n=3). The control compost, subsequently referred to as compost without antibiotics, was generated using manure collected from dairy cows with no antibiotic exposure during the previous lactation cycle. Dairy manure from cows treated with pirlimycin or cephalixin at therapeutic levels was collected and mixed to generate the raw manure with antibiotics and the compost with antibiotics amendments (Ray et al., 2017; Wind et al., 2018). Static composting and manure stockpiling methods were carried out at the Virginia Tech Kentland Farm in Blacksburg, Virginia. Thermophilic conditions (US FDA Food Safety Modernization Act (FSMA)-recommended temperature of $> 55^{\circ}\text{C}$) was reached in both the composts on day 2. All manure-derived amendments were applied at appropriate agronomic rates (6.72 Mg ha^{-1} ; 0 d) to plots (soil type: Remus fine sandy loam) during the 2016 growing season (March-July) to support the growth of lettuce (*lactuca sativa*) planted on 28 d. Inorganic fertilizer (N-P-K) was added to the inorganic chemical fertilizer control soils at rates recommended for the optimal growth of lettuce ($140\text{-}112\text{-}112 \text{ kg ha}^{-1}$; Virginia Cooperative Extension, 2015).

3.4.2 Soil Collection and DNA Extraction

Soil samples were collected at four time points (-1, 0, 67, 120 d) that coincided with critical points in the cultivation process: background soil conditions (-1 d), immediately following amendment application to the soil (0 d), lettuce harvest (67 d), and the USDA National Organic Produce's recommended wait-period following raw manure application to soils growing crops for human consumption (120 d; USDA, 2012). Briefly, a 2 cm wide x 5 cm deep soil core was randomly taken from each quadrat of the plot using a sterilized (70% ethanol) soil auger (Agrimart). On site, the four quadrat soil cores were homogenized into one sample per plot. The utmost precautions were used to prevent cross-contamination between soil plots and sample during collection periods (Wind et al., 2018). From each homogenized soil sample taken in the field, three biological replicates were used for further DNA analysis. 0.5 g of the biological replicate soil sample DNA was extracted using the FASTDNA SPIN Kit for Soil (MP Biomedicals, Solon, Ohio). Inhibition was minimized using the ZYMO PCR OneStep PCR Inhibitor Removal Kit (ZYMO Research, Irvine, CA). DNA extracts were stored at -80°C for up to four months prior to subsequent molecular analyses.

3.4.3 Culture analysis

A suite of antibiotic-resistant fecal coliforms (ceftazidime, clindamycin, erythromycin, sulfamethoxazole, and tetracycline) were cultured using MacConkey agar at known minimum inhibitory concentrations (MIC) in parallel to the soil collection. In-depth methodology and results are available in Wind et al. (2018). Antibiotic-resistant fecal coliforms were detected in all soils regardless of amendment or control, suggesting that these culture-based assays capture a substantial background source of AR (Wind et al., 2018). These antibiotic-resistant fecal coliform data are used for comparison to the molecular measurements made in the present study.

3.4.4 Molecular analysis

Three target ARGs, *sul1*, *tet(W)*, and *ermB*, and a class 1 integrase-integron gene, *intI1*, were selected in this study as these genes are associated with antibiotics critical to human health and known markers of anthropogenic sources of antibiotic resistance (Gillings et al., 2015; Pruden et al., 2012; WHO, 2017). In addition to being associated with resistance to macrolides, which are

designated as highest priority critically important antimicrobials; *ermB* was chosen as a target ARG to enable comparison with parallel microcosm, greenhouse, and runoff studies (Chen et al., 2019b; Giron et al., 2019; Jacobs et al., 2019). Genes were quantified from each soil biological DNA replicate extract in analytical triplicate at times 0, 67, and 120 d using known, published quantitative polymerase chain reaction (qPCR) protocols (Table S1). At background (-1 d), one DNA biological replicate from each plot was analyzed for target genes in analytical triplicate under the assumption that all background soils were homogenous across the field plots prior to amendment (0 d). All analyses relied on SsoFast EvaGreen Supermixes as the reaction mixture with amplification reactions in a Bio-Rad CFX96 RT-PCR Thermocycler (Bio-Rad, Hercules, CA). Analytical triplicates were performed on each sample and copy abundances were averaged for statistical analysis. A subset of samples was analyzed at six dilutions (1:5, 1:10, 1:25, 1:50, 1:100, and 1:500) to determine the correct DNA dilution required to minimize inhibition. Dilutions varied depending on targeted gene sequence (16S rRNA, 1:1000; *sul1*, 1:100; *tet(W)*, *ermB* and *IntI1*, 1:10). Standard curves ranging from 10^2 to 10^8 gene copies μl^{-1} for 16S rRNA and 10^1 to 10^7 gene copies μl^{-1} for ARGs, a class 1 integron gene, and a negative control were analyzed in triplicate on each 96-well plate. The limit of quantification was established as the lowest amplified standard from the curve for the associated 96-well plate. The minimum acceptable standard curve R^2 was 0.97. Relative abundance, a proxy for indicating conditions that select for and/or enrich AR, was calculated by normalizing ARG copy numbers to 16S rRNA copy numbers. Due to the natural complexity of soil as a sample media, inhibition was inevitable in some samples and melt curves were further analyzed to clarify C_t values and assure correct melt temperature was reached within the protocol.

3.4.5 Shotgun metagenomic sequencing analysis

A subset of samples ($n = 27$) was analyzed via shotgun metagenomic sequencing to provide a “snapshot” of the agricultural soil resistome at key time points. From one replicate plot, the extracted soil DNA, mentioned above, was sequenced in triplicate for inorganic chemical fertilizer control, compost with antibiotics, and raw manure with antibiotics soils at time points 0 and 67 d (i.e. immediately following application and at harvest). One DNA extract from each amended soil at -1 d (background) was sequenced, as soils were assumed to be homogenous prior to amendment

application. The non-amended control soils were sequenced in triplicate for -1 and 67 d, and not on 0d, as no amendments were applied to these soils and thus assumed to be equivalent to samples collected the previous day (i.e. background). The DNA extract 260/280 nm absorption ratios were recorded, and at least 400 ng DNA was submitted per sample for all but six samples that did not have sufficient mass; in these cases, all remaining DNA extract was submitted. Samples were prepared using the Nextera XT library prep (Illumina, San Diego, CA) and sequenced on an Illumina NextSeq 500 (2x75 bp, paired-end). Raw reads and associated metadata were uploaded to the NCBI BioProject: PRJNA506850. Raw sequences were processed using the online metagenomics analysis tool, MetaStorm (Arango-Argoty et al., 2016). In brief, raw FASTQ files were uploaded to the MetaStorm server for marker gene analysis, and on average, each sample contained 23,610,275 bp reads. 0.21% of reads, deemed low quality by TRIMMOMATIC (Bolger et al., 2014), were trimmed and filtered out (Table S2). Functional annotation of ARGs, with at least 90% identity and E-value<1e-10, were annotated against the Comprehensive Antibiotic Resistance Database (CARD v2.0.1; Jia et al., 2017) using the DIAMOND BLASTX (Buchfink et al., 2014) aligner with a representative hit approach (Arango-Argoty et al., 2016). This analysis solely focused on read-matching sequences, as 0.1-5.3 % of the sequences were assembled into scaffolds, which is similar to other soil resistomes studies (Myrold et al., 2014). Known housekeeping genes, cellular maintenance genes typically expressed at constant levels in all cell types (Eisenberg and Levanon, 2013), were removed prior to gene abundance analysis. Metastorm MGE v1, which combines NCBI's RefSeq non redundant database (O'Leary et al., 2016) and class I integrons and integrases from the integron-intergrase database (Zhang et al., 2018), was used to identify intI1 presence via intI1 class I integron-associated proteins relative abundance (Arango-Argoty et al., 2016). To account for variable read and gene lengths, gene counts analyzed from MetaStorm were normalized to 16S rRNA gene copy number to report overall gene relative abundance using Greengenes (DeSantis et al., 2006; Li et al., 2015).

3.4.6 Statistical analyses

All statistical analyses were conducted using R (v. 3.6.1, R Core Team, 2019) with an alpha of 0.05 ($p < 0.05$) applied as the level of significance. Via qPCR, abundances of *sul1*, *tet(W)*, and *intI1* were below the limit of quantification (LOQ) in 40.6 %, 78.6 %, and 37.7 % of the samples, respectively. Therefore, censored statistical analyses were conducted, as appropriate (Helsel,

2005). The Shapiro-Wilk test identified that absolute and relative abundances quantified via qPCR and the relative abundances annotated via shotgun metagenomics were non-normal (all gene abundances $p < 0.05$). ARG relative abundance varied based on gene amplified and a Kruskal-Wallis rank sum test followed by a post-hoc Dunn's test assessed differences among treatment and time for ARG relative abundance quantified via qPCR and metagenomics. For metagenomic sequencing statistical analysis, the effects of soil amendment (i.e. treatment) and day(s) after soil amendment (i.e. time) were analyzed in triplicate sequences, except for the amended soils at background (-1 d). On the assumption that prior to amendment application the field plots were homogenous, the -1d samples for inorganic chemical fertilizer control, compost with antibiotics, and raw manure with antibiotics amended soils were grouped with the no amendment control soil samples at the time of background, which resulted in six replicate samples during the statistical analysis. Analysis of similarities (ANOSIM) and nonmetric multidimensional scaling (NMDS) plots were performed to compare ARG profile resemblance among the soil samples by treatment and time (Bray-Curtis). Unweighted Bray-Curtis distances for metagenomic ARG profiles (ARG type and relative abundance) was used for one-way ANOSIM. A heatmap visualizing maximum ARG relative abundance $>1\%$ in the agricultural soils determined by Bray-Curtis distance was created using the R package 'Superheat' (Barter and Yu, 2018). Figures were produced using R packages 'ggplot2' and 'VennDiagram' (Wickham, 2009, Chen, H., 2018). Spearman's rank correlation was used to compare the potential linear associations between the culture, qPCR, and NGS-based measures. As each method employed in this study has varying limits of detection and quantification (potential for censored data) and additional potential for true zeros within the measures, the assumption is that non-detects are accounted for with a tied-rank within the Spearman correlation when p is calculated.

3.5 Results

3.5.1 Trends in qPCR measures of AR targets

Total bacterial abundance, estimated as 16S rRNA gene copy number, increased in all soils over the 120 d study, never returning to background (-1 d) levels (Kruskal-Wallis, $p < 0.0001$, Dunn's test, $p < 0.01$; Figure 3-1A). Bacterial abundance was significantly lower in the no amendment

control soils compared to the soils amended with either compost without antibiotics or raw manure with antibiotics (Kruskal- Wallis, $p < 0.0001$, Dunn's test, $p < 0.01$ and $p < 0.0001$; respectively).

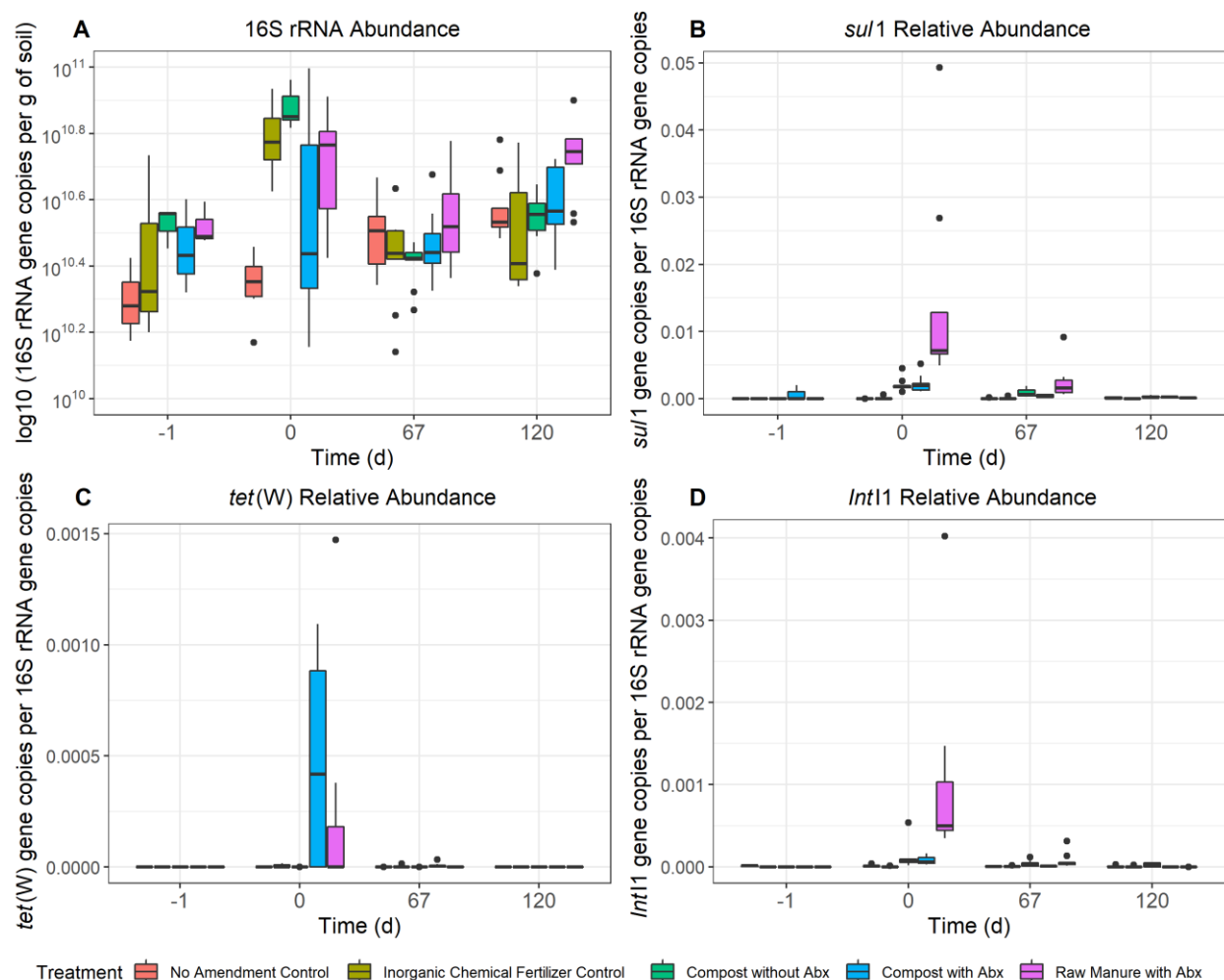


Figure 3-1. Treatment and time effects on the relative abundance (i.e., normalized to 16S rRNA genes) of genes quantified via qPCR in amended and un-amended soils over the 120 d study in Blacksburg, VA. (A) Total bacterial abundance, 16S rRNA gene copies, increased over the 120d; (B) *sul1* (C) *tet(W)* and (D) *int11* increased in the soils amended with manure or compost and returned to background levels by 120d. As *sul1*, *tet(W)* and *int11* were quantified at different magnitudes, relative y-axis ranges are adjusted accordingly.

The effect of amendment type and time had a significant effect on 16S rRNA, *sul1*, *tet(W)*, and *int11* abundances, both absolute and relative, in the agricultural soils (Table S3). Relative abundances of all AR indicator genes significantly spiked following amendment of soil (0d) with manure or compost, and returned to background levels or were <LOQ by the end of the 120 d

study (Dunn's test, $p < 0.05$ for all iterations; Fig. 3-1B-D). *sul1*, *tet(W)*, and *intI1* relative abundances between the time of application and harvest were similar (Dunn's test, $p > 0.05$). Absolute abundances of all quantified genes followed the same trend (Figure S1). *ermB* was not detected in any of the soils over the 120 d study.

The highest relative abundances for *sul1* (4.9×10^{-2} gene copies per 16S rRNA gene copies) and *intI1* (4.0×10^{-3} gene copies per 16S rRNA copies) were both measured in raw manure amended soils at the time of application. Additionally, the relative abundances of *sul1* in the raw manure- (6.6 *sul1* copies per 16S rRNA copies) and compost- (5.5 *sul1* copies per 16S rRNA copies) amended soils were almost 10-20% greater than that measured in the inorganic fertilizer and no amendment controls (1.2 *sul1* copies per 16S rRNA copies; Kruskal-Wallis, $p < 0.0001$). Observed patterns with time were similar for *intI1* and *sul1* relative abundance, with significantly greater abundance in the manure- and compost-amended soils compared to the inorganic fertilizer - amended soils at time of application (Kruskal-Wallis, $p < 0.001$). However, compost addition did not significantly influence soil *sul1* or *intI1* relative abundance levels when compared to raw manure-amended soils (Dunn's, $p > 0.05$ for all iterations). The majority of samples (78.6%) were below the LOQ for the *tet(W)* gene, although qualitative analysis suggests a spike in abundance occurred at 0 d in the compost with antibiotics and raw manure with antibiotic amended soils, and immediately returned to $< \text{LOQ}$ and non-detectable by the time of harvest (67 d; Figure 3-1C).

3.5.2 Shotgun metagenomic profile of agricultural soil resistomes

Amendment type and time did not affect the total relative abundance of annotated ARGs in the agricultural soils as detected through shotgun metagenomics (Kruskal-Wallis, $p = 0.064$ and $p = 0.590$; respectively; Figure 3-2). However, when the ARG profiles were characterized by the relative abundance of each annotated ARG per soil sample, distinct effects of soil amendment and time became apparent for the resistome profile. Bray-Curtis mean ranked dissimilarity distance analysis of the ARG profiles indicated a significant effect of amendment type (ANOSIM, $R = 0.178$, $p = 0.005$) and time (ANOSIM, $R = 0.127$, $p = 0.041$; Figure S2) over the course of the study.

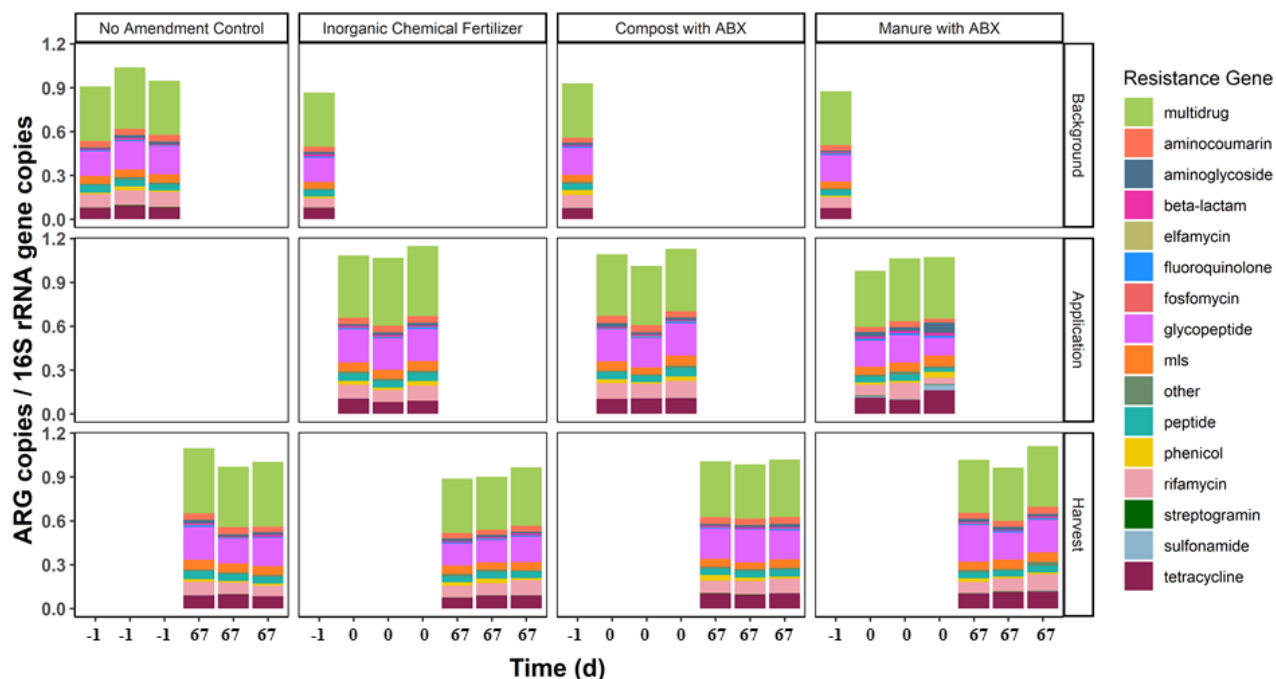


Figure 3-2. Total ARG relative abundance in soils represented according to class of antibiotic to which they encode resistance and grouped by treatment ($n=4$) for each column and by time ($n=3$) for each row annotated using CARD v2.0.1 via shotgun metagenomic sequencing. Each stacked bar represents one shotgun metagenomic sequence replicate among soil samples collected within the same soil plot, except for the amended soils at background (-1 d), which includes samples across plots/conditions ($n=3$) based on the assumption that the soil was homogenous across plots prior to amendment. No Amendment Control soils were not sequenced at application (0 d) as those soils were assumed to be the same as at the time of background (-1 d). MLS= macrolide-lincosamide-streptogramin; Other = not in the top 14 annotated drug classes, refer to Text S1 for detailed description of “other”.

Across all soil samples subject to metagenomic sequencing, 216 of the 511 total annotated ARGs were detected in all samples (Figure 3-3, Figure S3). Soils amended with raw manure with antibiotics contained the widest variety of ARGs ($n=394$) among the soils, i.e., 82 more ARGs detected compared to the soils amended with compost derived from the same manure ($n=312$). The most abundant ARGs present in all soils included: *vanRO* which confers resistance to glycopeptides; *mtrA* which confers resistance to beta-lactams and macrolides (a multidrug resistance gene); and *novA* which confers resistance to aminocoumarins (Figure S3, Figure S4). Overall, *vanRO* had the highest relative abundance of all ARGs in the no amendment control soils (0.178 copies per 16S rRNA copy) at the time of harvest (i.e., 67 d). The least relative abundant ARG was *abcA*, a multidrug resistance transporter protein, found in the raw manure with antibiotic

amended soils at the time of amendment application (i.e., 0 d). Multidrug was the most prevalent class among all annotated ARGs (n= 137); and *muxB*, *muxC*, and *mdtB*, were the top three detected multidrug resistance genes in all sequenced soils (Fig. S3). Glycopeptide associated ARGs (n=52) followed as the second most identified drug class among all sequenced soils; followed by tetracycline (n=46) and MLS (n=45) associated ARGs.

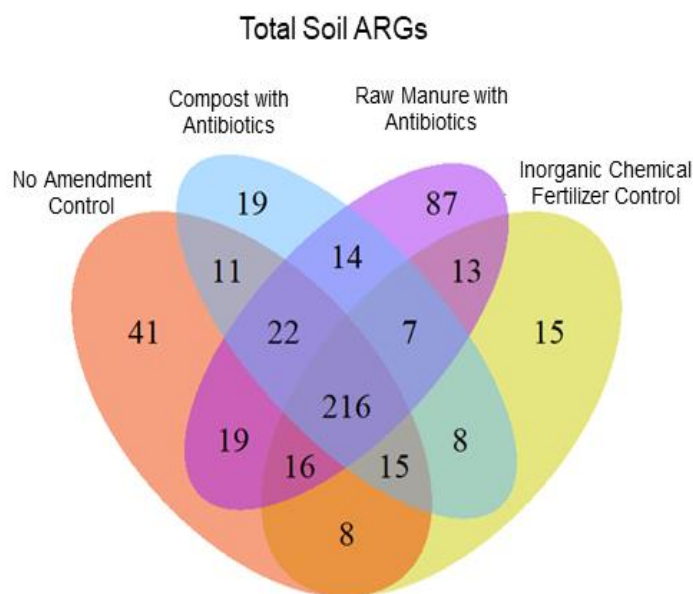


Figure 3-3. Total number of ARGs annotated via the CARD v2.0.1 database for each amended soil treatment. All background soils sampled on day -1 (n=6) were grouped in the No Amendment Control treatment based on the assumption that prior to amendment application the soil was homogenous among the amended soils. Of the 511 ARGs annotated, 216 ARGs were detected in all soil treatments. Individually the raw manure with antibiotics, no amendment control, compost with antibiotics, and inorganic chemical fertilizer amended soils yielded 87, 41, 19, and 25 unique ARGs, respectively.

3.5.3 Annotation of class 1 integrons in metagenomic data

For comparison to qPCR, integron-associated genes were also annotated and quantified in metagenomics data. Of the twenty-seven metagenomic sequenced soils, *intI1* was annotated in the raw manure and compost amended soil replicates at 0d and 67d (n= 6, Table S3). In total, there were ten distinct *intI1* class I integron-associated proteins. Eight unique *intI1* class 1 integron-associated proteins were detected in the raw manure amended soils at time of application (0 d)

with varying relative abundances ranging from 1.75×10^{-3} to 3.19×10^{-4} copies per 16S rRNA gene copies (Table S3). Further, three *intI1* class I integron-associated proteins were identified in two compost with antibiotic and one raw manure with antibiotics amended soil samples at time of harvest (67 d). At the time of harvest (67 d), *intI1* class I integron-associated proteins relative abundance was on average 20 % less than at the time of amendment.

3.5.4 Comparison of culture-, molecular-, and metagenomic-based measures of resistance

Relative abundances of *sul1*, *tet(W)*, and *intI1*, as annotated in the shotgun metagenomic data, were compared to measurements of these same genes via qPCR in the 27 sample subset examined via both strategies (Table 2, Figure S5). Although *sul1* measurements were positively correlated between the two methods (Spearman's Rho, $p=0.017$), *sul1* was detected in only 12/27 samples via qPCR and 5/27 soil samples via shotgun metagenomics. In contrast, metagenomics detected *tet(W)* in 4 more samples than qPCR, but the measured relative abundances were not correlated (Spearman's Rho, $p=0.76$). Relative abundance of *intI1* measured via qPCR and metagenomics were positively correlated (Spearman's Rho, $p=0.025$), though it is worth noting that metagenomics was only able to identify *intI1* in 6 of the 27 soil samples, while qPCR quantified *intI1* in 21 samples, which excluded background (-1d) and inorganic fertilizer soils at time of amendment (0 d). *ermB* was not annotated within the shotgun metagenomic data, which is consistent with its lack of detection via qPCR.

Table 3-2. Correlation of antibiotic-resistant fecal coliforms quantified by culture-based technique, antibiotic resistance genes (ARGs) quantified by quantitative polymerase chain reaction (qPCR), and relative ARG abundance annotated in shotgun metagenomic data (Spearman's rho (r_s), $p < 0.05$ significance).

Resistance Quantification Method Used												
Culture-Based (CFU)							Molecular- Based (qPCR) (ARG Gene Copies)					
ARB/ARG quantified	Sulfonamide- resistant Fecal Coliforms		Tetracycline- resistant Fecal Coliforms		MLS ^a - resistant Fecal Coliforms		<i>sul1</i>		<i>tet(W)</i>		<i>int11</i>	
	-r _s -	-p-	-r _s -	-p-	-r _s -	-p-	-r _s -	-p-	-r _s -	-p-	-r _s -	-p-
Comparison Method												
qPCR (CFU/ g soil)	0.262	0.001	0.109	0.189	N/A	N/A	-	-	-	-	-	-
Shotgun Metagenomics (ARG ² / 16S rRNA)	0.446	0.012	0.320	0.103	0.619	0.0006	0.626	0.0005	-0.061	0.761	0.438	0.025

^a Within the MLS drug class group, there were 55 ARGs and two antibiotics (i.e., clindamycin and erythromycin) combined for the relative and absolute abundances of MLS resistance correlations, respectively. There was no correlation between culture and qPCR due to the MLS ARG target, *ermB*, being nonquantifiable in the soils.

sul1 and *tet(W)* absolute abundances quantified via qPCR were compared to culturable sulfonamide and tetracycline-resistant fecal coliform levels reported by Wind et al. (2018; Table 2, Figure S6). Although sulfonamide-resistant fecal coliform levels were significantly correlated with *sul1* absolute abundance quantified via qPCR (Spearman's Rho, $p=0.001$), tetracycline-resistant fecal coliforms were not correlated with *tet(W)* (Spearman's Rho, $p=0.19$). Sulfonamide and tetracycline-resistant fecal coliforms were only quantifiable in the soils at the time of application (0 d). However, *sul1* and *tet(W)* ARGs were detected and quantified at low levels in the soils at the time of harvest (67 d) both by qPCR and metagenomics.

Lastly, the relative abundance of *sul1* and *tet(W)* from metagenomics and the absolute abundance of culturable sulfonamide and tetracycline-resistant fecal coliforms were compared in the 27 pairwise sequence soil samples (Table 2, Figure S7). Comparable to the previous method detection correlations, sulfonamide resistance was significantly correlated between metagenomics and culture techniques (Spearman's Rho, $p=0.012$), and tetracycline resistance was not (Spearman's Rho, $p=0.103$).

3.6 Discussion

3.6.1 Detection of resistance markers in agricultural soils

Varying detection of *sul1*, *tet(W)*, and *intI1* via qPCR and metagenomics highlights the potential for overlooking the presence of an ARG (i.e., false negatives) and underestimating the relative abundance of an ARG when a single quantification method is used. *sul1* and *intI1* have been demonstrated as sensitive anthropogenic markers of AR that have been linked to additional genes conferring antibiotic, disinfectant, and heavy metal resistance in the environment (Cytryn, 2013; Gillings et al., 2015; Storteboom et al., 2010). Based on shotgun metagenomic sequencing, the total ARG relative abundance between the time of application and the time of vegetable harvest was not significantly altered by the type of organic (manure and compost) or inorganic (chemical fertilizer) amendment applied to the soil. However, both qPCR and culture data indicated significant changes in resistance patterns over time. Targeted ARGs returned to background or <LOQ levels by the time of vegetable harvest for most amended soils, and ultimately decreased to nondetectable by the USDA National Organic Program's wait time recommendations (120 d, USDA, 2012). When comparing to ARB, patterns varied based on the corresponding antibiotic drug class for which resistance was assayed (Wind et al., 2018). In the case of sulfonamide resistance, the pattern of sulfonamide-resistant fecal coliforms detection in the soils across conditions at harvest were similar to that of *sul1* determined by qPCR.

The return of all soils to background levels comparable to the unamended control soil microcosms after a few months echoes recent microcosm study findings by Pérez-valera et al. (2019), which suggested that native soil bacteria may mitigate the inputs of ARGs in manure amendments. Competition with native soil bacteria, especially in the absence of selection pressure from antibiotics, is thought to be a key mechanism driving the soil as an ecological buffer to AR (Cytryn, 2013); while still acknowledging this is not the case within all agricultural soils (Heuer et al., 2011; Jechalke et al., 2013).

Although metagenomic analysis revealed similarities in ARG profiles among the soils collected from all test plots, it did provide a comprehensive profile of the soil resistome, which, in total, was comprised of 511 ARGs. Such a comprehensive profile is not possible with any other available

method and presents a critical opportunity to identify key AR markers of concern. The resistomes of soils receiving manure, compost, or inorganic fertilizer were comprised mostly of multidrug ARGs (n= 137). Multidrug resistance genes are of particular interest as a potential public health threat since they convey resistance to at least three classes of antibiotics. In a microcosm study using the same dairy-derived manure and compost as the present study, a similar soil resistome pattern was identified that highlighted the dominance of multidrug resistance genes (Chen et al., 2019a). The raw manure amended soils carried more than double the relative abundance of total ARGs compared to the control soils at time of amendment application and there were distinct differences between the manure- and compost-amended soil resistomes (Chen et al., 2019). At the microcosm scale, amendment type appeared to alter ARG abundance and soil resistome diversity to a greater extent than what was observed in the agricultural soils at field-scale. This may relate to the fact that microcosms are closed systems, whereas this field-scale study was open system and had more opportunity for loss or attenuation of resistance markers.

3.6.2 Differences in detection of antibiotic resistance by associated drug class

Macrolide-lincosamide-streptogramin (MLS) class drugs are critical to human and animal health (WHO, 2017). Although *ermB* is not considered an anthropogenic marker of resistance, it is listed as an important Rank II ARG that is loosely associated with clinical antibiotics (Zhang et al., 2019). *ermB* was not detectable or quantifiable in the soils via qPCR and shotgun metagenomics, which was surprising both because an MLS drug (pirlimycin) was administered to the dairy cows and because culturable erythromycin-resistant fecal coliforms were elevated in samples collected from soils that received manure-based amendments (Wind et al., 2018). While a parallel greenhouse study of bulk and rhizosphere soil using the same dairy cows to generate compost and manure was also unable to detect *ermB* in soils (Chen, H. et al., 2018), the absence of *ermB* abundance in the soils was unexpected because *ermB* was detectable, albeit at very low levels, (10^{-11} to 10^{-4} copies per 16S rRNA copies), in associated surface water runoff that was collected following natural storm events over the same growing season in these study field plots (Jacobs et al., 2019). Very low or nondetectable levels of *ermB* were also observed by McKinney et al. (2018) in samples from dairy-derived manure fertilized fields in Idaho, USA. Difficulty in *ermB* detection in soils (as opposed to runoff water) may reflect the more complex nature of the soil sample matrix

and resistance mechanisms. Observed erythromycin-resistant fecal coliforms recovered via culture may have carried other MLS or multidrug-associated ARGs, rather than *ermB*. To test this hypothesis, relative abundance of all MLS associated ARGs identified via metagenomics were correlated with the combined absolute abundance of all MLS associated ARB. Within the MLS drug class, there were 55 ARGs and two antibiotics (i.e., clindamycin and erythromycin) combined for the relative and absolute abundances of MLS resistance correlations, respectively. MLS resistance detection was significantly correlated between metagenomics and culture techniques (Spearman's Rho, $p=0.0005$; Table 3, Figure S7). Further, cephalosporin-associated ARGs (i.e. ARGs that may be related to the cephalin drug administered to dairy cows) were not targeted via qPCR in the present study as a parallel study did not quantify cephalin in the amended field soils (Wind et al., 2018). Cephalin relevant ARGs were quantified via metagenomics, which indicated beta-lactam resistance genes were the most abundant in the raw manure amended soils. As agricultural soil resistome research continues to evolve, quantification of key target ARGs using qPCR may be more useful to researchers and ultimately policy makers by focusing efforts on known clinical ARGs (Zhang et al., 2020) and MDR genes as per this study findings.

3.6.3 Metagenomic diversity and subsequent limitations in agricultural soils

Metagenomic results did suggest important changes in diversity in terms of the abundance and spectrum of ARGs present in the manure and compost amended soils compared to the control soils. Although the total ARG abundance in the manure- and compost-amended soils were similar, composting decreased ARG diversity by 21 % compared to soils amended with raw manure containing antibiotics. However, compared to the no amendment control soils, manure amendments increased ARG diversity in soil whereas compost amendments decreased diversity in the soil. Compost-amended soils also had lower levels of *sul1* and *intI1* as compared to levels observed in soils amended with raw manure, supporting the best management practice of composting prior to application to reduce the risk of ARG dissemination in the environment and/or farmworker exposure.

Analysis via qPCR was able to capture distinct differences between the two composts, inorganic fertilizer control, and raw manure amended soils with respect to ARG abundance, whereas shotgun

metagenomics did not. However, metagenomics was able to identify 162 unique ARGs across the four amended soil plots that targeted qPCR could not have as efficiently (Fig. 3). Distinguishing nuances in soil AR patterns from either method ultimately is due to the detection limit. For qPCR, a relatively sensitive quantification method, the limit of detection (LOD) is calculated precisely based on standard calibration curves (Forootan et al., 2017; Kralik and Ricchi, 2017). The limit of detection for shotgun metagenomics is not as sensitive and is reliant on sequencing depth and contig length (Bharti and Grimm, 2019). Despite the advancements in environmental metagenomic sequencing technology over the past decade, it is not possible to sequence all DNA from every microorganisms in a sample (Ni et al., 2013; Techtmann and Hazen, 2016). In soil samples, previous studies emphasize that metagenomic sequencing depth is currently not reaching the rarefaction curve necessary to cover the broader metagenome within the samples (Myrold et al., 2014; Zaheer et al., 2018).

3.6.4 Moving forward: Recommendations for AR detection in agricultural soils

It is neither realistic nor efficient to test for every potential marker of resistance (i.e., antibiotics, ARG, ARB, MGE, etc.) with every available analytical strategy (i.e., culture, qPCR, shotgun metagenomics, etc.) in agroecosystems. Through this study, we were able to characterize AR in agricultural soils amended with manure and compost and provide further evidence that manure management practices do impact dissemination of AR in agricultural soil. We intentionally chose culture, qPCR, and shotgun metagenomics to compare the differences between organic (manure and compost), inorganic (chemical fertilizer) and no amendment control soils; and discovered differences in AR patterns by technique that may lead decision makers (i.e., farmer, livestock handler, consumer, policy maker, etc.) to different conclusions. For example, if only the shotgun metagenomic results were analyzed, one might assume that the amendment type has little effect on ARG profiles in agricultural soils, particularly when considering total ARGs.

Agricultural stakeholders need to be aware that targeting a specific ARB, ARG, and/or the soil resistome may lead to different conclusions when analyzing soil at the field-scale. This is not to be unexpected given the complexity of resistance ecology. Moving forward, selection of methods for characterizing AR in agricultural soils must consider the intended output, i.e. the discovery of novel ARGs vs detection of a specific antibiotic-resistant pathogen of concern, if the AR

quantification is needed quickly (i.e. qPCR in a few hours or culture in a few days). Here we suggest a methodological decision-tree for factoring in which AR detection method may be most informative based on the objectives when tracking AR in agricultural soils (Figure 3-4).

Antibiotic Resistance Detection Methodological Decision Tree

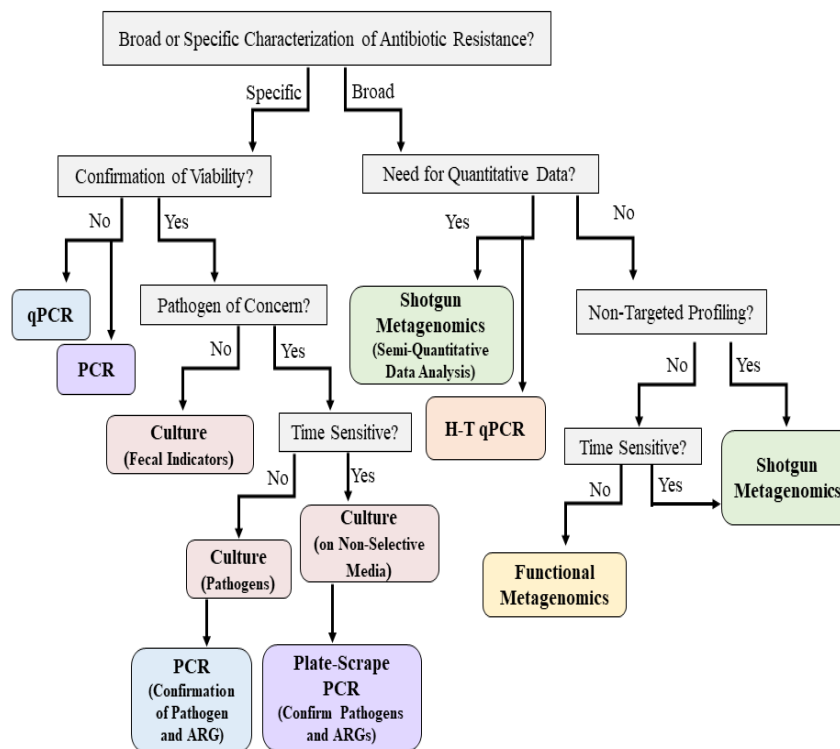


Figure 3-4. A methodological decision-tree for factoring in which detection method is most useful based on specific objectives for analyzing antibiotic resistance in soils. Time sensitive is intended to be descriptive of if results of detection is needed rapidly to make public health and management decisions.

3.6.5 Limitations

Although this study focused on culture, qPCR, and shotgun metagenomics, we are aware that many other AR detection methods available. However, any successful method must recognize the complexities of soil and be able to capture important nuances in AR patterns. When employing multiple culture-, molecular-, and metagenomic-based techniques to quantify and characterize resistance in agricultural soils, sample size can be a dilemma, particularly as costs are rarely unlimited. For example, only 27 shotgun metagenomic vs 150 qPCR samples were analyzed, resulting in a much smaller sample size and the loss of one soil amendment (control compost) and one time point (120 d) from ARG resistome identification, which limited ability to cross-compare.

Encouragingly, as the cost of sequencing continues to decrease (Boolchandani et al., 2019) shotgun metagenomic sample sets would be expected to increase, allowing for increased statistical power. Further, we recognize that there are many different computational methods for examining the soil resistome at greater which include: functional gene analysis through specific homology searches (Techa-Angkoon et al., 2017), discriminatory ARGs (Gupta et al., 2019), co-occurrence (Pal et al., 2015), and potential resistome risk (Oh et al., 2018) analyses. Future work intends to incorporate these additional computational methods to examine the soil resistome at a deeper level than the broad resistome profiling of the current study. Based on our experience and that of others, we suggest the decision-tree approach in Fig. 3-4. For example, culture-based methods focusing directly on culturing fecal pathogens (e.g., *E. coli*, *Salmonella*) or pathogens present in soils that can commonly colonize on vegetables (e.g., *Pseudomonas*) is the norm in the field of food safety. In the environmental sciences, fecal indicators are often more broadly monitored, but they have limited ability to directly inform human health risk models. On the other hand, for research purposes in which the goal is a sensitive method to directly discern effects of different treatments, qPCR and/or metagenomics may be desirable. However, metagenomics can be limited in its application to soil due to the extremely high microbial diversity, which substantially raises the detection limit and may require highly costly deep sequencing to detect differences of interest.

3.7 Conclusions

This study provides key information for extending best practices for manure management towards mitigating the potential for antibiotic resistance to spread. While useful in identifying the broad and overall diverse soil resistome, metagenomic analyses did not identify significant differences in total ARG relative abundance over time following soil amendment. This may be partly the result of high detection limits in the complex soil environment. This contrasts with the patterns observed via culture or qPCR targeting specific genes: qPCR and culture-based techniques were able to detect differences between resistance markers consistent with expectations of elevated ARGs in manure- and compost-applied soils and over the course of 120 days. Generally, soil in this field study appeared to act as an ecological buffer to the dissemination of ARB and ARGs introduced by manure amendments, which is encouraging.

Concurrent examinations of AR via culture, molecular, and metagenomics techniques is ideal, but likely not broadly practical. Given that human and animal health is of utmost concern the focus of investigations of best management practice impacts should identify means to mitigate both direct human health risk (i.e., resistant pathogens) and the risk of contributing to the evolution and spread of AR. Therefore, we suggest (1) screening for target ARGs efficiently using qPCR; (2) if ARGs persist, screen for relevant food safety and soil pathogens as viable organisms that carry quantified resistance through culture-dependent methods; and (3) implement best management practices to eradicate the quantified AR threats in agricultural soils that grow food for consumption. If resources allow, (4) then one can employ NGS techniques to characterize the broader resistome and ultimately discover novel ARGs within the system that could pose health risk to humans and animals in the future.

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Supplemental Information

Text S1. ARGs associated with amino salicylate, bacitracin, fusidic-acid, isoniazid, macrocyclic, mupirocin, nitrofurantoin, nucleoside, oxacillin, polymyxin, quinolone, triclosan, and trimethoprim drug classes were grouped as “other” throughout the manuscript. These drug classes were not in the top 14, and grouped as “other” to make legends more visible.

Table S1. Rationale for selected gene targets and primer sequences used for qPCR

Target Gene	Primer Sequence (5'→3')	Gene Characterization Rationale	Source
16s rRNA	CGGTGAATACGTTT ^a GGWTACCTTGTTAC ^b	Small-subunit rRNA genes in all prokaryotes. Provides measure of total bacteria and normalization denominator.	(Suzuki et al., 2000)
<i>sul1</i>	CGCCCGGAAACATCGCTCCAC ^a TGAAGTTCCGCGCAAGGCTCG ^b	Encodes resistance to sulfonamides, the 1 st class of antibiotics to be marketed. An established marker of anthropogenic sources of resistance.	(Pei et al., 2006)
<i>tet(W)</i>	GAGAGCCTGCTATATGCCAGC ^a GGGCGTATCCACAATGTAAAC ^b	Encodes resistance to tetracycline class drugs used in humans and animals and commonly found in manure.	(Aminov et al., 2001)
<i>ermB</i>	GATACCGTTTACGAAATTGG ^a GAATCGAGACTTGAGTGTGC ^b	Encodes resistance to MLS class antibiotics of importance to treating clinical resistance in humans and also administered to the cows in this study.	(Chen et al., 2007)
<i>int1</i>	CTGGATTTCGATCACGGCACG ^a ACATGCGTGTAATCATCGTCG ^b	The integrase gene of class 1 integrons, which broadly capture ARGs and facilitate dissemination and are exclusively clinical/anthropogenic associated.	(Hardwick et al., 2008)

^a forward

^b reverse

Table S2. QA/QC summaries for the metagenomic sequencing reads matching pre- and post-processing data from MetaStorm.

Sample	Sample Type	Treatment	Time (d)	Rep	Raw Reads	Reads QC	Fwd Surviving	Rvs Surviving	Dropped	% Dropped
1	Soil	No Amendment Control	-1	1	20,659,604	19,798,255	596,094	212,265	52,990	0.26
2	Soil	No Amendment Control	67	2	22,401,388	21,689,541	431,972	235,732	44,143	0.20
3	Soil	No Amendment Control	-1	1	22,145,101	21,502,235	360,845	244,871	37,150	0.17
4	Soil	No Amendment Control	-1	1	24,624,173	23,795,355	510,393	263,161	55,264	0.22
5	Soil	No Amendment Control	67	1	24,534,603	23,677,213	569,331	227,493	60,566	0.25
6	Soil	No Amendment Control	67	3	21,973,422	21,260,947	445,737	219,296	47,442	0.22
AVERAGE					22,723,049	21,953,924	485,729	233,803	49,593	0.22
7	Soil	Inorganic Chemical Fertilizer	-1	1	21,657,794	20,986,191	387,606	240,716	43,281	0.20
8	Soil	Inorganic Chemical Fertilizer	67	2	24,718,879	23,976,014	433,429	265,576	43,860	0.18
9	Soil	Inorganic Chemical Fertilizer	67	3	22,456,689	21,767,292	400,543	247,021	41,833	0.19
10	Soil	Inorganic Chemical Fertilizer	0	3	24,885,670	24,133,384	460,402	240,942	50,942	0.20
11	Soil	Inorganic Chemical Fertilizer	0	2	26,418,408	25,610,060	497,634	255,206	55,508	0.21
12	Soil	Inorganic Chemical Fertilizer	0	1	25,505,509	24,775,848	427,968	255,234	46,459	0.18
13	Soil	Inorganic Chemical Fertilizer	67	1	23,756,283	23,052,661	431,877	224,981	46,764	0.20
AVERAGE					24,199,890	23,471,636	434,208	247,097	46,950	0.19
14	Soil	Compost with ABX	0	2	22,683,806	21,759,418	626,929	235,667	61,792	0.27
15	Soil	Compost with ABX	-1	1	25,853,125	25,078,686	450,601	276,035	47,803	0.18
16	Soil	Compost with ABX	67	2	23,787,045	22,997,478	486,710	253,149	49,708	0.21
17	Soil	Compost with ABX	67	1	21,945,957	21,251,568	440,353	205,556	48,480	0.22
18	Soil	Compost with ABX	67	3	23,861,824	23,138,239	441,855	234,759	46,971	0.20
19	Soil	Compost with ABX	0	1	24,962,727	23,962,440	703,404	230,312	66,571	0.27
20	Soil	Compost with ABX	0	3	23,994,068	23,245,501	457,428	241,234	49,905	0.21
AVERAGE					23,869,793	23,061,904	515,326	239,530	53,033	0.22
21	Soil	Manure with ABX	67	2	22,214,289	21,209,646	7,078,016	234,272	62,555	0.28
22	Soil	Manure with ABX	0	3	22,379,951	21,678,139	416,354	241,585	43,873	0.20
23	Soil	Manure with ABX	67	1	21,952,981	21,215,381	445,406	246,748	45,446	0.21
24	Soil	Manure with ABX	-1	1	21,632,223	20,980,799	382,140	229,938	39,346	0.18
25	Soil	Manure with ABX	0	2	24,034,276	23,314,186	419,468	258,290	42,332	0.18
26	Soil	Manure with ABX	0	1	27,354,209	26,495,907	534,701	266,177	57,424	0.21
27	Soil	Manure with ABX	67	3	25,083,431	24,299,077	482,537	250,457	51,360	0.20
AVERAGE					23,521,623	22,741,876	1,394,089	246,781	48,905	0.21

Table S3. Significance of amendment type and time (d) on the absolute and relative gene abundances quantified in the soils using qPCR (Kruskal-Wallis, **p<0.05**).

	Absolute Abundance (gene copies)				Relative Abundance (gene copies per 16S rRNA copies)		
	16S rRNA	<i>sul1</i>	<i>tet(W)</i>	<i>Int11</i>	<i>sul1</i>	<i>tet(W)</i>	<i>Int11</i>
Amendment							
chi-squared	17.364	82.495	13.46	23.52	81.342	19.39	22.66
p-value	1.64e-03	2.2e-16	9.21e-03	9.97e-05	2.2e-16	6.57e-04	1.47e-04
Time							
chi-squared	23.058	21.241	20.68	60.44	20.8	27.25	58.15
p-value	3.98e-05	9.38e-05	1.26e-04	4.72e-13	1.15e-4	5.27e-06	1.45e-12

Table S4. *IntI1* relative abundance (gene copies per 16S rRNA) detected in six amended soil samples.

Treatment	Time (d)	Replicate	intI1 (relative abundance)
Manure with ABX	0	1	1.879e-03
Manure with ABX	0	2	5.176e-04
Manure with ABX	0	3	3.871e-03
Compost with ABX	67	1	5.613e-04
Compost with ABX	67	2	4.565e-04
Manure with ABX	67	1	4.752e-04

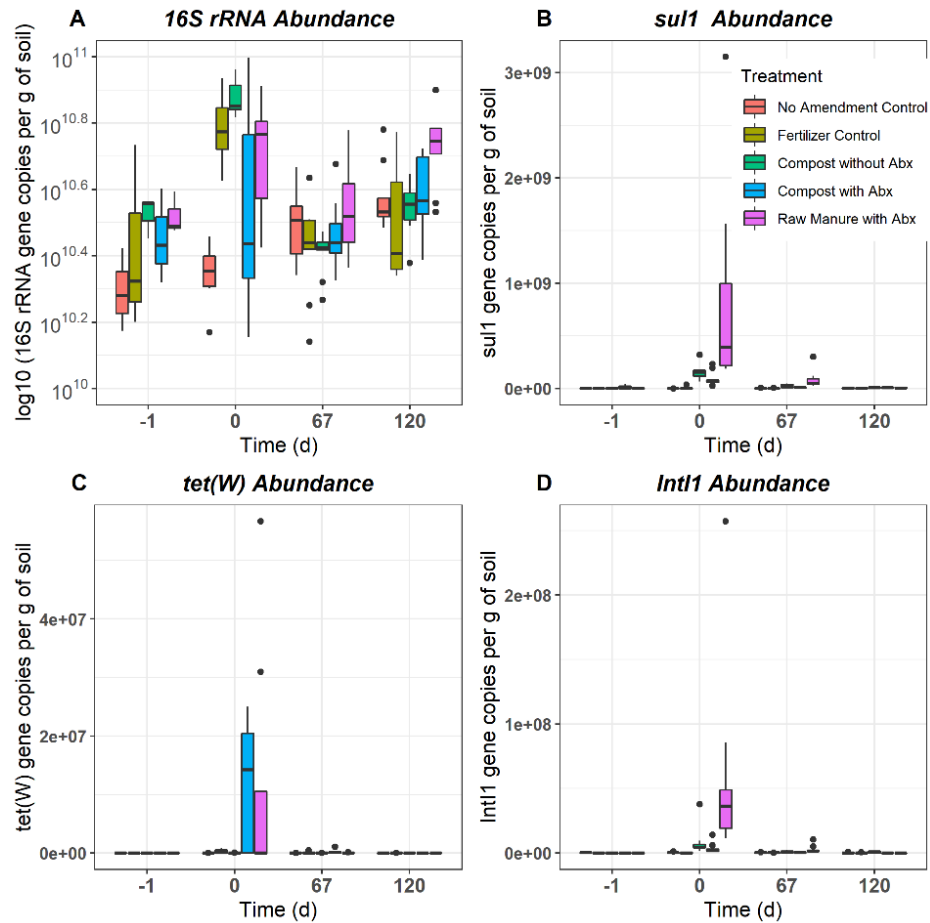


Figure S1: Absolute abundances of 16S rRNA, *sul1*, *tet(W)*, and *intI1* gene copies quantified in the treated soils over at the four sampling times (d: -1, 0, 67, 120). Abx = antibiotics. Time -1d represents background levels in the soils before amendment at time 0d.

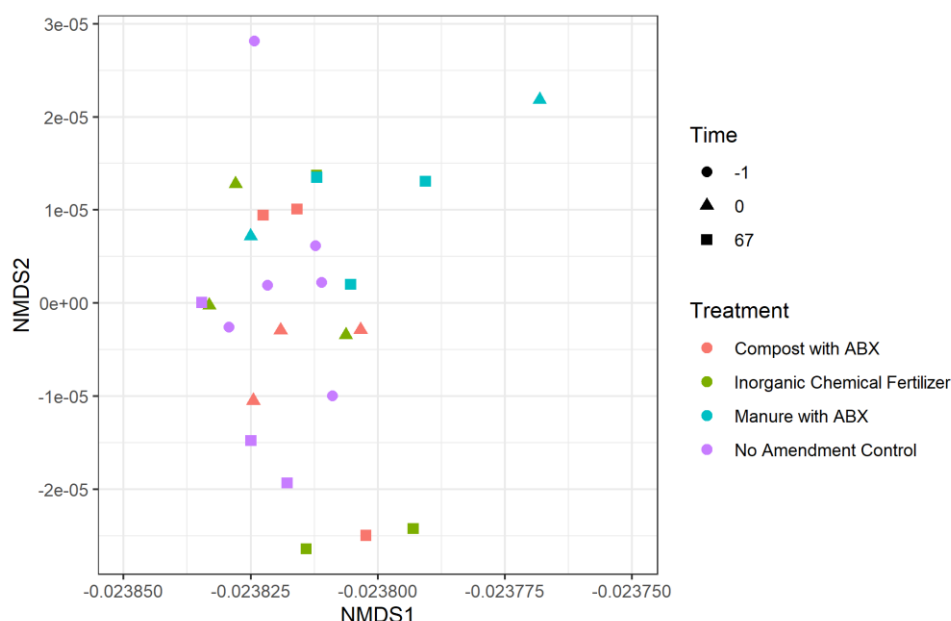


Figure S2. NMDS comparison of Bray-Curtis distance matrix of the resistomes in each amended soil over the course of 67 days. ANOSIM analysis indicated that amendment type and time had a significant effect on soil resistome profiles (ARG type and relative abundance) (ANOSIM, $R = 0.178$, $p = 0.005$ and $R = 0.127$, $p = 0.041$, respectively). ABX= antibiotics.

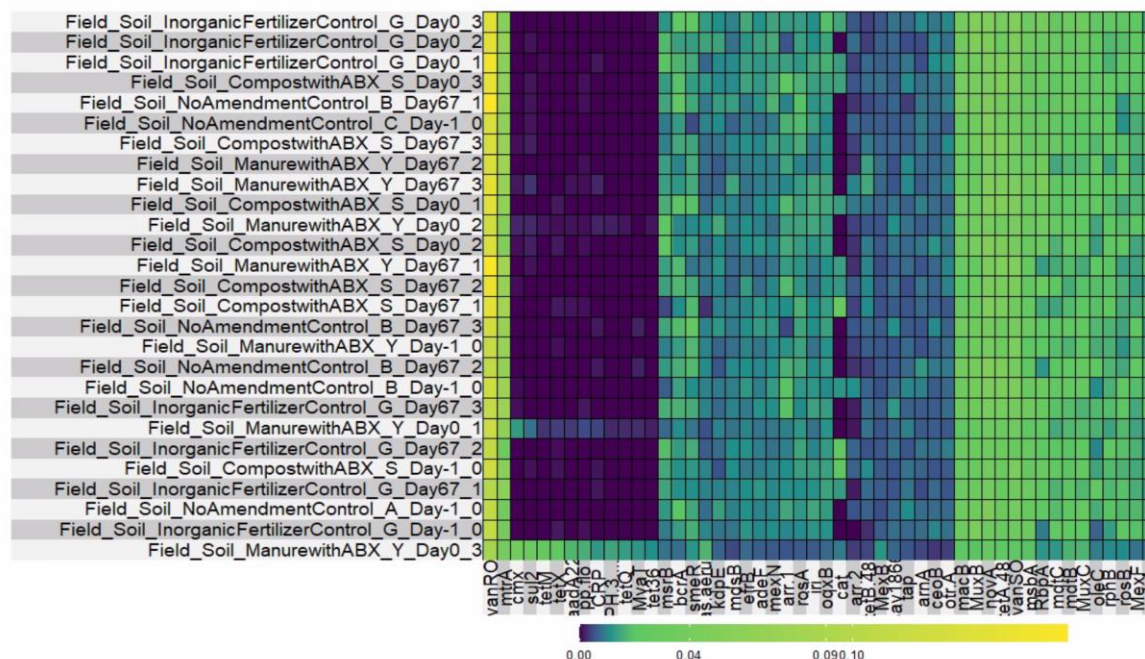


Figure S3. Heatmap visualizing genes that have a maximum relative abundance >1% in the agricultural soils determined by bray-curtis distance. Yellow color depicts low relative abundance, where red indicated high relative abundance of the gene in soils.

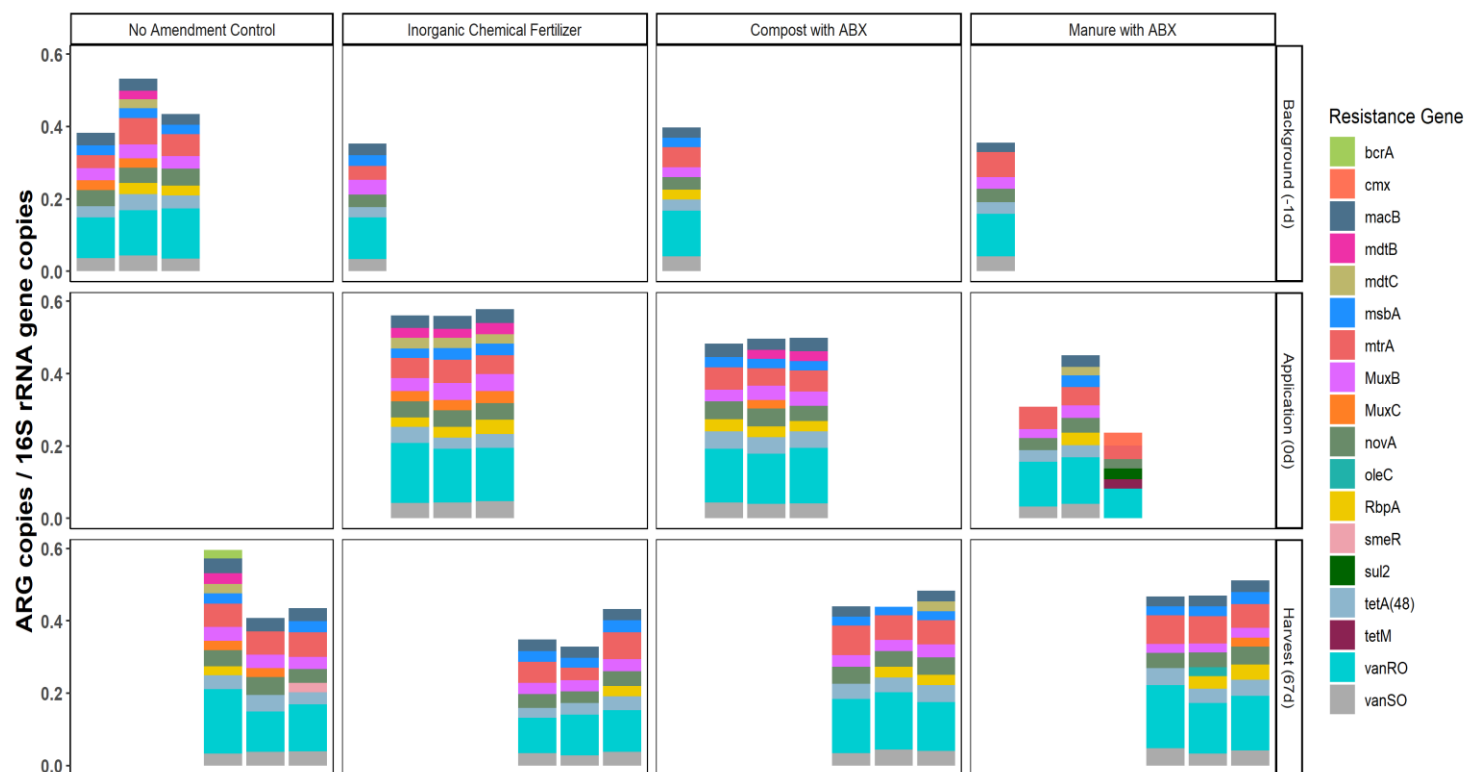


Figure S4. Relative abundances of the top 3.5% (n=18/511) of ARGs in soils grouped by treatment (n=4) and time (n=3) annotated via shotgun metagenomic sequencing. Samples were run in triplicate for all treatments and time points, except for the amended soils at background due to the assumption that prior to amendment application the soil was homogenous. ARGs were identified via alignment to CARD database v.2.0.1, and relative abundance were measured based on GreenGenes 16S rRNA annotations.

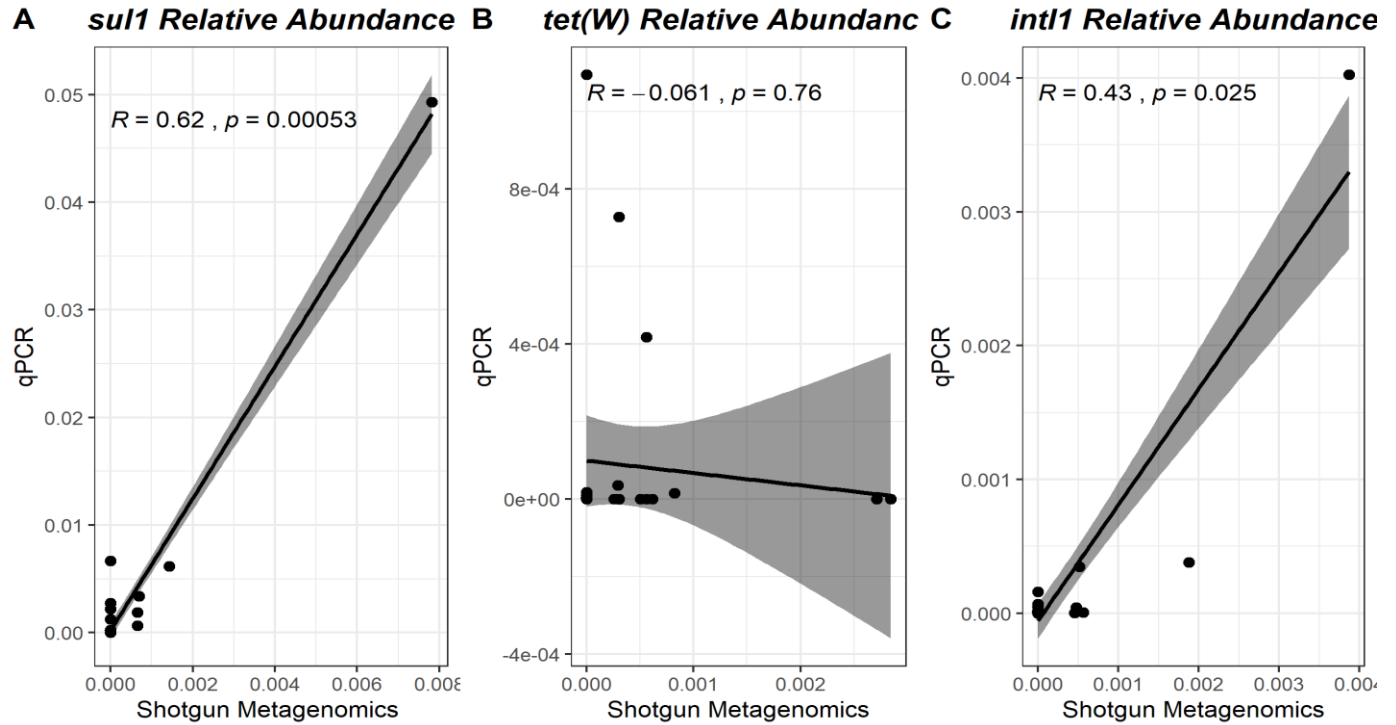


Figure S5. Correlations between qualitative polymerase chain reaction (qPCR) and shotgun metagenomics relative abundances of: (A) *sul1*, (B) *tet(w)*; and (C) *intI1*. *sul1* relative abundance were highly correlated between methods (Spearman Rho, $p = 0.017$). *tet(W)* relative abundance was not correlated between methods (Spearman Rho, $p = -0.061$). *intI1* relative abundance were highly correlated between methods (Spearman Rho, $p = 0.025$). The grey-shaded area indicates the 95% confidence interval.

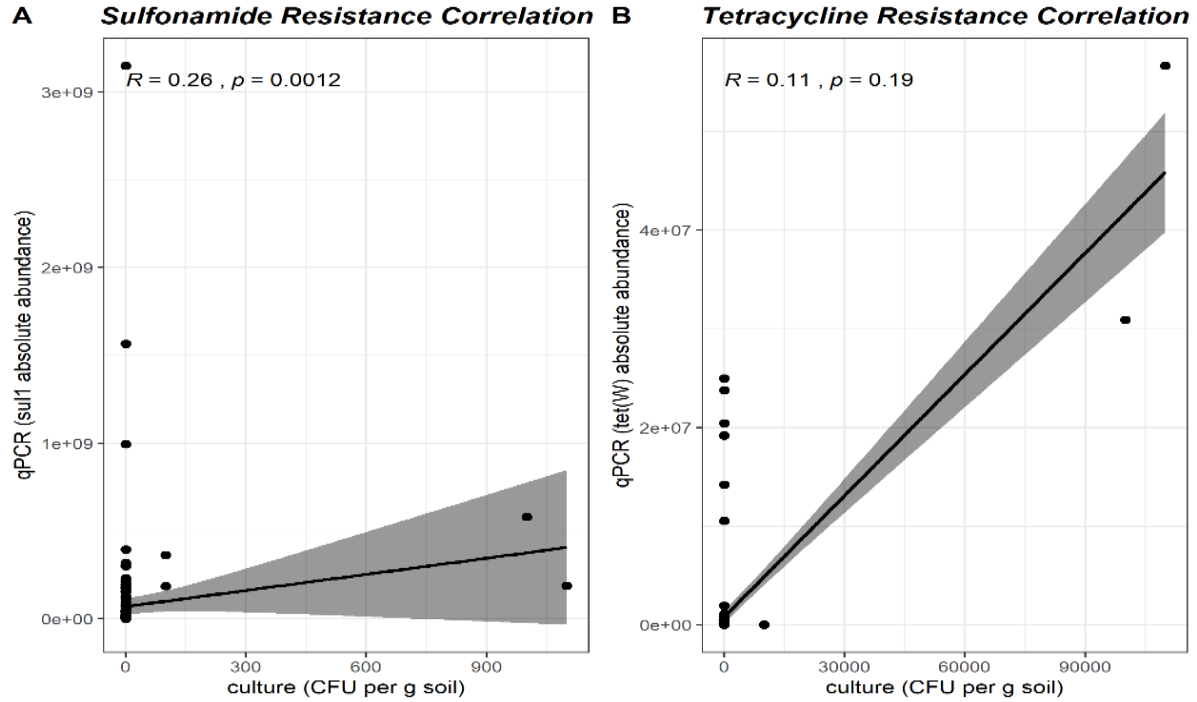


Figure S6. Correlations between qualitative polymerase chain reaction (qPCR) and culture-based methods of: A) the absolute abundance of *sul1* compared to colony forming unit (CFU) of sulfonamide-resistant fecal coliforms and B) the absolute abundance of *tet(W)* compared to colony forming unit (CFU) of tetracycline-resistant fecal coliforms. Sulfonamide associated resistance was highly correlated between methods (Spearman Rho, $p=0.0012$). Tetracycline associated resistance was not correlated between methods (Spearman Rho, $p=0.19$). The grey-shaded area indicates the 95% confidence interval.

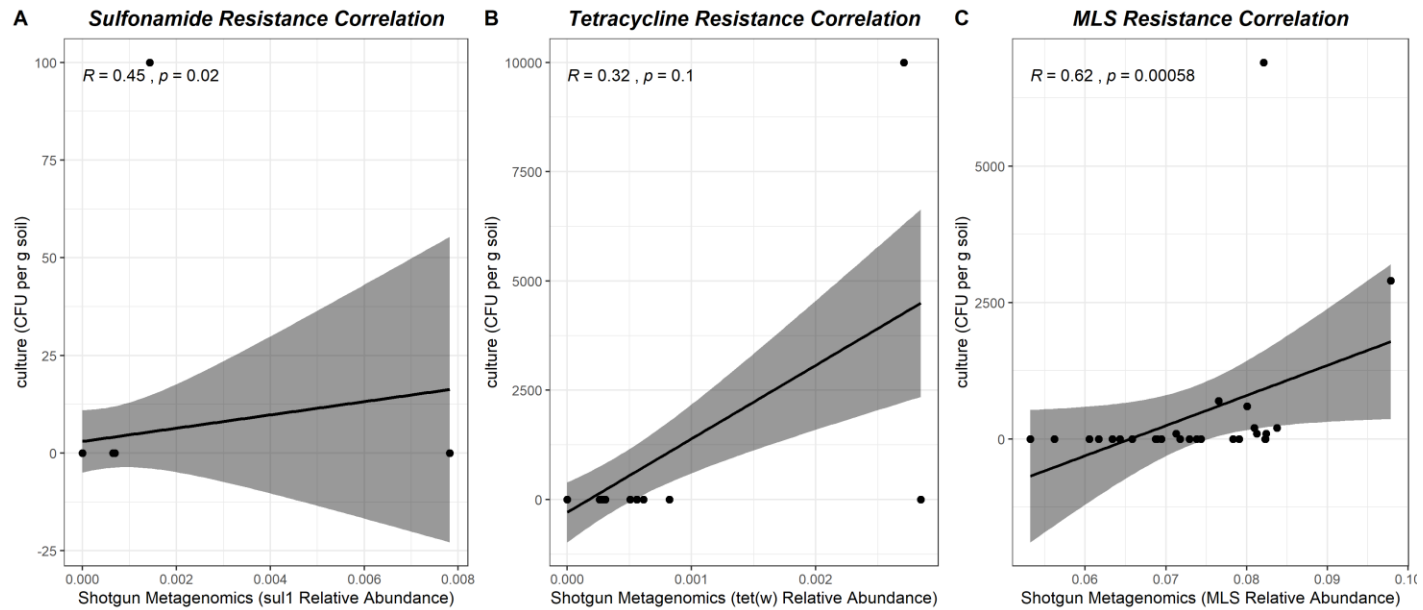


Figure S7. Correlations between shotgun metagenomics relative abundances and culture-based methods of: (A) sulfonamide resistance, (B) tetracycline resistance, and (C) MLS associated resistance. *sul1* relative abundance and sulfonamide-resistant fecal coliforms were highly correlated between methods (Spearman Rho, $p = 0.02$). *tet(W)* relative abundance and tetracycline-resistant fecal coliforms was not correlated between methods (Spearman Rho, $p = 0.01$). MLS associated relative abundance were highly correlated between methods (Spearman Rho, $p = 0.0005$). The grey-shaded area indicates the 95% confidence interval.

Chapter 4. Examination of metal presence and metal resistance within inorganically and organically fertilized agricultural soil microbial communities

A research article in preparation for Applied & Environmental Microbiology.

Wind, L.*, Krometis, L-A., Hession, W.C., Badgley, B., and Pruden, A. Cross-resistance of metal and antibiotic resistance genes within agricultural soil microbial communities. (submission anticipated Winter 2020).

4.1 Abstract

Agricultural soils are often associated with heavy metal contamination from inorganic and organic fertilization, which can alter native soil microbial community composition and functions. Metal contamination may increase the incidence of metal resistance genes (MRGs), which can promote co-selection with antibiotic resistance genes (ARGs). In the present study, we analyzed the effects that inorganic (chemical) and organic (manure, compost) fertilizers have on soil metal concentration throughout the duration of one growing season. Heavy metal presence was compared with soil microbial community diversity as determined via 16S rRNA amplicon sequencing. Relative abundances of MRGs and ARGs relative were quantified for all soils via shotgun metagenomics. All amended soils, regardless of amendment with inorganic or organic fertilizers, had increased concentrations of Cd and As compared to background control soils. Immediately following amendment, there were statistically significant differences between soils by fertilizer type. However, by the end of the growing season all soil microbial communities were statistically indistinguishable from background compositions, regardless of initial treatment. Total MRG relative abundance was highest in soils amended with inorganic fertilizers as compared to manure and compost amended soils. Cu-resistance genes were most prevalent among the amended soils. Cross-resistance analysis between MRGs and ARGs suggest that multidrug efflux pump complexes are the most likely to confer resistance to both metals and antibiotics in these agricultural soils. MRG and ARG relative abundances were strongly correlated, however metal presence and MRG relative abundances were not. Surprisingly, these results suggest a shift from inorganic to organic fertilization of agricultural soils may lead to MRG reduction and ultimately mitigate cross-resistance of MRGs and ARGs.

4.1.1 Importance

Heavy metal contamination is an unintended consequence of applying manure and livestock-derived composts to agricultural soils. Within the soil microbial community, metal resistance genes (MRG) are often selected via the same mechanisms as antibiotic resistance genes (ARGs) through co- or cross-resistance. This work compares patterns of MRG and ARG detection and microbial community diversity between soils amended with varying inorganic (chemical) and organic (manure, compost) fertilizer using shotgun metagenomics. Total MRG relative abundance was highest in soils amended with inorganic fertilizers as compared to manure and compost amended soils. MRG relative abundance was twice that of ARG relative abundance, with evidence that cellular efflux pumps were a common cross-resistant mechanism for these soil samples. Given that MRGs were elevated in agricultural soils post fertilization, regardless of amendment type, the ecological impact of metals addition should be considered when designing best management practices to reduce both ARG and MRG dissemination in agroecosystems.

4.2 Introduction

Agricultural practices including fertilization, irrigation, and livestock manure management strategies can result in environmental heavy metal and metalloid accumulation in soils, potentially presenting risks within food chain systems (Lopes et al., 2011). Livestock feeds intentionally include trace inorganic minerals and metals at levels designed to inhibit the growth of less desirable microorganisms, prevent disease, and promote animal growth (Ji et al., 2012; Seiler and Berendonk, 2012; Yu et al., 2017). Up to 90% of the metals used in feed are ultimately excreted by the livestock (Imran et al., 2019), and therefore animal manure and also inorganic fertilizer addition has been identified as the most prominent source of heavy metal input to soils (Yu et al., 2017; Shi et al., 2018a). Given that heavy metals become bioavailable when changes in their chemical form occur (Han et al., 2001), can be absorbed by plants and crops (Rattan et al., 2005; Ismail and Moustafa, 2016), and are widely recognized as toxic (Järup, 2003); the accumulation of metals in agricultural soils presents potential food safety concerns (Tóth et al., 2016).

In addition to direct inputs of metals, inorganic and organic amendments can also alter soil microbial communities (Philippot et al., 2013; Hartmann et al., 2015; Francioli et al., 2016; Lopatto et al., 2019). Long-term (i.e. 20-100+ years) fertilization studies indicate that bacterial abundance and diversity in soils fertilized with organic inputs (manure, compost) is greater than that of soils amended with inorganic fertilizers (Hartmann et al., 2015; Francioli et al., 2016). Beyond the effects of nutrients alone, heavy metal enrichment can also alter the composition and functions of autochthonous microbial communities (Shi et al., 2002; Gołębiewski et al., 2014; Li et al., 2017) through the inhibition of enzymatic activity, direct denaturing of proteins, and/or damage to cell membrane integrity (Leita et al., 1995; Khan et al., 2010).

As in all environments, some microorganisms acquire resistance mechanisms that permit survival in the presence of elevated levels of toxic heavy and/or essential metals (Igiri et al., 2018). These abilities are well-documented in environmental science applications, such as the bioremediation of polluted environments to convert heavy metals into less toxic states (Abbas et al., 2014). Because of this selective pressure, soil microbial communities exposed to elevated levels of heavy metals are generally less diverse (Yao et al., 2003; Gołębiewski et al., 2014) and mediate lower rates of nutrient cycling, respiration, and enzyme activity (Shi et al., 2018b). Bacteria adapted to heavy metal enrichment rely on strategic resistance defense mechanisms that include limiting metal uptake, inactivation/sequestration, and targeted use of efflux pumps (Beaber et al., 2004; Davies and Davies, 2010; C Reygaert, 2018; Johnson et al., 2019), which are coded for via metal resistance genes (MRGs). As soil microbiomes have direct effects on crop (phylosphere) microbiomes (Jacoby et al., 2017), focus must be given to manure management and amendment addition which may ultimately effect downstream crop microbiomes.

Heavy metal enrichment may also stimulate or support the dissemination of antibiotic resistance, which has been identified as a pressing environmental health concern (Ashbolt et al., 2013; Berendonk et al., 2015). A long-term field-scale study analyzing the impact of pig manure application on agricultural soils in China observed a positive correlation between multiple sulfonamide and tetracycline-resistance genes relative abundances quantified via qPCR and Cu, Zn, and As levels (Guo et al., 2018). Antibiotic resistance genes (ARGs) often co-occur with metal resistance genes (MRGs) via co- or cross-resistance, which appears to be common when microbial

populations are under biological and heavy metal stress (Baker-Austin et al., 2006; Di Cesare et al., 2016). Co-resistance occurs when MRGs and ARGs are physically co-located in the cell's genome (plasmid, mobile genetic element, transposon; Pal et al., 2015). Cross-resistance refers to mechanisms (i.e., efflux pump, enzymatic inactivation, cell permeability) that simultaneously confer resistance to both metals and antibiotics through the same gene (Chapman, 2003).

Previously, observations and/or quantification of co- and cross-resistance depended on susceptibility testing, microarrays, and mutation frequencies for individual plasmids, integrons, and transposons (Cantón and Ruiz-Garbajosa, 2011). These techniques are labor-intensive and rely on the presence of culturable microorganisms actively expressing the resistance genes of interest. The advent of shotgun metagenomic techniques offers a relatively efficient means to broadly evaluate the potential of co-occurrence within agricultural soil microbial communities (Pal et al., 2015; Luby et al., 2016; Yu et al., 2017). Recent studies have employed metagenomic techniques to demonstrate that heavy metal exposure exerts a selective pressure on antibiotic resistance in soils (Zhou et al., 2016; Kang et al., 2018). Examination of soils from a vegetable farm in Australia at the mesocosm level determined that adding gradual levels of Cu resulted in the co-occurrence of ARGs and Cu-associated MRGs (Kang et al., 2018). Analysis of manure and soils collected from a Chinese dairy farm demonstrated significant correlations between ARG and MRG presence and heavy metal contamination (Zhou et al., 2016). Understanding patterns of heavy metal and MRG presence to identify environmental co-selection pressures within microbial communities (Zhu et al., 2013) may prove useful in preserving the usefulness of antibiotics and metals in medicine, agriculture, and public health applications (Peralta-Videa et al., 2009; Grass et al., 2011).

Composting, a manure best management practice, is currently recommended by the USDA to mitigate infectious disease risks (USDA Organic, 2012), and there is encouraging evidence that composting manure prior to application also reduces ARGs. However, because heavy and trace metals are nondegradable, they persist through the composting process and are inevitably applied to agricultural fields during soil amendment (Lopes et al., 2011). Predicting the efficacy and sustainability of manure management practices requires an examination of the potential for co-selection of metals and antibiotic resistance under these conditions. In the present study, inorganic

(chemical) and organic (manure or livestock-derived composts) fertilizers were applied to agricultural soil to compare the effects of amendment on metal presence. Subsequently, the soil heavy and trace metal concentrations were compared to microbial community diversity, and MRG/ARG presence to broadly evaluate cross-resistance potential in agricultural soils collected from a field-scale experiment. This study aimed to quantify the effects of organic (manure or livestock-derived composts) vs. inorganic fertilizer on (1) soil metal concentration, (2) soil microbial community structure, and (3) soil ARG and MRG relative abundances. Additionally, this work aimed to understand specific cross-resistance mechanisms which occur between the ARGs and MRGs in the agricultural soils.

4.3 Materials and Methods

4.3.1 Experimental Design

All field work was conducted at the Virginia Tech Urban Horticulture Center (UHC) in Blacksburg, Virginia, and has been described in detail previously (Wind et al., 2018). In brief, lettuce was cultivated on land free from known livestock grazing, manure application, or other potential sources of heavy metal contamination for at least a decade. Soils were pre-treated with glyphosphate one summer prior to lettuce cultivation to clear vegetation, and 3.36 metric ton ha⁻¹ of hydrolyzed lime was added to raise the soil pH to 6.5 to ensure optimal growing conditions for lettuce in accordance with best practice.

At the beginning of the growing season (March 2016), soil amendments were applied at appropriate agronomic rates (6.72 Mg ha⁻¹) to the top 5 cm of soil within a series of 3 m x 3 m plots. Five different amendment practices were tested using fifteen plots in a complete block design: (1) no amendment control (n=3 plots); (2) inorganic chemical fertilizer control (n=3 plots); (3) dairy-derived control compost (antibiotic-free) (n=3 plots); (4) dairy-derived compost (cows treated with pirlimycin or cephalixin, i.e. compost with antibiotics) (n=3 plots); and (5) stockpiled dairy manure (cows treated with pirlimycin or cephalixin, i.e. stockpiled manure with antibiotics) (n=3 plots). In depth descriptions of antibiotic administration, manure collection, composting procedures, and amendment application are available in Wind et al. (2018) and Ray et al. (2017). Supplemental inorganic fertilizer was applied to the stockpiled manure and compost amended plots

to ensure minimal nutritional requirements for lettuce growth due to insufficient nutritional value of the manure generated amendments (Wind et al., 2018). Lettuce transplants obtained from a local grower were planted 28 d following amendment application per vegetables spacing recommendations (Virginia Cooperative Extension, 2015).

4.3.2 Soil Sampling and DNA Extraction

Soil samples were collected following aseptic technique at four time points throughout the growing season and stored at -80 °C until DNA extraction and post-processing (approx. 24 months). Sample collection times were selected in keeping with critical points in the lettuce cultivation process: background/pre-planting (-1 d), amendment application (0 d), lettuce harvest (67 d), and the USDA National Organic Produce's recommended wait-period following raw manure application to soils growing crops for human consumption (120 d; USDA Organic, 2012). Detailed soil collection and DNA extraction methods can be found in Wind et al. (2020). DNA was extracted using the FASTDNA SPIN Kit for Soil (MP Biomedicals, Solon, Ohio), with inhibition minimized through use of the ZYMO PCR OneStep PCR Inhibitor Removal Kit (ZYMO Research, Irvine, CA).

4.3.3 Metals Analysis

Prior to analysis, soil samples were removed from deep freeze at -80 °C and air dried until moisture content was effectively zero (i.e., no further change in weight). At time point -1 d, one soil sample was analyzed from each plot to represent background conditions (n=15), as the soil was assumed to be homogenous across all plots prior to amendment application (0 d). At time points 0, 67, and 120 d, three soil samples from each plot were analyzed in triplicate (n=135). Once dried, 10 g of soil was ground using a mortar and pestle until particle size was equivalent to very fine sand (<0.1 mm diameter, confirmed via sieve). Soil samples were acid digested in triplicate using the MARS6 Batch Microwave Digestion System with MARSExpress Vessels (CEM Corporation, Mathews, NC, USA). In keeping with EPA Method 3051A (USEPA, 2004), 2 g of soil, 9 ml nitric acid, and 3 ml hydrochloric acid were microwaved for 10 minutes. After a 15 min cooling period, samples were diluted to 50 ml with deionized (DI) water. The sample supernatant was decanted into sterile tubes following 24 hours of settling. Each sample was diluted to a 1:10 solution using deionized

water prior to analysis via inductively coupled plasma-mass spectrometry (ICP-MS) on a Thermo Electron X-Series (Thermo Scientific, Waltham, MA, USA) following standard methods 3030D and 3125B (APHA/AWWA/WED, 1998). The heavy metals and metalloids associated with the most prevalent MRGs annotated (Co, Ni, Cu, Zn, As) and of the most environmental concern (Cr, Fe, Cd, Pb) were focused on in this study for downstream analysis. DI blanks and analytical replicates were run every twelve samples, and DI blanks were subtracted from each sample to account for variability within dilution (DI concentration <0.01% for each metal analyzed). Analytical replicates were averaged before statistical analysis.

4.3.4 Microbial Community Analysis

Soil microbial community analysis was paired with the soil metal analysis, resulting in 150 16S rRNA amplicon sequencing samples to characterize relative taxon abundance within and between treatments and over time. DNA extracts were barcoded in triplicate using the 515f (Parada et al., 2016) and 926r (Quince et al., 2011) primers to sequence the V4-V5 regions. The triplicate PCR amplicon products were pooled and analyzed on a 1.2 % agarose gel to ensure quality and absence of primer dimers. Amplicon quantity was measured via the Qubit Fluorometer 2.0 using dsDNA HS assay kit (ThermoFisher Scientific, Waltham, MA). 240 ng of each PCR amplicon product was composited, and the final sample was purified using QIAquick Purification Kit (QIAGEN, Germantown, MD). Samples were sequenced on an Illumina MiSeq v3 (2x300 bp, paired-end) and data were generated based on the Earth Microbiome Protocol (EMP; Caporaso et al., 2012). Due to low quality of reverse reads, single-end reads from 5' to 3' were used for soil microbial community analysis. Processing of demultiplexed reads was conducted using the QIIME 2 microbiome bioinformatics platform (Bolyen et al., 2019), and annotated against the GreenGenes database (DeSantis et al., 2006) to cluster 99% similar Operational Taxonomic Units (OTUs) as a proxy for microbial species. Additional description of QIIME 2 workflow can be found in Supplemental Text S1. Downstream taxonomic analysis was carried out using the 'vegan' (Oksanen et al., 2015), 'phyloseq' (McMurdie and Holmes, 2013), and 'DESeq2' (Love, 2020) R packages (R Core Team, 2016).

4.3.5 Metagenomic Analysis

Shotgun metagenomic analysis was conducted on a subset of soil samples to provide a snapshot of potential MRG presence (n= 27). Briefly, the subset of samples included: no amendment control soils at -1 d (n=3) and 67 d (n=3) and the inorganic chemical fertilizer control, compost with antibiotics, and raw manure with antibiotics amended soils at -1 d (n=1), 0 d (n=3) and 67 d (n=3) for each group respectively. 260/280 ratios to confirm DNA purity were recorded. At least 400 ng DNA was submitted per sample, except in the case of six samples where total mass was below 400 ng where all available mass was submitted. Samples were prepared using the Nextera XT library prep (Illumina, San Diego, CA) and sequenced on an Illumina NextSeq 500 (2x75 bp, paired-end). Paired-end sequences were uploaded and processed using MetaStorm (Arango-Argoty et al., 2016), and quality filtering was achieved using trimmomatic default settings to provide 80% nucleotide coverage (Bolger et al., 2014). Functional annotation of MRGs for the read matched metagenome sequences were annotated using BacMet: antibacterial biocide and metal resistance gene database (BacMet v1.1-4 Unique Metals; Pal et al., 2014). To account for variable read and gene lengths, gene counts analyzed from MetaStorm were normalized to 16S rRNA gene copy number to report overall gene relative abundance using Greengenes (DeSantis et al., 2006). Each sample contained 23,610,275 bp reads on average after quality filtering (removal of 0.21 %). In-depth read matching sequencing statistics are available in Wind et al. (2020). Cross-resistance was evaluated among the MRGs annotated and the ARGs annotated in the same soil metagenomic samples. *de novo* assembled scaffolds averaged 504 bp and the associated assembled reads ranged between 41,853 and 1,149,128 for all sequences. Due to low assemblies (i.e. 0.1-5.3 % in the soil shotgun metagenomic samples), co-occurrence of MRGs and ARGs via assembly analysis not practical for this analysis. Therefore, cross-resistance (i.e. coexistence of MRGs and ARGs in same sample) was used to compare the potential for co-resistance driven mechanisms in the agricultural soils.

4.3.6 Statistical Analysis

R v3.6.1 (R Core Team, 2016) was used for all statistical analyses, with $p < 0.05$ used as the level of significance. On the assumption that prior to amendment application the field plots were

homogenous, background (-1d) inorganic chemical fertilizer, compost(s), and manure amended soil samples were treated as no amendment control soils for statistical analysis.

A two-way analysis of variance (ANOVA) comparing treatment and time effects on metal concentrations in the amended soils was used for Fe and As as the data were normally distributed, and confirmed via Shapiro-Wilks test. A post-hoc Tukey HSD test was used to determine differences in metal concentrations among the treatment and time groups. In contrast, Mg, Ag, Cd, Cu, Ni, Zn, and Co datasets were not normally distributed, and therefore the Kruskal-Wallis non-parametric test was employed followed by a post-hoc Dunn's Test to determine directionality using 'FSA' package in R (Ogle et al., 2020).

Within the metagenomic dataset, the relative abundances of total MRGs were compared by using the Kruskal-Wallis test, followed by a post-hoc Dunn's Test. Alpha and Beta diversity metrics (Shannon, Evenness, and Bray-Curtis, Jaccard) were used to analyze microbial community composition shifts in the amended soils. Additionally, unweighted and weighted UniFrac distances were used for one-way analysis of similarities (ANOSIM) for both microbial community and MRG profile characterization. R-value cutoffs as defined by Clarke and Warwick (2001) were used ($R > 0.75$) when well separated. Cross-resistance between MRGs and ARGs was evaluated by presence of identical genes that are known to confer resistance to both metals and antibiotics using the same resistance mechanisms within the same soil sample. Observations of MRG presence and abundance were compared to previously reported observations of ARG presence and abundance (Wind et al., 2020). Potential relationships between soil metal concentration and corresponding MRG relative abundances were analyzed using Spearman's rank correlation coefficient in the amended agricultural soils (Knapp et al., 2017).

4.3.7 Data Availability

Shotgun metagenomic gene sequences, 16S rRNA amplicon sequences, and associated metadata from this study are accessible in NCBI BioProject PRJNA506850.

4.4 Results

4.4.1 Metal Concentrations in Soils

Concentrations of the heavy metals (Ni, Cu, Zn, Co, Cd, Fe, Mg, Ag) and metalloid (As) in soil differed significantly by amendment type ($p < 0.0001$, Table 4-1, Table S1). The organically amended soils (i.e., compost without antibiotics, compost with antibiotics, raw manure with antibiotics) had the highest levels recovered of all the metals. Concentrations of most metals remained constant over the course of the 120 d study, with the exception of Fe, As, Cd, and Ag concentrations ($p = < 0.001$, < 0.0001 , 0.039, 0.046; respectively). Concentrations of Cd and As increased at the time of amendment application and stayed elevated over the study in all amended soils compared to the control soils. Ag concentrations increased in soils amended with inorganic fertilizer and stockpiled manure but decreased in the compost amended soils at the time of application. By the end of the study (120 d), concentrations of Fe decreased in all the amended soils compared to the control soils (Table S2).

The highest levels of metals in the soils were all quantified at the time of amendment application (0 d), except for Zn which reported the highest level quantified at the end of the study (120 d; Table 4-1). Soils amended with raw manure had the highest levels of As (8.73 ppm at time of amendment) and Zn (6.63 ppm at the end of the study, Table S2). Soils amended with compost with or without antibiotics had the highest levels of Fe (5.27 ppm), Cu (9.69 ppm), Co (26.26 ppm), Cd (0.12 ppm), Ni (22,586 ppm); respectively. The largest increase (43.88 % Ag) in soil metal concentration relative to the control (i.e. no amendment soil) was observed in the compost without antibiotic amended soils. The largest decrease (-21.37 % Co) relative to the control was observed in the inorganic chemical fertilized soils.

Table 4-1. Summary statistics of the top metals associated with annotated MRGs characterized within the soils.

	Fe ^{*+}	Co	Ni	Cu	Zn	As ^{*+}	Cd [*]	Mg	Ag [*]
Background Concentration (ppm)	19165.18	5.38	4.97	6.84	19.82	4.15	0.09	559.71	0.02
% Change in Soil Metal Concentration compared to background levels in No Amendment Control Soils									
Treatment applied to Soils									
Inorganic Chemical									
Fertilizer	-19.78%	-21.37%	-6.84%	-13.25%	0.20%	-17.43%	4.47%	-18.89%	-0.10%
Compost without Abx	-15.18%	-5.34%	1.51%	-5.63%	7.72%	-9.30%	27.73%	-16.29%	43.88%
Compost with Abx	-3.07%	32.61%	8.30%	10.97%	17.74%	8.68%	10.12%	24.59%	25.09%
Raw Manure with Abx	-8.22%	-6.77%	13.44%	14.38%	18.61%	2.49%	13.65%	33.09%	29.04%

⁺ 2-way ANOVA used for Fe and As statistical analysis, all other metal concentrations were evaluated using the non-parametric Kruskal-Wallis followed by a post-hoc Dunn Test. Significance of **p<0.05** is indicated in bold.

*Time was a significant variable for metal soil concentration

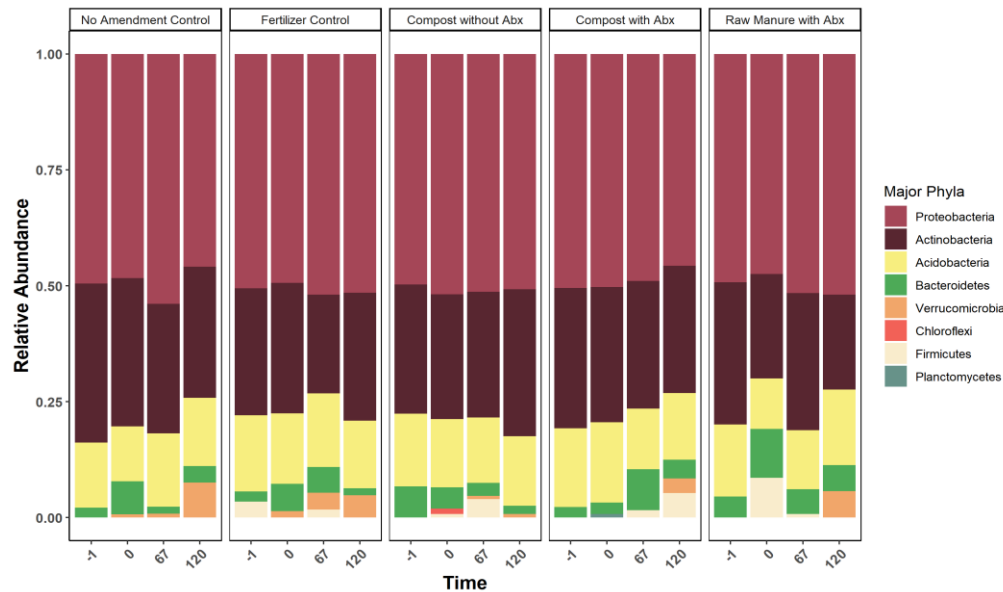
4.4.2 Soil Microbial Community Composition

Proteobacteria, *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Verrucomicrobia*, *Chloroflexi*, *Firmicutes*, and *Planctomycetes* were the most abundant phyla, comprising over 95% of all the soil microorganisms characterized (Figure 4-1A). Relative abundances of *Actinobacteria* and *Firmicutes* in the soils differed significantly by treatment (Kruskal-Wallis, $p<0.002$; 0.02, respectively). Additionally, *Proteobacteria* and *Verrucomicrobia*, in soils differed significantly by treatment (Kruskal-Wallis, $p<0.001$; 0.007, respectively) and over time (Kruskal-Wallis, 0.042; <0.001 ; respectively). *Verrucomicrobia* relative abundances significantly increased among all the soils between each time point (post-hoc Dunn; $p<0.001$ for all iterations), where *Proteobacteria* relative abundances varied from background to end of study (post-hoc Dunn; $P<0.001$). Relative abundances of *Verrucomicrobia* in soils amended with compost without antibiotics were significantly less than that observed in the no amendment control and inorganic chemical fertilizer amended soils. Soils amended with raw manure had significantly more relative abundance of *Firmicutes* than the no amendment control soil (post-hoc Dunn; $p=0.013$).

Within all 150 soil samples, 133 unique classes containing 418 unique families were identified across the soil microbial communities (Figure 4-1B). At the time of application, there was a spike

in *Moraxellaceae* (phyla; *Proteobacteria*) in the raw manure amended soils. At the time of lettuce harvest (67d), there was a spike in all the inorganic and organic amended soils of *Chitinophagaceae* (phyla; *Bacteroidetes*) (Figure 4-1B).

A.



B.

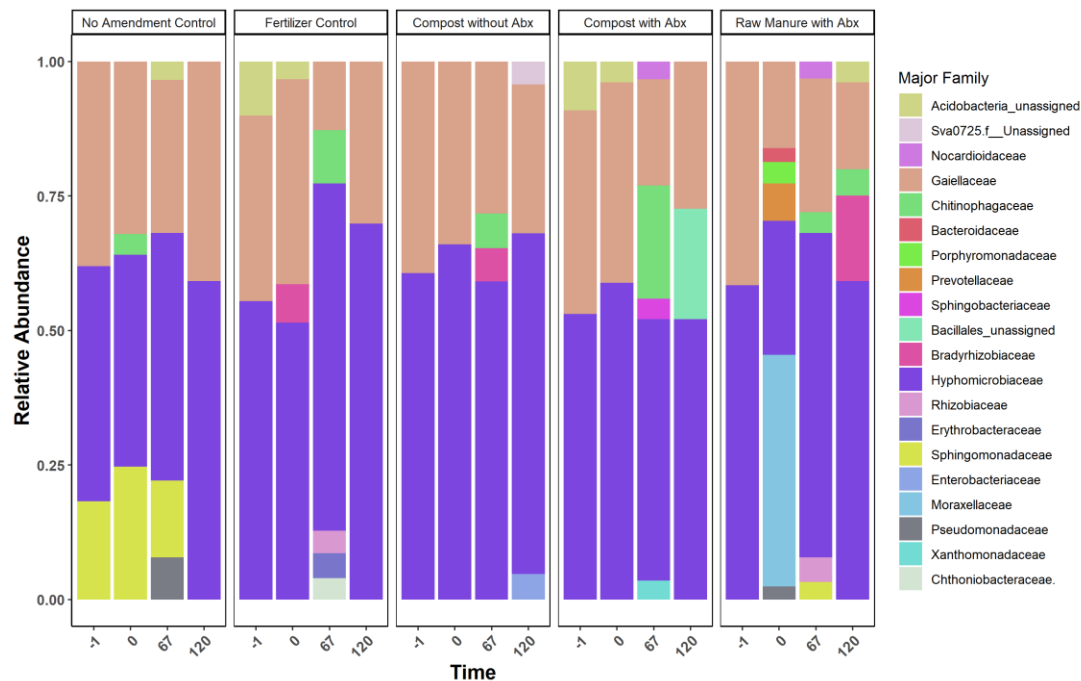


Figure 4-1. Relative abundance of major phyla (A) and family (B) that composed more than 95% of each soil microbial community grouped by treatment over the course of the study (background, -1; amendment application, 0; crop harvest, 67; and study completion, 120d). For the inorganic fertilizer control, composts and manure amended soils, each bar represents three biological replicates in triplicate (i.e., n=9). Based on the assumption that prior to amendment application the soil was homogenous, three analytical replicates of one biological replicate were sequenced for the no amendment control soils at background for each amended soil treatment (i.e., n=3).

Treatment had a significant effect on the alpha diversity (Shannon, Evenness) between the control and amended soils (Kruskal-Wallis, $p < 0.005$; Figure S1). The no amendment control soil samples were more diverse (i.e., had more OTUs) than the inorganic and organic amended soils, and were significantly different from each of the other amended soils (post-hoc Dunn: < 0.005 for each iteration). For each treatment effect, the soil microbial community profiles were distinctly different from each other according to Bray-Curtis dissimilarity beta diversity analysis (PERMANOVA, $p < 0.001$, Figure 4-2).

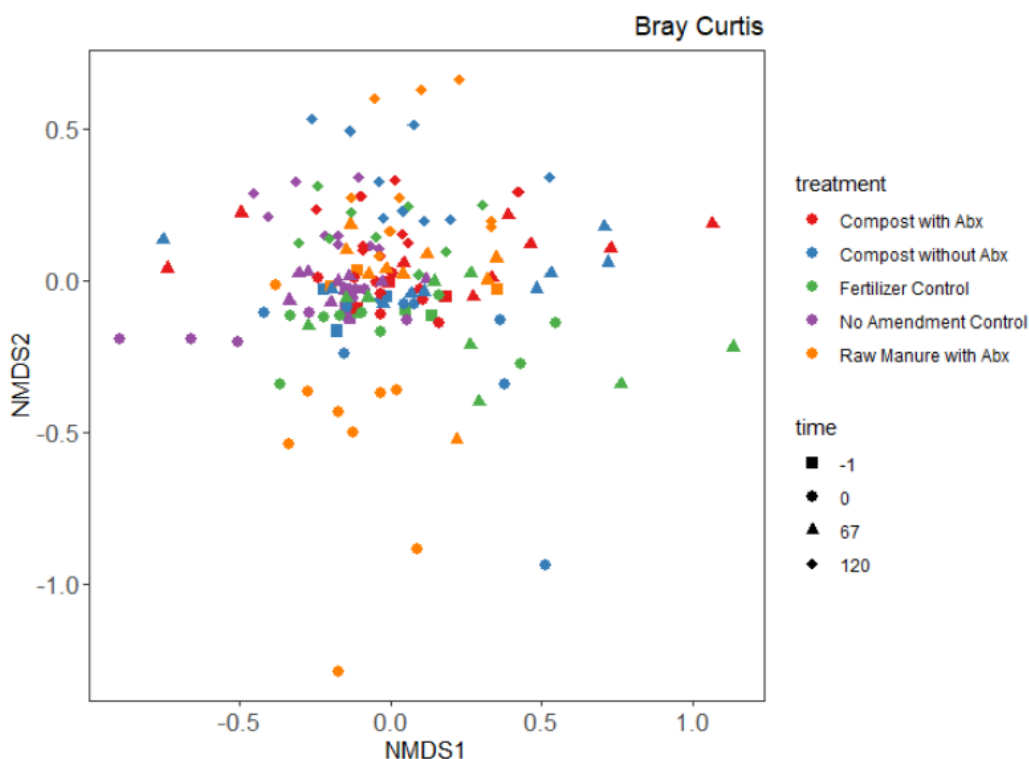


Figure 4-2. Non-metric dimensional scaling (NMDS) plot using the Bray-Curtis Dissimilarity distance matrix to compare the bacterial microbiota profiles grouped by treatment and over the course of the study (background, -1; amendment application, 0; crop harvest, 67; and study completion, 120d) using 16S rRNA gene amplicons annotated against the GreenGenes database with 99% similar OTU.

4.4.3 Metals Resistance Genes in Soils

The relative abundances of MRGs within each soil were significantly different based on which amendment had been applied (Kruskal-Wallis; $p = 0.0378$), and over the course of the growing season (Kruskal-Wallis; $p = 0.027$; Figure 4-3, Figure S2). The inorganic chemical fertilized soils

had the greatest total relative abundances of MRGs, with an average of 2.36 MRG copies per 16S rRNA gene copy; and they were significantly different from the stockpiled manure amended soils (average 2.16 MRG copies per 16S rRNA gene copy; Dunn's Test, $p=0.039$, Figure S2). There was no significant difference between soil MRG levels at the time of the amendment (0 d, average 2.33 MRG copies per 16S rRNA gene copy) and at the time of harvest (67 d, average 2.28 MRG copies per 16S rRNA gene copy); however, MRG levels were significantly greater compared to the background levels (-1 d, average 2.09 MRG copies per 16S rRNA gene copy; post-hoc Dunn, $p=0.021$).

The 10 most abundant resistant metal groups detected among the soils, characterized by total MRG relative abundances and the diversity of distinct MRGs, were associated with Ni, Cu, Zn, Co, Cd, Fe, As, Mg, Ag and "others" defined as a group chemical compounds toxic to bacteria (Figure 4-3, Pal et al., 2014). Overall, the top three metals which corresponded with total MRG relative abundances among the soils were Cu, As, and Fe-resistance genes (12.3, 7.9, and 6.6 total MRG gene copies per 16S rRNA gene copy, respectively). A Cu related resistance gene, *dnaK*, was the most prevalent MRG among soils regardless of amendment type, and was found at its highest abundance in the stockpiled manure amended soils at the time of harvest (67d; 0.303 gene copies per 16S rRNA gene copy, Figure S3). Cu-resistance genes were on average 3.1 and 1.6 times more likely to be annotated within the soils compared to As- and Fe-resistance genes. Of the 38 unique Cu resistance genes across the soils, *arcD* accounted for almost 1/8th of total Cu-associated MRG relative abundance annotated. In addition to Cu-resistance genes, *ruvB* and *acn* were also notably abundant, which confer Cr and Fe resistance, respectively. In total, 226 MRGs were annotated among the soils, with 156 found in all amended soils (Figure S4). Stockpiled manure and the inorganic chemically fertilized amended soils each had 10 unique MRGs not found in other treatments, followed by 6 and 3 in the no amendment control and compost with antibiotic amended soils, respectively.

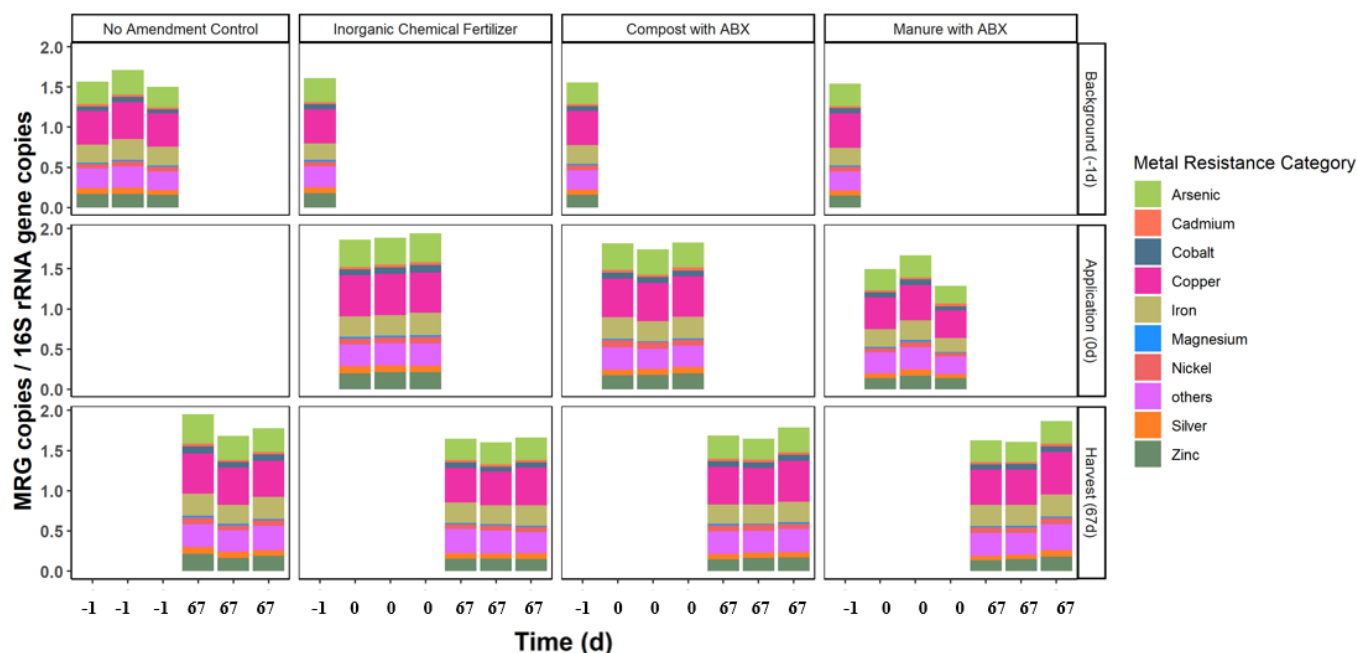


Figure 4-3. Total MRG relative abundances in soils represented according to the top 10 abundant metals to which they encode resistance and grouped by treatment (n=4) for each column and by time (n=3) for each row annotated using BacMet v1.1-4 via shotgun metagenomic sequencing. Each stacked bar represents one soil shotgun metagenomic sequence replicate among soil samples taken within the same treated soil plot for each sampling time. Treatment and time effects were measured in triplicate (i.e. three bars per box), except for the amended soils at background (-1d) based on the assumption that prior to amendment application the soil was homogenous to the No Amendment Control soils. No Amendment Control soils were not sequenced at application (0d) as those soils were assumed to be the same as at the time of background (-1d) due to lack of amendment. BacMet defines “others” as a group chemical compounds toxic to bacteria.

4.4.4 Cross-resistance of antibiotic- and metal resistance genes

Through comparative analysis of the annotated MRGs identified here and a previously published ARG dataset (presented in detail in Wind et al., 2020), ten genes were identified which confer resistance to both antibiotics and metals in the agricultural soils (Table 4-2, Figure S5). Nine resistance genes were multidrug resistant (*acrD*, *baeR*, *baeS*, *cmeB*, *golS*, *mdtA*, *mdtB*, *mdtC*, and *smeD*), and one was a macrolide-lincosamide-streptogramin resistant gene (*mgtA*). These genes conferred resistance to Cu, Zn, Au, Co, Mg, and the “other” chemical compounds toxic to bacteria. The majority of these genes are associated with efflux pump resistance mechanisms though *golS* is associated with regulator activation and *mgtA* with enzyme inactivation mechanisms.

Of these cross-resistant genes, *mdtB* and *mdtC* were the most abundant in these samples, i.e. on average 0.042 and 0.039 resistance gene copies per 16S rRNA gene copy across all the soils. When analyzing amendment type and time effects, *mdtB* was the only cross-resistance gene that was significantly different between amended soils (Kruskal-Wallis, $p=0.028$) and none of the genes were significantly different over time (Kruskal-Wallis, $p>0.05$). *mdtB* was significantly less abundant in the raw manure amended soils (0.036 *mdtB* copies per 16S rRNA gene copy) compared to the no amendment control soils (0.046 *mdtB* copies per 16S rRNA gene copy; Dunn Test, $p=0.008$).

Table 4-2. Summary of potential cross-resistant ARGs and MRGs detected and associated mechanisms in the amended agricultural soils.

Resistance Gene	Associated Antibiotic Drug Class ¹	Associated Metal ²	Resistance Mechanism(s)
acrD	Multidrug	Cu, Zn	Efflux pump
baeR	Multidrug	other	Efflux pump, cell division
baeS	Multidrug	other	Efflux pump, cell division
cmeB	Multidrug	other	Efflux pump
golS	Multidrug	Au	Regulator of mdtABC efflux complex
mdtA	Multidrug	Zn	Efflux pump
mdtB	Multidrug	Zn	Efflux pump
mdtC	Multidrug	Zn	Efflux pump
mgtA	M-L-S ⁺	Co, Mg	Enzyme inactivator
smeD	Multidrug	other	Efflux pump

⁺ M-L-S: Macrolide-Lincosamide- Streptogramin

* other:

¹ ARGs were annotated against the CARD v2.0.1 database

² MRGs were annotated against the BacMet v1.1.4 database

4.5 Discussion

4.5.1 Metals Enrichment in agricultural soils

The agricultural land in this study had not been used for agricultural production for at least 10 years prior to amendment application and therefore, observed increases in amended soils as compared to the control soil represent the impact of one growing season. Amendments were not

purposefully spiked, indicating increased metal levels were likely the result of typical feed and herd management. The cow feed was supplemented with Cu (26 ppm), Fe (179 ppm), Zn (109 ppm), and Co (0.87 ppm), and a 5 % CuSO₄ solution is flushed throughout the lactating commercial herd barn at the Dairy Science Complex (Blacksburg, VA) to ensure hoof health. Estimated calculations suggest at any given time based on herd size, lbs. of manure produce by each animal, four floor flushes a day, facility size there is 11 mg of Cu on the barn floor (Personal Communication with Dairy Science Researcher, Dr. Katherine Knowlton). Although raw manure and compost levels of metals prior to soil application were not collected, increases in metal concentration post-application implies that these amendments were a source.

While increases in some metal concentrations of organically amended soils were observed, the concentrations remained within the normal range expected in agricultural soils and were not at levels of environmental concern. For example, mean As levels in these soils ranged between 5.71-8.73 ppm, and the mean As levels throughout the contiguous US was found to be 5 ppm (Smith et al., 2014). The As concentrations within these agricultural soils are not high enough to affect crop uptake (Punshon et al., 2018); however, there is no safe level of As for human consumption and the potential bioaccumulation into crops is a known concern among the agri-food industry (Capita and Alonso-Calleja, 2013; Nguyen et al., 2019).

4.5.2 Treatment effect on soil microbial community

Multiple studies report that the introduction of new taxa via livestock-derived organic amendments does not disrupt the stable native microbial communities that already exist long-term (Hamm et al., 2016; Podmirseg et al., 2019). Although short-term changes in community composition may be observed following amendment, soils revert to the pre-amended state within a growing season (Ghosh et al., 2010). Zhang et al. (2018) hypothesized that any immediate microbial community shifts observed in agricultural soils following manure amendment are more likely related to changes in environmental or climate variables (e.g., temperature, soil respiration, etc.) that often occur concurrently with manure amendment. Francioli et al. (2016) suggested that the resiliency of native microbial communities in agricultural soils is due to the long-term application of organic livestock-derived fertilization that makes seasonal manure application marginal to insignificant in community structure changes.

Given that this was a 120 d study it is not surprising that while some distinctions in community composition were observed among soils of different treatments immediately following amendment, with the exception of raw manure amended soils, soil sample communities were statistically indistinguishable from the background soils by the study's conclusion (Figure 4-1, 4-2A,B). To analyze the soil microbial shifts on a deeper level, a follow up indicator family-level analysis was carried out. Of the 418 families detected, 180 were significant among the soils ($p < 0.001$). Of the most significant families associated within each soil treatment, there was no overlap at the family level (i.e. no amendment control: *Ktedonobacteria* (unassigned); inorganic fertilizer control (*Chthoniobacteraceae*); Control Compost (*Nocardiopsaceae*); Compost (*Bacillales* (unassigned)); and manure (*Corynebacteriaceae*). While the indicator analysis highlighted that inorganic and organic fertilizers may alter microbial communities at the family level, the soil remains similar at the phyla level over the course of a growing season. This may suggest the native soil microbial communities do not shift drastically when foreign communities (i.e., dairy-derived manure or compost) are added even when there is no recent history of agricultural use. In the future, characterizing known multi-resistant (i.e., metal and antibiotic) foodborne pathogens (i.e., *Salmonella* spp., *Staphylococcus aureus*, *Escherichia coli*) will be necessary to evaluate the potential risks associated with the application of manure-derived amendments on the soil microbial community population at the field-scale in the long-term (Perry and Wright, 2013; Wales and Davies, 2015).

4.5.3 Heavy metals, MRGs and ARGs correlation in agricultural soils

Cu and Zn are considered indicators of environmental pollution in agricultural systems (Imran et al., 2019); therefore, the increase in these metal concentrations in the soils may be indicative of potential enrichment of the MRGs detected in the soils amended with dairy-derived compost and manure. However, this was not the case in these agricultural soils as Cu-, Zn-, As, and Fe-resistance genes relative abundances was not correlated with Cu, Zn, As and Fe soil metal concentration (Spearman's Rank, $p = 0.545, 0.973, 0.998, \text{ and } 0.948$; respectively).

Metals-resistance appeared more widespread than antibiotic resistance in these soils, i.e. the relative abundance of annotated MRGs was 1.5x the relative abundance of ARGs, though MRGs and ARGs were positively correlated (Spearman's Rank; $p < 0.001$, $\rho = 0.73$; Figure S6). Multi-

resistance genes were recovered in both soils amended with inorganic fertilizer, manure, and compost, which may indicate that the addition of an organic amendment is not necessary to stimulate MRG proliferation. Previous studies have observed increases in MRGs (Wang et al., 2018) and metal concentrations (Atafar et al., 2010), in soils amended with inorganic fertilizers. Antibiotic and metals cross-resistance interactions have been characterized in specific bacterial genomes, e.g. *E. coli*, *Acinetobacter*, and *Salmonella* (Nashino et al., 2017; Nakajima et al., 1995; Imran et al., 2019). Given that soil amendment at typical agronomic rates appears to impact metals concentrations and MRG presence throughout the duration of one growing season, and the potential for co-selection of ARGs in human pathogens is of concern, metals management should be more actively implemented within best practices to support agroecosystems.

4.5.4 Potential agricultural soil assembly and biases

Due to low metagenomic assembly (0.1-5.3%), MRG/ARG co-occurrence analysis was limited in this study. Investigation of co-selection was not possible via assembled scaffolds, though cross-resistance was evaluated. The inherent diversity of soil metagenomes is recognized as a challenge for full sequencing depth coverage (Howe et al., 2014; Myrold et al., 2014). Additionally, MRG and ARG ontology databases must be taken account for when comparing resistance genes. For example, the prevalence in Cu-associated MRGs in this study may be related to the 13 % increase in Cu concentration in the raw manure amended soils. However, Cu resistance genes are also the most annotated group within the BacMet database: of the 420 MRGs within the database (BacMet v1.1-4), Cu had the most (n=60 Cu-resistance genes), followed by Zn (n=58 Zn-resistance genes) and As (n=51 As-resistance genes) (Pal et al., 2014). In the annotated agricultural soils, the groups containing the most unique MRGs were Ni, Cu, and Zn with 39, 38, and 38 MRGs, respectively. Although Ni, Cu, and Zn may have had the most unique MRGS; As, Fe, and Cu were the top metal resistance relative abundant genes on average.

4.6 Conclusions

Agricultural best management practices are increasingly focused on reducing the environmental spread of antibiotic resistance and associated potential risks posed by pathogenic microorganisms potentially found in manure and composts. Addressing the presence of heavy metals and the

potential for MRG/ARG co-selection is therefore critical in the development of holistic best management practices for agroecosystems designed to reduce the risk of antibiotic resistance dissemination. In this study we found that using organic amendments (i.e. derived from livestock) is beneficial for the reduction of total MRG relative abundances compared to the use of inorganic chemical fertilizer. However, compared to background soil levels, both inorganic and organic amendments increased MRGs over the growing season. These findings highlight that fertilization variance has the potential to increase MRGs, and there may be other ecological mechanisms driving functional gene dissemination within these agricultural soils. Although composting is a strategy to reduce antibiotic resistance mitigation strategies in agroecosystems, in this study there were no differences in MRG relative abundance between manure and compost amended soils which suggests additional best management practices may be necessary to mitigate MRG dissemination. Application of inorganic and organic amendments changed the soil microbial community composition at the time of application. After one growing season, 120 d, the community profiles were similar among all the amended soils, indicating that the native soil communities and intrinsic resistance profiles have more of an effect on what occurs within these systems than adding dairy-derived manure amendments (i.e., all returned to background levels).

Continued investigation of long-term effects of agricultural practices in relation to potential MRG presence, changes in microbial community composition, and MRG/ARG should be evaluated in soils with a focus on the potential application of these findings to best management practices. When evaluating agricultural soil MRG/ARG interactions for downstream livestock and manure management and policy actions, recognition of potential low assembly and database biases to assess potential co-occurrence mechanisms is needed, as this significantly limits the ability to understand the relative importance of management, climatic, and ecological drivers.

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Supplemental Information

Text S1. Processing of 5' to 3' forward reads was conducted using the QIIME 2 pipeline (Boylan et al., 2018). Raw single-end reads already demultiplexed by sequencing facility (The Scripps Center for Computational Biology & Bioinformatics) were imported into QIIME 2 as “SampleData[SequenceswithQuality]Q2 artifact files and used to create a single-end demux sequences .qza output file using “SingleEndFastqManifestPhred33” source format (Caporaso et al., 2012). DADA2, a model which predicts and corrects for errors across samples, was used to denoise and dereplicate single-end sequences while simultaneously filtering chimeras as the quality-filtering method prior to clustering (Callahan et al., 2016). A Q=0, i.e. no truncation, was used to keep all nucleotide sequences. Once the sequences were quality-filtered and the results were visualized as an additional quality check, and they were used to create an operational taxonomic unit (OTU) table which de-novo clustered the filtered sequences based on 99% sequence identity using VSEARCH (Rognes et al., 2016). The OTUs were assigned taxonomy using the known 99% naïve-bayes classifier from GreenGenes (DeSantis et al., 2006). After a 99% de novo clustering of the representative sequences (i.e., removal of singletons), there were 5068 unique operational taxonomic units (OTUs) among the soils. On average, each sample had 346 associated OTUs. Finally, as we are only interested in 16S rRNA from bacteria, mitochondria and chloroplast sequences were filtered out to produce the final OTU clustered .qza file to use for analysis. Processed OTU data was exported as .biom file for downstream alpha and beta diversity and phylogenetic analysis in R v3.0.1 (R Core Team, 2016).

Table S1. Detailed % change soil metal concentration compared to background levels.

		Fe	Co	Ni	Cu	Zn	As	Cd	Mg	Ag
	Background Concentration (ppm)	19165.18	5.38	4.97	6.84	19.82	4.15	0.09	559.71	0.02
Treatment	Time (d)	% Change in Soil Metal Concentration compared to background levels								
No Amendment Control	0	-4.52%	-32.65%	-13.59%	-4.20%	-13.39%	0.13%	2.34%	-24.17%	-25.42%
	67	8.57%	-25.36%	-2.91%	-1.31%	-5.65%	9.91%	-1.47%	-16.47%	22.99%
	120	3.56%	-31.77%	-14.76%	-1.42%	-4.00%	7.18%	-3.38%	-22.44%	11.52%
Inorganic Chemical Fertilizer	(0d)	-10.51%	-20.78%	-5.34%	-11.87%	-6.01%	-16.67%	-1.83%	-17.57%	-12.00%
	(67d)	-27.45%	-21.31%	-4.30%	-11.35%	4.34%	-19.74%	5.36%	-16.62%	11.77%
	(120d)	-21.37%	-22.01%	-10.89%	-16.53%	2.28%	-15.89%	9.88%	-22.48%	-0.07%
Compost without Abx	(0d)	17.85%	5.45%	-2.44%	1.38%	8.29%	18.44%	36.07%	-12.06%	93.93%
	(67d)	-31.70%	-30.47%	-14.47%	-8.47%	7.89%	-23.64%	24.12%	-23.63%	17.35%
	(120d)	-31.70%	8.99%	21.46%	-9.78%	6.98%	-22.68%	23.00%	-13.17%	20.37%
Compost with Abx	(0d)	10.19%	80.14%	16.60%	13.25%	20.46%	26.95%	7.60%	17.16%	60.22%
	(67d)	-11.16%	19.63%	-6.05%	6.88%	9.72%	-3.62%	7.89%	13.89%	-4.60%
	(120d)	-8.26%	-1.93%	14.35%	12.76%	23.04%	2.72%	14.88%	42.70%	19.64%
Raw Manure with Abx	(0d)	-5.33%	-3.86%	11.10%	27.57%	27.34%	5.60%	11.32%	31.88%	51.51%
	(67d)	-16.94%	-9.83%	-4.21%	12.75%	5.31%	-9.55%	20.48%	4.23%	-0.13%
	(120d)	-2.40%	-6.64%	33.42%	2.84%	23.18%	11.41%	9.14%	63.15%	35.74%

Table S2. In depth summary statistics of soil metal concentrations within the soils.

Treatment	Co [^]		Ni [^]		Cu [^]		Zn [^]		As*		Mg [^]		Fe*		Cd [^]		Ag [^]	
	mean	±sd	mean	±sd	mean	±sd	mean	±sd	mean	±sd	mean	±sd	mean	±sd	mean	±sd	mean	±sd
No Amendment																		
Control (-1d)	21.68	4.99	19165.18	3137.06	5.38	3.49	4.97	0.56	6.84	0.78	559.71	79.90	19165.18	3137.06	0.09	0.01	0.02	0.01
(0d)	18.35	6.15	18298.59	5814.56	3.62	0.64	4.30	0.67	6.56	0.50	655.79	105.44	18298.59	5814.56	0.09	0.02	0.03	0.01
(67d)	21.52	3.81	20807.15	2530.85	4.02	0.33	4.83	0.57	6.75	0.51	637.49	211.49	20807.15	2530.85	0.09	0.01	0.02	0.00
(120d)	21.03	4.11	19847.69	4498.53	3.67	0.29	4.24	0.43	6.75	0.61	798.73	290.68	19847.69	4498.53	0.09	0.00	0.02	0.01
Fertilizer Control																		
(0d)	17.88	5.38	17150.80	4731.36	4.26	0.73	4.71	0.75	6.03	0.72	492.19	86.15	17150.80	4731.36	0.09	0.01	0.03	0.04
(67d)	16.40	2.78	13904.85	2750.41	4.23	0.63	4.76	0.47	6.07	0.67	427.45	39.38	13904.85	2750.41	0.10	0.01	0.02	0.00
(120d)	16.80	5.07	15069.15	3200.84	4.20	0.52	4.43	0.35	5.71	0.80	485.98	56.60	15069.15	3200.84	0.10	0.01	0.02	0.01
Compost without Abx																		
(0d)	24.66	6.90	22586.91	5816.04	5.67	1.78	4.85	0.59	6.94	0.38	461.37	119.49	22586.91	5816.04	0.12	0.02	0.01	0.00
(67d)	15.27	2.53	13089.44	1325.75	3.74	0.22	4.25	0.36	6.26	0.46	466.71	63.15	13089.44	1325.75	0.11	0.03	0.02	0.00
(120d)	15.29	3.28	13089.38	2138.81	5.86	5.17	6.04	5.01	6.18	0.35	433.91	46.57	13089.38	2138.81	0.11	0.02	0.02	0.00
Compost with Abx																		
(0d)	26.26	7.68	21118.94	4375.57	9.69	6.45	5.80	1.08	7.75	0.62	424.46	47.49	21118.94	4375.57	0.10	0.01	0.01	0.00
(67d)	21.37	2.92	17027.02	2167.09	6.44	5.02	4.67	0.36	7.32	1.16	467.54	72.63	17027.02	2167.09	0.10	0.01	0.02	0.01
(120d)	23.00	6.83	17581.81	3235.72	5.28	0.80	5.68	1.46	7.72	1.12	434.11	52.30	17581.81	3235.72	0.10	0.01	0.02	0.00
Raw Manure with Abx																		
(0d)	23.48	6.20	18143.67	3143.45	5.17	0.53	5.52	0.80	8.73	1.18	738.17	171.08	18143.67	3143.45	0.10	0.01	0.03	0.01
(67d)	19.51	5.33	15917.86	3301.46	4.85	0.44	4.76	0.25	7.72	0.88	583.39	58.60	15917.86	3301.46	0.11	0.01	0.02	0.00
(120d)	23.45	6.32	18705.15	4608.31	5.02	0.55	6.63	1.24	7.04	0.89	913.17	210.28	18705.15	4608.31	0.10	0.01	0.02	0.01

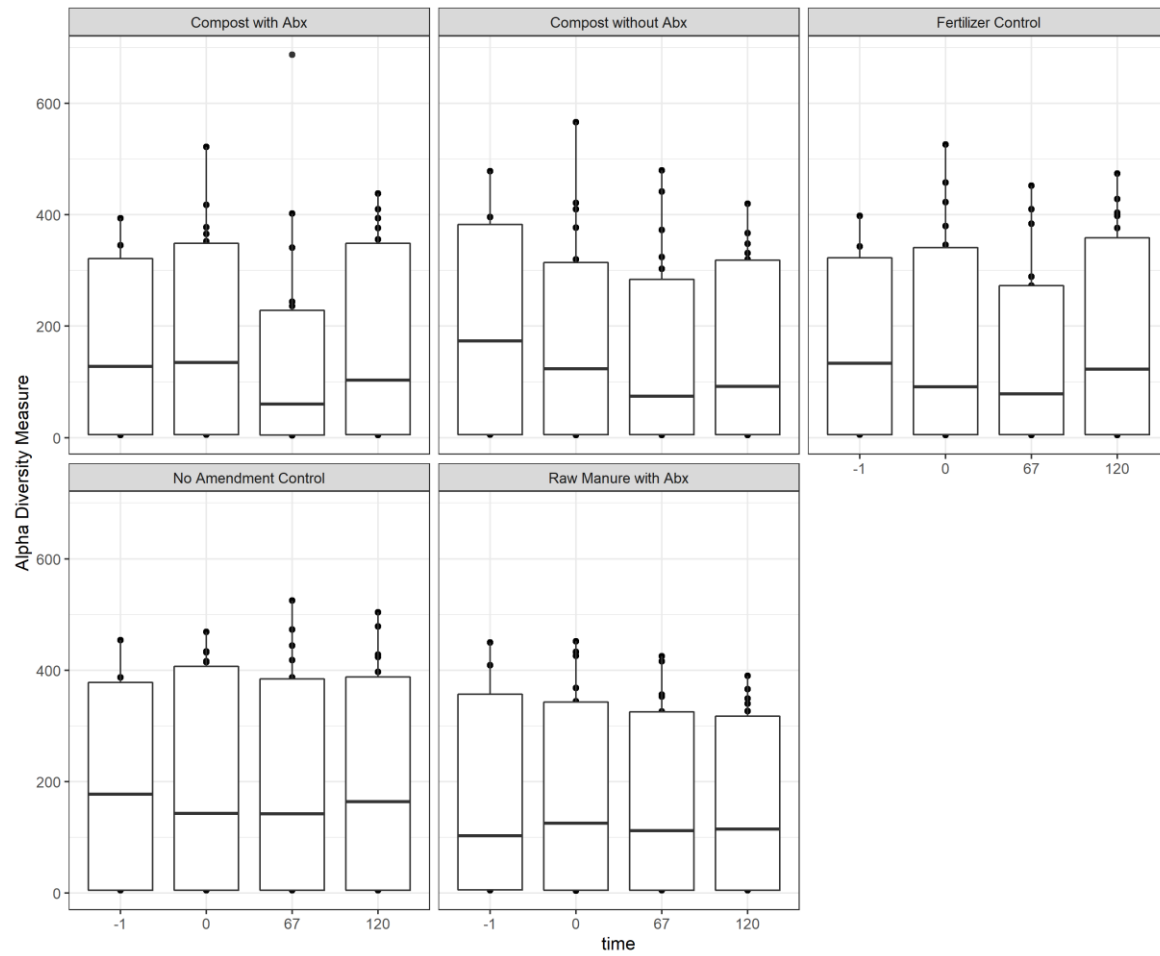


Figure S1. Alpha diversity measure, visualized as clustered operational taxonomic unit (OTU), for the soils in each treatment over the course of the study (background, -1; amendment application, 0; crop harvest, 67; and study completion, 120d).

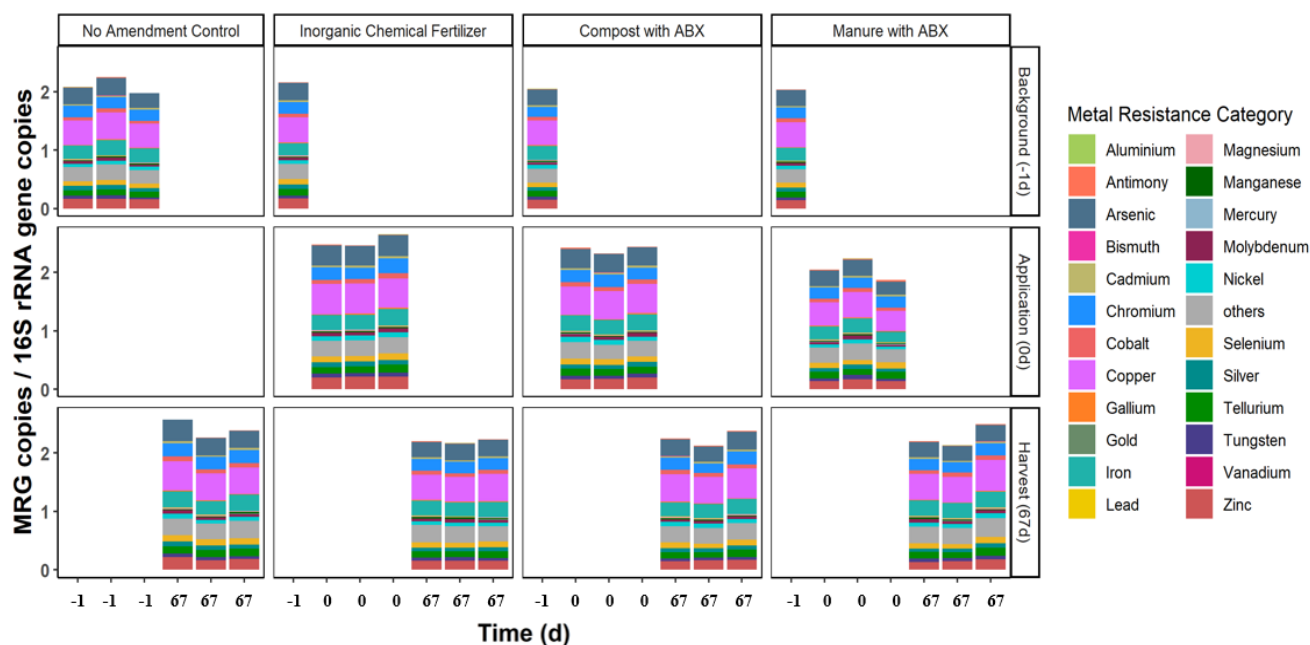


Figure S2. Relative abundances of total MRGs in soils represented according to the metal to which they encode resistance grouped by treatment (n=4) for each column and by time (n=3) for each row annotated using BacMet v1.1-4 via shotgun metagenomic sequencing. Each stacked bar represents one soil shotgun metagenomic sequence replicate among soil samples taken within the same treated soil plot for each sampling time. Treatment and time effects were measured in triplicate (i.e. three bars per box), except for the amended soils at background (-1d) based on the assumption that prior to amendment application the soil was homogenous to the No Amendment Control soils. No Amendment Control soils were not sequenced at application (0d) as those soils were assumed to be the same as at the time of background (-1d) due to lack of amendment. BacMet defines “others” as a group chemical compounds toxic to bacteria

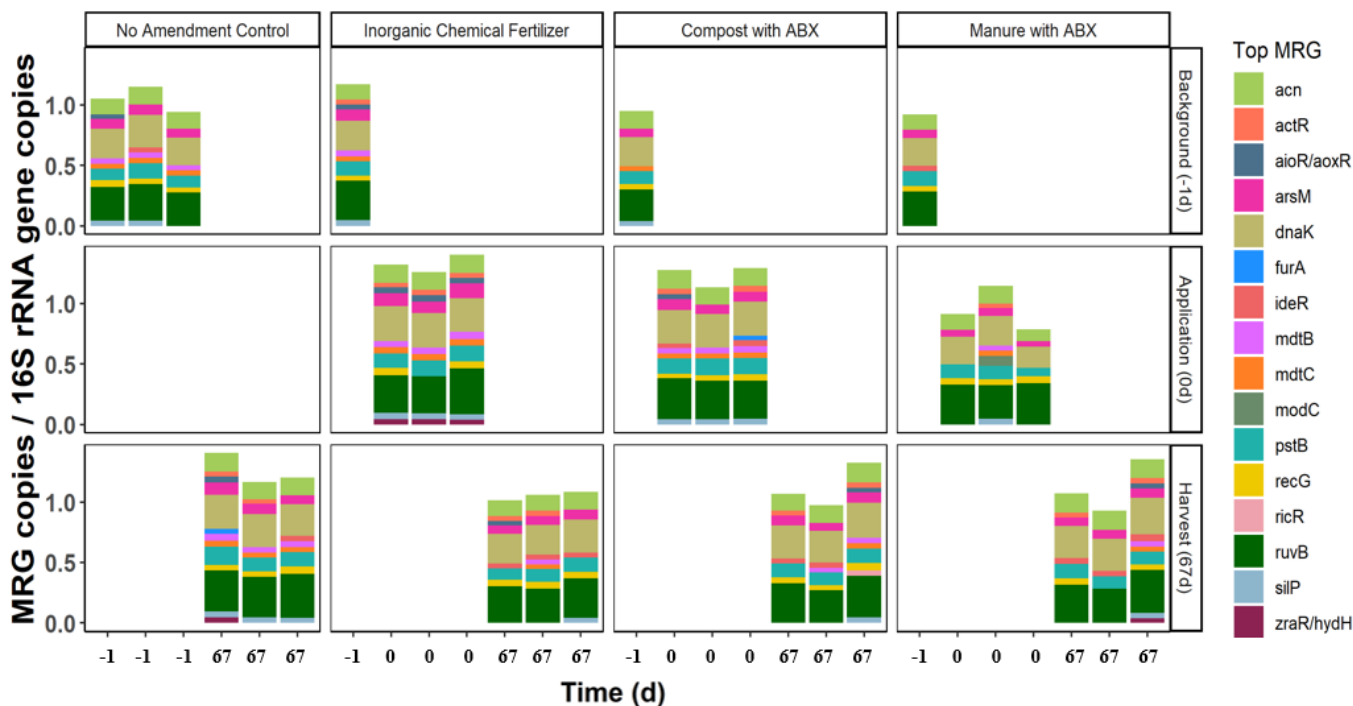


Figure S3. Relative abundances of the 95th percentile of MRGs in soils grouped by treatment (n=4) for each column and by time (n=3) for each row annotated using BacMet v1.1-4 via shotgun metagenomic sequencing. Each stacked bar represents one soil shotgun metagenomic sequence replicate among soil samples taken within the same treated soil plot for each sampling time. Treatment and time effects were measured in triplicate (i.e. three bars per box), except for the amended soils at background (-1d) based on the assumption that prior to amendment application the soil was homogenous to the No Amendment Control soils. No Amendment Control soils were not sequenced at application (0d) as those soils were assumed to be the same as at the time of background (-1d) due to lack of amendment.

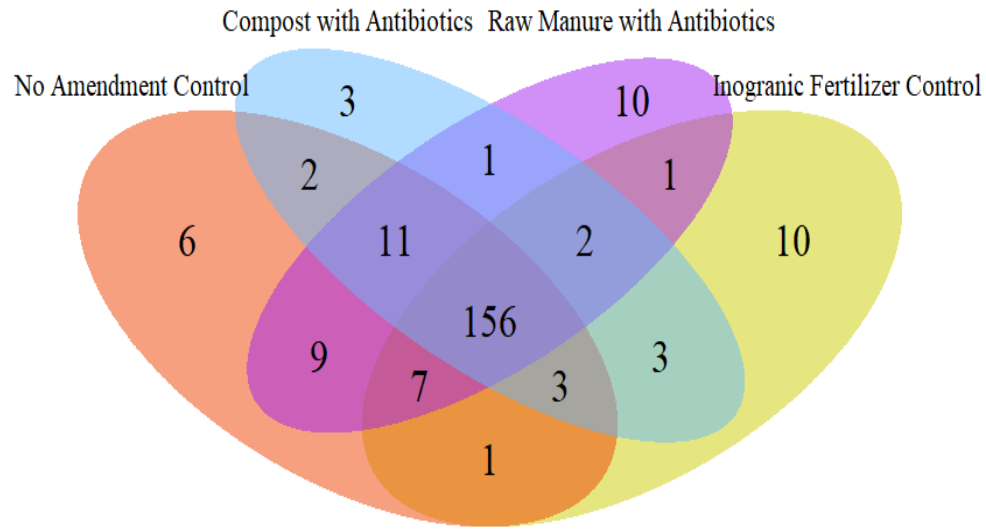


Figure S4. Total number of MRGs annotated via the BacMet v1.1-4 database for each amended soil treatment. All background soils sampled on day -1 (n=6) were grouped in the No Amendment Control treatment based on the assumption that prior to amendment application the soil was homogenous among the amended soils. Of the 226 MRGs annotated, 156 MRGs were detected in all soil treatments. Individually the raw manure with antibiotics, no amendment control, compost with antibiotics, and inorganic chemical fertilizer amended soils yielded 10, 6, 3, and 10 unique MRGs, respectively.

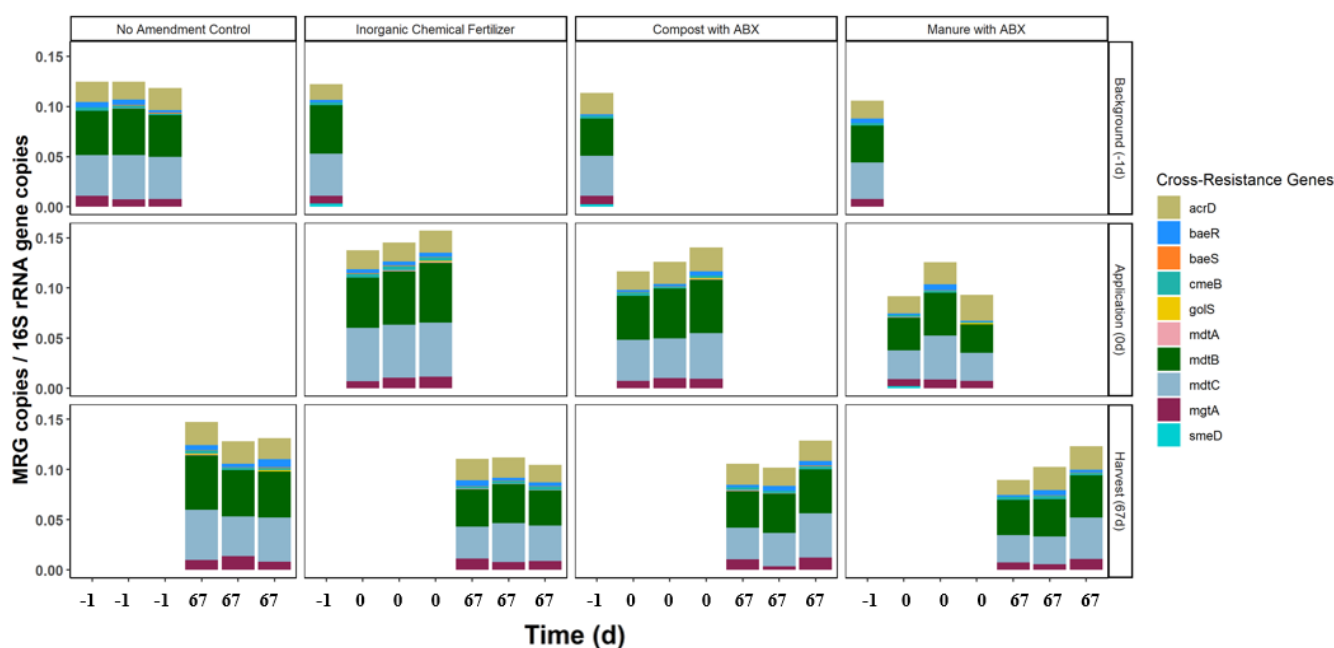


Figure S5. Relative abundances of the ten resistance genes identified conferring resistance to both metals and antibiotics in soils grouped by treatment (n=4) for each column and by time (n=3) for each row annotated using BacMet v1.1-4 via shotgun metagenomic sequencing. Each stacked bar represents one soil shotgun metagenomic sequence replicate among soil samples taken within the same treated soil plot for each sampling time. Treatment and time effects were measured in triplicate (i.e. three bars per box), except for the amended soils at background (-1d) based on the assumption that prior to amendment application the soil was homogenous to the No Amendment Control soils. No Amendment Control soils were not sequenced at application (0d) as those soils were assumed to be the same as at the time of background (-1d) due to lack of amendment. BacMet defines “others” as a group chemical compounds toxic to bacteria.

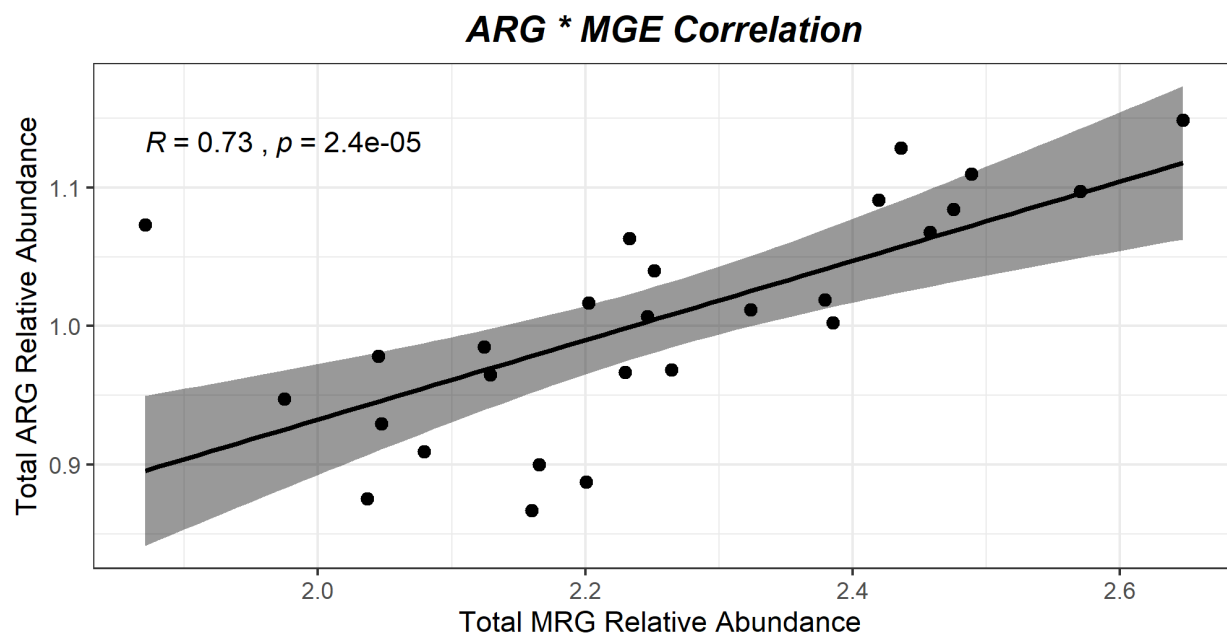


Figure S6. Correlation of total ARG and MRG relative abundances among the amended agricultural soils.

Chapter 5. Characterizing the antibiotic resistome and associated microbial communities at multiple points along the vegetable production chain

A research article in preparation for Frontier Special Issue: Exposure, Risks, and Drivers of the Mobile Antimicrobial Resistance Genes in the Environment- a Global Perspective.

Wind., L.*, Keenum, I., Krometis, L.A., Hession, W.C., Pruden, A., et al. Integrated Metagenomic Assessment of Multiple Preharvest Control Points on Lettuce Resistomes at Field-Scale. (submission anticipated Fall 2020).

5.1 Abstract

An integrated understanding of factors influencing the occurrence, distribution, and fate of antibiotic resistance genes (ARGs) in agroecosystems is critical for the design of strategies for mitigating the spread of antibiotic resistance. The overarching objective of this study was to identify and compare trends in resistome (i.e., total ARGs) and taxonomic profiles via shotgun metagenomic sequencing across the agricultural production chain at the field scale. Samples were collected and analyzed from a controlled field study to examine hypothetical control points, including: antibiotic use, composting, soil amendment soil with organic (manure or compost) versus chemical fertilizer, and lettuce cultivation and harvest. Resistomes and microbial communities were unique across control points according to analysis of similarity. The highest alpha diversity (i.e., unique ARGs, $n=642$) was detected in the amendment samples, but composted manure had the lowest total ARG relative abundance (i.e., 16S rRNA gene-normalized). Regardless of amendment type, soils acted as an apparent ecological buffer: soil resistome and taxonomic profiles returned to background conditions after 120d. However, effects of amendment conditions were still apparent in the phyllosphere resistomes recovered from lettuce, with the highest relative ARG abundances recovered on lettuce grown in organically-fertilized soils. Co-occurrence analysis identified 55 unique ARGs present in both the soil amendments and on lettuce surfaces. Among these, *bacA*, *arnA*, and *pmrF* were the most abundant ARGs co-occurring with mobile genetic elements, and thus may prove useful targets for monitoring agroecosystems. This study highlights the value of holistically assessing impacts across multiple control points on final post-harvest products.

5.2 Introduction

Antibiotic resistance (AR) is recognized as a critical threat to human health, food security, and global development. In 2015, the WHO strongly encouraged the development of holistic action plans to preserve drug efficacy for usage in humans and animals (European CDC, 2015). Partially in response to this directive, the US promulgated the Veterinary Feed Directive (final rule #213), which requires veterinary authorization and oversight, rather than simple over-the-counter distribution, for livestock use of antimicrobials (FDA, 2013; FDA, 2015; FDA, 2017). The use of antibiotics as growth promoters on US farms was banned completely in 2017 (FDA, 2018). Despite this progress, it is worth noting that even though European nations banned sub-therapeutic usage of antibiotics for growth promotion in 2006, resistance markers are still regularly recovered from continental livestock herds (Aarestrup et al., 2001; EC, 2005). In addition, antibiotics remain widely used to promote livestock growth in other nations beyond the US and EU. As the need for food and agricultural products (e.g. crops, livestock, and livestock associated products) increases to meet the demands of a growing global population (FAO, 2020), there is concern that the emergence and dissemination of environmentally-linked antibiotic resistant infections could intensify, especially in nations that have not yet adopted environmental AR mitigation policies (Van Boeckel et al., 2015).

A variety of agricultural best management strategies are employed to prevent contamination of consumer products with pathogens (e.g. manure management, post-harvest washing), though these practices were primarily developed to be effective against standard culturable sentinels of disease risk (e.g. coliforms, *Salmonella* spp.), rather than specifically designed to control the spread of AR. For example, the US Food and Drug Administration (FDA) Food Safety and Modernization Act (FSMA) recommends composting to decrease the transfer of pathogens to crops from soils amended with manure (FDA, 2015). Previous research emphasizes that composting can effectively reduce fecal indicator organisms, bacterial pathogens, and up to 70% of antibiotics (Sharma et al., 2009; Storteboom et al., 2007; Wu et al., 2010). However, observed reductions of pathogens and/or indicator bacteria do not necessarily ensure a subsequent decrease in antibiotic resistant bacteria (ARB) and/or antibiotic resistance genes (ARGs) (Edrington et al., 2009; Storteboom et al., 2007; Xu et al., 2019). The presence of mobile genetic elements (MGEs) in soil amendments is of particular concern due to their ability to facilitate horizontal gene transfer (HGT) of ARGs among

ARBs and non-ARBs (Partridge et al., 2018). Some MGEs are considered markers of the mobilization potential of ARGs in agroecosystems (e.g. class 1 integrons, Gillings et al., 2015). Additional MGEs have been linked to HGT of ARGs through co-occurrence analysis of agricultural soils (Xie et al., 2018).

The movement of AR throughout environmental systems is complex (Finley et al., 2013; Graham et al., 2019), with characterization complicated by a lack of standard tracking strategies. Multiple quantification methods are used to evaluate AR in agroecosystems (i.e., culture-based, qPCR, next-generation sequencing (NGS)) and, as a consequence, analytical endpoints are not directly comparable (Pelley, 2020; Rocha et al., 2019; Suttner et al., 2020; Zhang et al., 2019; Davies and Davies, 2010; Wind et al., 2020). Further, there is biological complexity in defining AR, as, in addition to characterized and curated ARGs, there are also precursor genes (e.g. cryptic embedded genes, genes encoding protein resistance) associated with all viable, viable but non culturable, and recently lysed bacteria (Wright, 2007). Shotgun metagenomic sequencing is therefore an attractive approach for capturing such complexity, as it has the potential to identify the full range of ARGs present in the bacterial community, along with other key taxonomic and functional genes of interest, including pathogen, MGE, and metal resistance gene markers and associated regulatory genes. Further, metagenomic sequencing avoids the biases of a prior selection of targets necessary for quantitative polymerase chain reaction or culture (Chambers et al., 2015).

Previously, metagenomic techniques have characterized resistomes in agricultural ecosystems at specific, and mostly single, points along the vegetable production chain (Fang et al., 2015; Gou et al., 2018a; Chen et al., 2019a; Guron et al., 2019). The majority of these studies focus on a single harvest cycle. Gou et al. (2018) observed that composting livestock-manure decreased resistome diversity as compared to raw (source) manure (Gou et al., 2018a) prior to land application. However, examinations of soils amended with either inorganic and organic-derived fertilizers indicate that, although there may be an initial increase in some measures of resistance, agricultural soil resistomes tend to return to background levels after a growing season (Gou et al., 2018a; Han et al., 2018; Chen et al., 2019b; Zhang et al., 2019b). After 25-years of repeated pig manure fertilization to agricultural soils in Hunan Province, China, Xie et al. (2018) did find that organic fertilization altered soil resistome abundance and diversity of ARGs compared to control and

inorganically fertilized soils. However, there was no observed α -diversity (i.e., total unique operational taxonomic units) difference in soil bacterial communities between the different soil treatments (Xie et al., 2018). Examinations of interactions between agricultural soils and crop surfaces via NGS have only considered a single growing season, and do suggest that soil amendment type can affect the resistomes recovered from crop surfaces (Tien et al., 2017; Guron et al., 2019). For example, Guron et al. (2019) observed that lettuce grown in soils amended with manure carried 2.3 and 11.1 times more aminoglycoside- and triclosan-associated ARGs on their surfaces as compared to those grown in soils amended with compost. Field-scale examination of the fate and transport of resistance markers from manure collection and management through soil amendment and crop harvest is essential in order to identify critical control points that represent the greatest risk of AR spread and further refine management practices as necessary (Bengtsson-Palme, 2017; Vikesland et al., 2017; Larsson et al., 2018).

Use of livestock-derived organic fertilizer (i.e. manure, slurry, compost), which can introduce significant quantities of ARGs into global agroecosystems, is anticipated to continue to increase in order to support increasing global agricultural demands. Although multiple studies have examined AR through individual points within agricultural production systems, no previous work has provided an integrated assessment of multiple critical control points and their influence on corresponding resistomes across a given pre- to post-harvest vegetable production chain. The aim of the present study was to characterize the antibiotic resistome at hypothetical critical control points associated with field-scale lettuce cultivation in soils amended with dairy manure and associated compost: antibiotic use, composting, soil amendment soil with organic (manure or compost) versus chemical fertilizer, and lettuce cultivation and harvest. Shotgun metagenomic sequencing was used to determine and/or compare: (1) the ARG resistomes (abundance and diversity); (2) ARG-MGE co-occurrences; and (3) indicator taxonomy connections, and (4) relative potential for ARGs to mobilize to pathogens (i.e., resistome risk) among amendments, amendment soils, and lettuce phyllosphere samples. We hypothesized that manure treatment would reduce ARG recovery throughout the agricultural chain, and that raw manure would present the most risk of ARGs penetrating critical control points, compared to compost and inorganic chemical fertilizer controls. The integrated analysis carried out in this study provides a means to directly

compare and optimize the roles of various on-farm management practices in mitigating the spread of antibiotic resistance.

5.3 Materials and Methods

5.3.1 Study Overview

The aims of this study were achieved through integrated analysis of previously published soil and lettuce phyllosphere resistome datasets described in Wind et al. (2020) and Fogler et al. (2019), as well as amendment resistomes. The soil and crop surface shotgun metagenomic sequences used in this study are publicly available from the NCBI BioProject: PRJNA506850. The following sections include brief summaries of the relevant experimental design and analysis described in detail in the prior published studies, along with the metagenomic analyses applied in this study.

5.3.2 Field Study Design and Treatment Description

All experiments were conducted at the Virginia Tech Urban Horticulture Center in Blacksburg, VA, USA on land that was previously fallow for at least a decade. Dairy manure was collected and subsequently amended to soils to support the growth of lettuce (*lactuca sativa*) during the 2016 growing season (March-July). Further study details including descriptions of plot construction, field preparation, and patterns of culturable fecal ARB, heavy metals, and metal resistance genes in soils, release of targeted ARGs quantified via quantitative polymerase chain reaction in surface runoff during natural storm events, and recovery of ARGs from harvested vegetables have been previously reported (Wind et al., 2018; Fogler et al., 2019a; Jacobs et al., 2019).

Two prior studies compared the effects of organic and inorganic soil amendments on the antibiotic resistomes of amended soils (Wind et al., 2020) and the phyllosphere of cultivated lettuce (Fogler et al., 2019) (Figure S1). Three organic amendments were examined: control compost, compost, and raw manure. The raw manure was collected from dairy cows treated with pirlimycin or cephalixin at therapeutic levels with compost was derived from this raw manure (Ray et al., 2017;

Wind et al., 2018). A control compost was derived from dairy cows that were not treated with pirlimycin or cephalixin, i.e. animals without antibiotic exposure. Static composting and manure stockpiling methods were carried out over the course of 63 days before soil application. Thermophilic conditions (US FDA FSMA-recommended temperature of $> 55^{\circ}\text{C}$) were reached in both the composts on day 2 (FDA, 2015). All organic amendments were applied at appropriate agronomic rates (6.72 Mg ha^{-1} ; Day 0) to $3\text{m} \times 3\text{m}$ individually bordered soil plots as previously described in Wind et al. (2018). In total, four treatments were examined: soils amended with (1) compost from animals treated with antibiotics ($n=3$); (2) raw manure from animals treated with antibiotics ($n=3$); (3) inorganic chemical fertilizer control ($n=3$); and (4) a no amendment control ($n=3$). Inorganic fertilizer (N-P-K) was added to the inorganic chemical fertilizer control soils at rates recommended for the optimal growth of lettuce ($140\text{-}112\text{-}112 \text{ kg ha}^{-1}$; Virginia Cooperative Extension, 2015). Samples of lettuce grown and harvested within the inorganic and organically amended soils were analyzed. No lettuce was grown in the no amendment control soils, due to insufficient nutrients for crop growth.

5.3.3 Amendment, Soil, and Vegetable Sample Collection

Stockpiled raw manure and compost samples were collected at two time points: initial compost/stockpile mixture (0 d) and at the time of compost completion (63 d). Samples were collected from the middle of the manure and compost piles ($3\text{-}4 \text{ m}^3$). Soil samples were collected at three time points (-1, 0, and 67 d) that coincided with critical points in the cultivation process: background soil conditions (-1 d), amendment application (0 d), and lettuce harvest (67 d). In-depth description of soil collection and processing are available in Wind et al. (2020). In brief, at each sampling time, four 2 cm soil cores from a 5 cm depth were collected from each soil plot at a randomly generated location and homogenized into one sample on site. To create a snapshot of the phyllosphere resistome, lettuce plants were collected from each plot at the time of harvest (67 d). Further details on recovery of surficial microbes from the phyllosphere can be found in Fogler et al. (2019).

5.3.4 DNA Extraction

DNA was extracted from 0.5 g compost or manure samples of the homogenized amendment samples using the FASTDNA SPIN Kit for Soil (MP Biomedicals, Solon, Ohio) which followed the same extraction methods used that for that of the soil DNA previously described (Wind et al., 2020). Inhibition was minimized using the ZYMO PCR OneStep PCR Inhibitor Removal Kit (ZYMO Research, Irvine, CA). Lettuce were subject to DNA extraction immediately upon harvest (< 2 hr). An in-depth description of crop collection and processing can be found in Fogler et al. (2019). Briefly, lettuce leaves were washed in a sterile 0.1 % peptone and 0.1 % Tween solution and hand massaged in order to remove surficial phyllosphere bacteria, while avoiding release of plant DNA (Fogler et al., 2019). The subsequent lettuce wash diluent was filtered through 0.22- μ m filters (EMD Millipore, Merck Group, Darmstadt, Germany), which were subject to the same DNA extraction and clean-up procedures noted above for manure, compost, and soil. All DNA extracts were stored at -80 °C for up twenty-four months prior to subsequent metagenomic analyses.

5.3.5 Shogun Metagenomic Sequencing

Amendment (n=14), soil (n=27), and lettuce samples (n=12) were analyzed via shotgun metagenomic sequencing to provide a “snapshot” of the agricultural soil resistome at key time points along the vegetable production chain (Figure S1, Table S1). For most of the treatment and time factors across the vegetable production chain, samples were sequenced in triplicate. However, the amendments prior to manure management (0 d) and the soils prior to amendment (-1 d) were considered homogenous across the treatments and were grouped together as the control replicates. Samples and corresponding replicates were selected for sequencing depending on treatment and time effects of interest. Details on samples subject to metagenomic sequencing are reported in Table S1.

Amendment metagenomes were prepared using the Nextera XT library prep (Illumina, San Diego, CA) and sequenced on an Illumina NextSeq 500 (2x75 bp, paired-end) at the Scripps Center of Computational Biology and Bioinformatics (La Jolla, CA), which is the same preparation and

sequencing methods of the amended soils. The lettuce samples were prepared using the Accel-NGS 2S DNA kit (SwiftBio, Ann Arbor, MI) and sequenced on an Illumina HiSeq 2500 (2x100 bp, paired-end) at the Biocomplexity Institute of Virginia Tech (Blacksburg, VA). Complete sequencing preparation details are available in Wind et al. (2020) and Folger et al. (2019).

5.3.6 Antibiotic Resistome Analysis

Raw reads and associated metadata were uploaded to the NCBI BioProject: PRJNA506850 for each point along of the vegetable production chain compared in this study (Table S2). Paired-end raw sequences were processed using MetaStorm using the read-matching pipeline (Arango-Argoty et al., 2016). On average, each amendment, soil, and lettuce sample contained 24,650,830, 23,610,275, and 12,292,992 bp raw reads, respectively after TRIMMOMATIC quality filtering (<0.16% reads dropped via filtering, Bolger et al., 2014). ARGs were annotated against the Comprehensive Antibiotic Resistance Database (CARD v2.0.1; Jia et al., 2017) with at least 80 % identity using the read match sequences pipeline of MetaStorm. Known housekeeping genes were removed prior to gene abundance analysis. To account for variable read and gene lengths, gene counts analyzed from MetaStorm were normalized to 16S rRNA gene copy number to report relative abundance using Greengenes (DeSantis et al., 2006; Li et al., 2015).

5.3.7 ARG-MGE Co-Occurrence and Resistome Risk Analysis

de novo assembled scaffolds annotated using the MetaStorm assembly pipeline were used for downstream co-occurrence analyses (Arango-Argoty et al., 2016). In brief, MetaStorm uses IDBA-UD (Peng et al., 2012) to assemble scaffolds and then employs PRODIGAL (Hyatt et al., 2010) to predict genes within each scaffold. Downstream taxonomic and functional gene annotation details are described in-depth in Arango-Argoty et al. (2016). Assembled scaffolds averaged 1,102, 504, and 1,066 bp for amendments, soils, and lettuce surfaces, respectively. Assemblies for each sample matrix differed substantially, with a percent assembled range between 0.2–74.5 % after normalization by total number of contigs. On average, amendments and lettuce samples assembly was 36.8 % and 41 % respectively. This was much higher than the average assembly from soil samples, which was 0.64 % (see Table S2 for sequencing reads). Estimated average coverage of

the amendment, soil, and lettuce samples were 40 %, 20.62 % and 63 % as determined via Nonpareil, which assesses metagenomic data coverage levels (Rodriguez-R and Konstantinidis, 2014). Due to low assembly of the soil samples, which is to be expected giving its high complexity (Howe et al., 2014; Myrold et al., 2014; Chen et al., 2019a); the ARG-MGE co-occurrences along each point of the vegetable production chain are assumed to be a broad analysis, and relative only among each sample type (i.e. amendment, soil, vegetable surface). Using MetaCompare (Oh et al., 2018), ARG-MGE co-occurrences were analyzed from assembled contigs and the resistome risk scores were calculated for each metagenome. The risk score, Q, was calculated by evaluating the occurrence of a scaffold that contained one or more co-occurring ARG, MGE, or pathogen gene marker (Oh et al., 2018). To reduce influence of potentially incorrectly assembled contigs, a threshold was set in which a given ARG-MGE contig had to be identified in at least two sequence replicates per sample type (i.e. amendment, soil, vegetable surface). This threshold was not achieved for any soil samples and they were therefore excluded from further assembly-based analysis.

5.3.8 Microbial Community Analysis

Taxonomic labels were assigned to the metagenome samples via Kraken2 (Wood and Salzberg, 2014). Once taxonomy was assigned, genera abundance was determined via Bracken (Bayesian Re-estimation of Abundance with Kraken; Lu and Salzberg, 2020). Chloroplast sequences were removed from the lettuce surface raw reads to prevent inhibition of bacterial sequences in downstream analysis prior to Kraken analysis (Fogler et al., 2019b; Guron et al., 2019). Genera relative abundance files produced via Bracken were analyzed for microbial communities using R ‘vegan’ package (Oksanen et al., 2015).

5.3.9 Statistical Analysis

All statistical analyses were conducted using R (v. 3.6.1, R Core Team, 2019) with an alpha of 0.05 ($p < 0.05$) applied as the level of significance. All metagenomic datasets were confirmed as non-parametric via the Shapiro-Wilkes normality test ($p < 0.05$). ARG relative abundance varied based on gene amplified, and a Kruskal-Wallis rank sum test followed by a post-hoc Dunn’s test

assessed differences between time points, treatments, and among the amendment, soil, and lettuce surface metagenomes. On the assumption that prior to amendment application the field plots were homogenous, the background (-1 d) soil metagenomic samples from the inorganic chemical fertilizer control, compost with antibiotics, and stockpiled raw manure with antibiotics amended soils were grouped with the no amendment control amended soil samples at the time of background, which resulted in six replicate samples during the statistical analysis (Table S1). Unweighted Bray-Curtis distances and a one-way analysis of similarities (ANOSIM) statistical analysis was used to compare resistome profiles (ARG type and ARG abundance).

The microbial community alpha diversity was measured using Shannon diversity index which takes species proportional abundances into account for species richness. A one-way analysis of variance (ANOVA) followed by a post-hoc Tukey HSD test assessed differences among the amendment, soil, and lettuce surface microbial communities and subsequent treatment and time effects if needed at each point. Indicator species analysis of the significantly differentially abundant species was measured using point biserial correlation coefficient from the R package ‘indicspecies’ (Cáceres, 2013). Figures were produced using R packages ‘ggplot2’ and ‘VennDiagram’ and “networkD3” (Wickham, 2009; Chen, H., 2018; Allaire et al., 2017).

5.4 Results

5.4.1 Comparing ARG Profiles Across Amendments, Soil, and Lettuce in the Vegetable Production Chain

5.4.1.1 Comparison of Resistomes Across Sample Types

Profiling antibiotic resistomes according to number of unique ARGs encountered and their relative abundances revealed differences among the three sample types (i.e., amendment, soil, lettuce (ANOSIM, $p < 0.01$; Figure 5-1)) along the vegetable production chain. Of the 854 unique ARGs annotated across all metagenomes, 302 were detected in all three sample types (Figure 5-2). A total of 121, 55, and 114 ARGs were found to be unique to the amendments, soils, and lettuce samples, respectively.

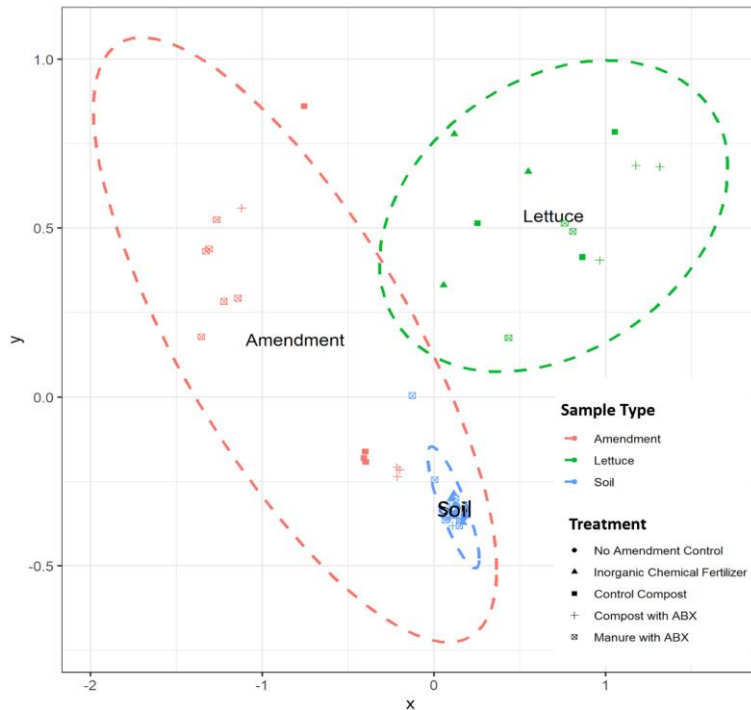


Figure 5-1. Non-metric multidimensional (NMDS) scaling of the Bray-Curtis dissimilarity distances highlight the distinct ARG profiles among amendment, soil, and vegetable samples representing the vegetable production ecosystem (ANOSIM, $p < 0.001$). ARGs were annotated against the CARD v2.0.1 database.

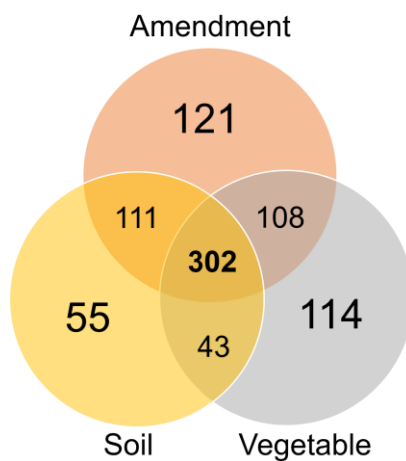


Figure 5-2. Total number of unique ARG types annotated via the CARD v2.0.1 database in each of the three sample types. Of the 854 ARGs annotated, 302 ARGs were common to each of the three sample types.

5.4.1.2 Comparison of Amendment Resistome with Time

Among the amendment samples, raw manure resistome profiles were distinct from those of the two composts (ANOSIM, $p < 0.001$; Figure S2). Significant differences between the start (0d) of the composting and stockpiling of raw manure compared to the end (63d) were expected (ANOSIM, $p < 0.011$; Figure S2). The greatest total ARG relative abundances were found in the compost derived from cows administered antibiotics, relative to the control compost or raw manure (Kruskal-Wallis, $p < 0.001$; post-hoc Dunn Test, $p < 0.001$) and across all time points (Kruskal-Wallis, $p < 0.001$). The control compost and raw manure were indistinguishable in terms of total ARG relative abundances (post-hoc Dunn test, $p = 0.63$; Figure S3).

The ARG classes with the greatest relative abundances among the amendments were multidrug, tetracycline, macrolide-lincosamide-streptogramin (MLS), and glycopeptide (Figure S3). The top ARGs in the compost generated from dairy cows treated with antibiotics and the control compost were *rpoB2*, *parY*, *mtrA*, and *vanRO*. The top ARGs in the stockpiled raw manure consisted of *tetW*, *InuA*, *efrB*, and *rpoB2*. The most abundant ARG (0.204 gene copies / 16S rRNA gene copy) was a rifamycin-resistance gene (*rpoB2*) in the compost with antibiotics post composting (63 d). Overall, 642 ARGs were annotated across the amendment samples and almost half ($n = 316$) were common among all samples (Figure S4). The control compost had the most unique ARGs types ($n = 555$), followed by the compost with antibiotics ($n = 475$) and the raw manure ($n = 405$). The control compost also had the most ARGs distinct to that sample type ($n = 88$), followed by the compost and raw manure with antibiotics with 43 and 34 distinct ARGs, respectively.

5.4.1.3 Comparison of Soil Resistome with Amendment Type and Time

At the time of soil amendment, there were distinct differences between the no amendment control and raw manure-amended soils resistomes. Samples collected from the inorganic fertilizer- and compost-amended soils were statistically equivalent to the control soil. By the time of lettuce harvest (67 d), all soil resistomes were statistically indistinct (Wind et al., 2020). The total relative abundance of ARGs in some soils were almost 2-fold greater than the corresponding amendments,

indicating that there were substantial naturally-occurring ARGs in the soil before the amendments were applied.

5.4.1.4 Comparison of Lettuce Resistomes Grown in Different Amendments

ARG relative abundances from surface samples of lettuce grown in soils amended with inorganic chemical fertilizer and the control compost were half the relative abundances level of surface samples from lettuce grown in composts or manure (Fogler et al., 2019). Total ARG relative abundances on the lettuce surfaces were up to 50% greater than in the amended soils in which they were grown.

5.4.2 Co-Occurrence of ARGs across the Vegetable Production Chain

Following assembly of metagenomic data, there were 734, 22, and 4,761 unique ARG-MGE co-occurrences among the amendment, soil, and lettuce samples, respectively. After applying the threshold of ARG-MGEs contigs occurring at least twice in a given data set (i.e., across metagenomes from the same sample type), there were 460, 2, and 1221 unique ARG-MGE co-occurrences among the assembled contigs from amendment, soil, and lettuce samples, respectively (Figure 5-3A-C).

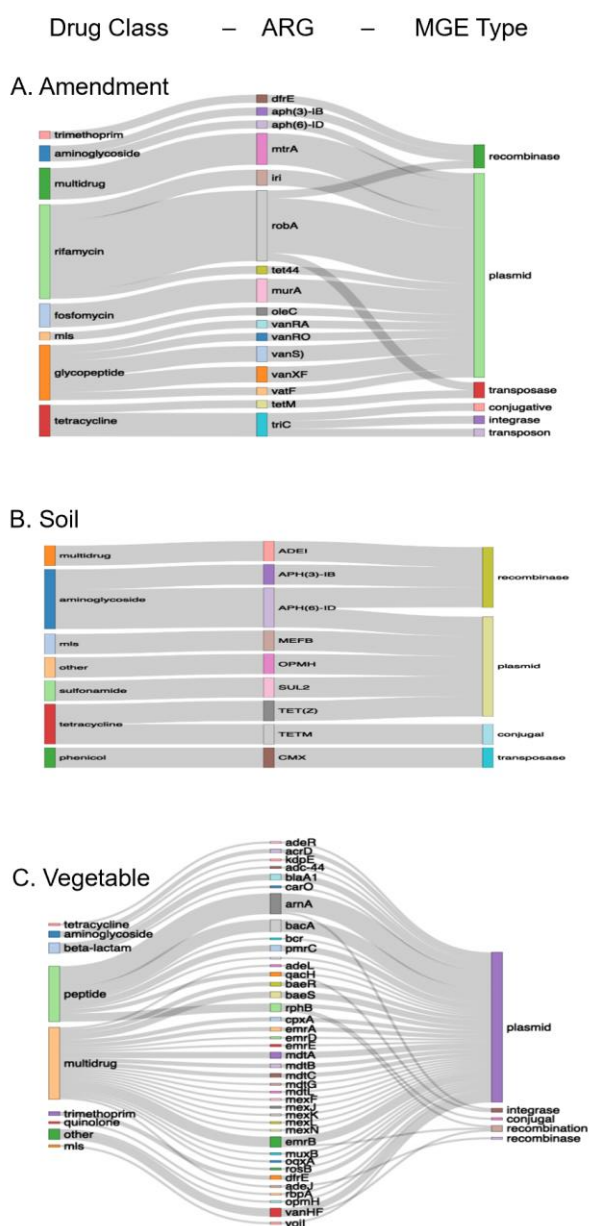


Figure 5-3. Unique ARG-MGE co-occurrences annotated among assembled contigs of the (A) amendment (n=460), (B) soil* (n=22), and (C) lettuce (n=1221) categorized by drug resistance class, ARG type, and MGE type. The relative height of the bars for each category is proportional to the number of times the category was annotated in the samples and the thickness of the connecting ribbons corresponds to the frequency of the combination. For visualization purposes, the amendments and lettuce represent 460 and 1221 unique ARG-MGE co-occurrences, respectively, meeting the threshold of having been annotated on at least two contigs; however, the soil (B) represents all 22 unique ARG-MGE co-occurrences (without applying the threshold of 2+ common ARG-MGE contigs).

Of the 460 unique ARG-MGE co-occurrences assembled within the amendment metagenomes, the most frequent occurred between the rifamycin drug class and plasmids. *robA* was the most prevalent ARG on contigs also annotated with plasmid markers (Figure 5-3A). Due to low assembly of the soil samples compared to the other points along the vegetable production chain, only 2 unique ARG-MGE co-occurrences were detected within the annotated soil samples. Although significantly less ARG-MGEs were annotated, the small co-occurrences still highlight the impact amendments may have on soils. Specifically, the unique ARG-MGE co-occurrences were only annotated with the raw manure amended soils (Figure 5-3B). The most frequent soil ARG-MGE co-occurrences occurred between the aminoglycoside drug class and plasmids. APH(6)-id, resistance through antibiotic inactivation, was the most prevalent ARG on plasmids annotated in the soils. Of the 1221 unique ARG-MGE co-occurrences assembled on the lettuce surfaces, the most frequent occurred between the multidrug class and plasmids. *amA*, *bacA*, and *ermB* were the most prevalent ARGs co-occurring on the plasmids annotated on the lettuce surfaces (Figure 5-3C).

5.3.3 Relative Resistome Risk Comparison

All amendment and lettuce samples contained scaffolds that were annotated with co-occurring ARGs, MGEs, and human- pathogen markers, which increased their resistome risk scores. Overall, the resistome risk scores were significantly different among the three sample types (Kruskal-Wallis, $p < 0.001$; post-hoc Dunn, $p < 0.001$ for all iterations; Figure S5). The average resistome risk scores were 26.9, 22.6, and 60.1 for the amendments, soils, and lettuce, respectively (Table S3). On average, there were 375, 13, and 1,420 contigs annotated with pathogen gene markers among the three sample types, respectively. Within each sample type, the resistome risk scores were comparable, regardless of associated experimental conditions or time points (Kruskal-Wallis, $p > 0.05$ for all iterations; Figure S6).

5.3.4 Taxonomic Composition Across the Vegetable Production Chain

The three sample types were also distinct in terms of taxonomic composition across the vegetable production chain (ANOVA, $p < 0.001$; ANOSIM, $p = 0.0104$; Figure 5-4, Figure S7) and Shannon diversity (post-hoc Tukey HSD, $p < 0.001$ for all iterations). Compost and manure were also distinct

from each other in terms of Shannon diversity (ANOVA, $p=0.0114$), but there was not a significant difference among the soil samples or among the lettuce samples in terms of the amendment conditions (ANOVA, $p=0.0687$ and 0.287 , respectively) or at different time points (ANOVA, $p=0.795$).

Beta diversity analysis, by means of Bray-Curtis dissimilarity matrixes, also indicated vegetable production chain differences in taxonomic profiles among the three sample types. Further, differences could be discerned among the amendment samples and soil on samples according to corresponding experimental conditions (ANOSIM, $p<0.001$ for both). There was no difference between the taxonomic composition of the microbial communities before versus after amendment treatment (ANOSIM, $p=0.613$) or in the soils over the growing season (ANOSIM, $p=0.147$).

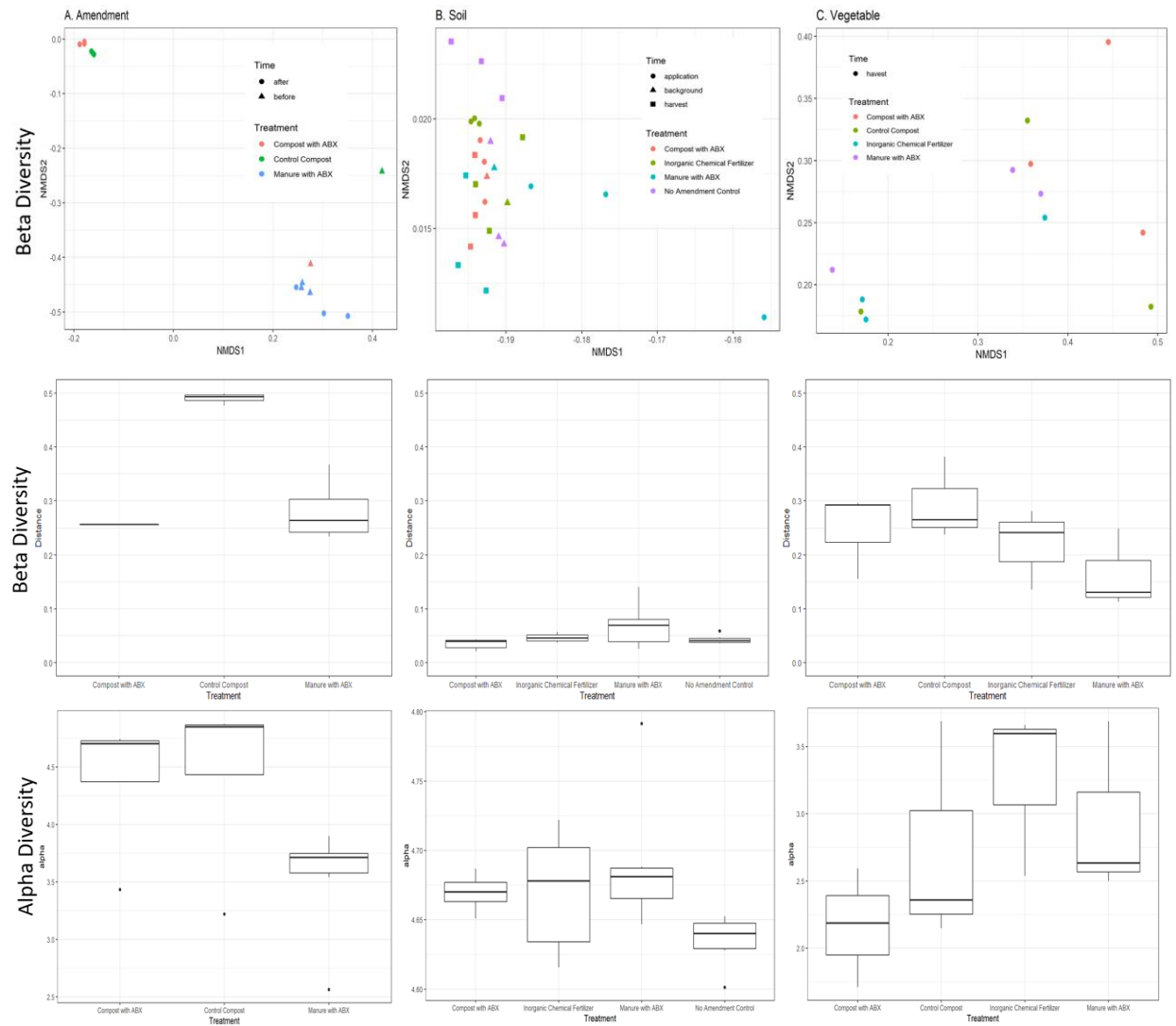


Figure 5-4. Taxonomic alpha (bottom row) and beta (top and middle rows) diversity among the microbial community's vegetable production chain for the (A) amendment, (B) soil, and (C) lettuce. Alpha diversity was calculated as Shannon diversity. Beta Diversity was determined from Bray-Curtis dissimilarity matrices and visualized as NMDS (top panel) and distance to euclidean centroid (middle panel). Taxonomy was annotated using NBCI taxonomy id using Kraken2 as the metagenomic classification algorithm and Bracken2 to compute species abundance.

Among all the agricultural metagenomes, 567 unique genera were identified, with 25% uniquely found in each sample type. Indicator species analysis using point biserial correlation coefficient highlighted the significant differentially abundant genera at and between each point ($p < 0.01$ for all iterations, Supplement Table S4). Amendments had the most diverse set of differentially abundant genera, including 197 unique genera not found at the other points. Soil and lettuce samples contained 129 and 31 genera that were differentially abundant, respectively. The top

indicator genera of the amendments were *Parageobacillus*, *Aneurinibacillus*, and *Belliella* ($p < 0.0001$ for all). The top indicator genera of the amended soils were *Bradyrhizobium*, *Mycobacterium*, and *Rhodoplanes* ($p < 0.0001$ for all). The top indicator genera of the lettuce surfaces were *Pseudomonas*, *Pantoea*, and *Massilia* ($p < 0.0001$ for all).

5.4 Discussion

5.4.1 The Agricultural Resistome

Overall, total ARG relative abundances increased from amendment to soil to lettuce surface following the organic (manure and compost) pathways (Figure 5-2). The amendments and lettuce surfaces had more diverse ARG profiles than the soils (Figure 5-2). Highlighting the benefit of manure management throughout the vegetable production chain, lettuce grown in control compost amended soils had the least ARG relative abundance of the organic treatments. However, lettuce grown in soils amended with manure or compost had 2-3 times the ARG abundance of lettuce grown in soils amended with inorganic (chemical) fertilizer.

Specific antibiotic resistance mitigation strategies (i.e., antibiotic administration, and composting versus stockpiling manure) had varying effects across the vegetable production chain. For example, composting raw manure in this study elevated ARG abundances compared to stockpiled raw manure. Other compost studies have observed the proliferation of ARG abundance following composting compared to manure (Xu et al., 2019), which highlights that although composting is recommended to mitigate pathogens (FDA, 2015), ARGs remain at elevated levels. Further, the control compost was the most diverse, in terms of number of ARGs annotated (Figure 5-2), compared to the other amendments, but that did not carry over to the lettuce grown in the amended soils. Lettuce grown in the control compost amended soils had the least total ARG relative abundance of all the organically grown lettuce.

Previous research reported that there were differences between the raw manure amended soil and the no amendment control soil resistomes, but all other amended soils were statistically indistinguishable from the control (Wind et al., 2020). The time point (background, application,

harvest) did not have a significant impact on the resistomes. From this finding, we hypothesize that one growing season was not enough time to predict the long-term impact of potential amendment addition of ARGs in agricultural soils. Recent studies have identified similar patterns after a single growing season, with ARG levels retuning to background levels (Zhou et al., 2017), and that soil resistome proliferation begins after a few years of manure application (Liu et al., 2021). The lettuce grown in organically amended soils had significantly greater total ARG relative abundances compared to the lettuce grown in the inorganic chemically fertilized soils. Zhang et al. (2019) similarly found that manure (poultry) application to the soil increased ARG abundance on lettuce surfaces at the greenhouse scale.

Across the vegetable production chain at every time point, multidrug-associated ARGs were the most abundant. However, the second most abundant drug class varied among the vegetable production chain. For example, the amendments had Macrolide-Lincosamide-Streptogramin (M-L-S), the amended soil had glycopeptide, and the lettuce surfaces had peptide as the second most abundant drug classes following multidrug. These differences highlight that although multidrug resistance dominates agroecosystems, each point has specific nuanced resistance patterns that were not carried along the production chain. In total, 302 ARGs were found at all points of the vegetable production chain. The highest abundance of clinically relevant ARGs were found in the lettuce surfaces samples. This suggests that even with manure management and time lapse in agricultural systems, ARGs can still be found on crop surfaces and further mitigation strategies will need to be in place before consumption. Recent studies have focused specifically on vegetable surface resistomes and have found that post-harvest sanitization practices, including irradiation and sanitizer in water wash, reduces surface ARGs (Dharmarha et al., 2019); our results suggest these are an essential barrier to consumer exposure.

5.4.2 Distinct Agricultural Microbial Communities

The microbial communities were distinct at each point along the agricultural production chain (Figure 5-4). The top amendment genera were from the *Firmicutes* and *Bacteroides* phyla, which is unsurprising as these are often associated with gut microbiomes and fecal bacteria. The top soil genera were from the *Proteobacteria* and *Actinobacteria* phyla, which are environmental groups of pathogenic bacteria and known to be found in agricultural and terrestrial systems. The top

vegetable genera were also *Proteobacteria*. Although the vegetables surfaces have the least indicator genera (n=31), it should be noted that many are considered opportunistic pathogens, e.g., *Pseudomonas* (nosocomial infections), *Panteo* (septic arthritis), and *Massilia* (malaria). The amendment and soil microbial communities were the most similar, containing 124 indicator genera that were significantly associated between the two points ($p < 0.001$). The top three genera between amendment and soils included *Spriochoeta*, *Acetobacter*, and *Leptospirillum*. Soil and vegetable microbial communities were the least similar, containing only 5 indicator genera that were significantly associated between the two points ($p < 0.001$). The top three genera between the soil and vegetable surfaces included *Nocardioide*s, *Methylobacterium*, and *Pseudarthrobacter*. The common genera between the agricultural production chain points may be indicative of a transfer of microbiomes within agroecosystems. Further microbial community analyses are needed to connect microbial community diversity and ARG degradation or proliferation along the vegetable production chain. Other studies have indicated this specifically in agricultural soils (Cycon et al., 2019, Lopatto et al., 2019) and on vegetable surfaces (Zhang et al., 2019).

5.4.3 ARG-MGE Co-Occurrence highlights integral focus areas

The majority of ARG co-occurrences were linked to plasmids (i.e. MGEs). Within the raw manure amendment samples, the *TETM* to transposon (*Slostridious difciles*) co-occurred 12 times and was the most abundant ARG-MGE. The *TriC* to plasmid (*Ralstonia solanacearum*) co-occurred 10 times on the lettuce surfaces grown in the compost, and was the second most abundant ARG-MGE. Only 22 ARG-MGE co-occurrences were annotated in the soil samples compared to the 734 and 4761 annotated within the amendment and lettuce surface samples, respectively. Although this was much less, likely due to lower percent assembly of contigs, it is important to note that all ARG-MGE co-occurrences were annotated within the raw manure amended soils. This suggests that the raw manure amended soils may ultimately contain more ARG-MGE co-occurrence than the other soils if deeper sequencing is utilized in the future to explore the amended soils. Ultimately, the higher number of ARG-MGE, and at times pathogen detection on the lettuce surface contigs (Table S3) is concerning from a human health perspective and should be focused on in future studies.

5.4.4 Assembly Difference along the Agricultural Production Chain

It is interesting to note that although soil is generally considered one of the most microbiologically diverse of environments, the amended soils had the lowest assemblies of the shotgun metagenomic data, and subsequently had the least annotated ARGs (n=511) compared to the amendments (n=642) and the lettuce surfaces (n=567). Soil is known to have low sequencing coverage from shotgun metagenomic data due to its exponentially and widespread diversity (Myrold et al., 2014; Zaheer et al., 2018). The low sequence coverage and percent assembled contigs from the soil metagenomic samples may have resulted in missing the key ARG-MGE co-occurrences, which ultimately lacks the ability to track ARG-MGEs co-occurrence between each vegetable production chain point. The low soil assembled contigs not only produced two to three-fold less ARG-MGE co-occurrences, it also was a contributing factor to the low resistome risk score calculated using assembled contigs via MetaCompare (Oh et al., 2019). To account for the varying percent assembled along the vegetable production chain, the resistome risk score data were normalized using percent of contigs assembled (Figure S7). However, this normalization did not improve the results; therefore, the resistome risk scores should only be recognized as one potential piece of the agricultural resistome to which future agricultural samples can be compared to for reference.

Additionally, although the vegetable samples averaged ~80% of the bp reads for the amendment (~18 million) and soil bp reads (~23 million); there was almost 2-fold more total ARG relative abundance on the lettuce surfaces than the soils and 3-fold more than the amendment. Additionally, the increase in assembled contigs subsequently produced more ARG-MGE co-occurrences and subsequently increased resistome risk compared to the amendment and soil samples. A possible explanation of the increase in assembly is due to the higher sequencing depth (~40%) found for the lettuce samples. As the lettuce surface resistome and microbiome is inherently less diverse than the soils, these metagenomic samples may have detected a higher relative abundance of ARGs on the lettuce because there was a smaller 16s rRNA presence for the relative abundance normalization. In addition, for the microbiome analysis, there were no treatment effects observed between the lettuce surfaces grown in the different soils.

5.4.5 Limitations

Although this study was the first of its kind to bridge the vegetable production chain resistomes together at the controlled field scale, it was not without its limitations. First, shotgun metagenomic sequencing was carried out on varying Illumina instruments for the amendment and soil versus lettuce samples. It is well known that NGS instruments are variable not only from one another, but also between lanes. Ideally, future studies should rely on a single instrument and sequencing center. However, variability also occurs between sequencing lanes and that in some cases the same instruments are not available (phased out, under repair, etc.). Second, due to budget constraints we were unable to sequence the soils amended with the control compost (i.e., from cows that did not receive antibiotics). Although this soil treatment was lacking, the sequencing of the amendment and the lettuce grown in the control compost amended soils helped illuminate the potential risk that treatment had on the crops. As mentioned previously, the difference in metagenomic sample coverage and wide range of assembled contigs between the amendment, soil, and crop surfaces are indicative of the inherent resistome variability based on sample media. The soils, which are noticeably more biologically diverse, had the lowest sequencing coverage and percent assembly which resulted in fewer annotated ARG-MGE co-occurrences and ultimately a lower resistome risk. We hypothesize that as soil sequencing depth improves, a deeper characterization of soil co-occurrences will be possible at this point of the vegetable production chain.

5.5 Conclusions

Agroecosystems are a key reservoir of antibiotic resistance and appropriate mitigation strategies are needed in order to limit its potential to spread and negatively influence human health. Tracking antibiotic resistance along the vegetable production chain highlighted specific points that are critical for mitigation, while also uncovering potential gaps of resistome information that are still needed. This study employed metagenomic analysis to provide an integrated assessment of amendment, soil, and lettuce resistomes and the role of multiple hypothetical control points in shaping them at field-scale, including antibiotic use, composting manure prior to soil amendment, and cultivation of lettuce. The findings emphasize the importance of multiple agricultural best management practices to provide a multi-barrier approach for preventing the spread of ARGs.

Composting as a manure management technique had a measurable effect on the harvested vegetable resistomes. Soil was found to be highly stable in the composition of the resistome and corresponding resistomes and has the potential to act as a natural ecological buffer to ARG proliferation. Notably, amendment application at standard rates did not alter soil resistomes or microbial community taxonomic composition after one growing season compared to background levels. Further studies are recommended to determine if there is variance among soil types in their capacity to buffer the potential for ARGs to spread. Still, total ARG relative abundances were higher in the lettuce phyllosphere than in the soil or amendments and were also higher on lettuce grown in soils receiving organic compared to inorganic amendments. In addition, lettuce was characterized by the greatest number of ARG-MGE co-occurrences as well as resistome risk scores. Thus, further research examining effects of post-harvest practices, such as washing, packaging, and storage on vegetable resistomes, are warranted. Moving forward, it would be beneficial to start targeting multidrug resistance from a One Health lens (i.e., which multidrug ARGs are we most concerned about for humans, animals, and the environment) and then shift detection methods to identifying the most clinically important co-occurring ARGs to improve future agricultural antibiotic resistance mitigation strategies.

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Supplemental Information

Text S1. Table S1 summarizes the replication of samples for each condition. For the amendments, at the start of composting and stockpiling (0 d), raw manure was analyzed in triplicate; single samples of the control compost and compost with antibiotics initial mixtures were analyzed. Triplicate samples were collected and analyzed on day 63, post-composting and post-stockpiling. For the amended soils, at the time of background (-1 d) single samples were analyzed as soils were assumed to be homogenous prior to amendment application. The no amendment control soil samples were analyzed in triplicate at background and harvest (67 d). At the time of amendment (0 d) and harvest (67 d), the inorganic chemical fertilizer control, compost with antibiotics, and raw manure with antibiotics were analyzed in triplicate. Lastly, lettuce surface samples were analyzed in triplicate at the time of harvest (67 d) to correspond with the soil samples; by the means of biological replicates (i.e., one lettuce samples for each of the three plots amended).

Table S1. Detailed replication schematic of the metagenomes.

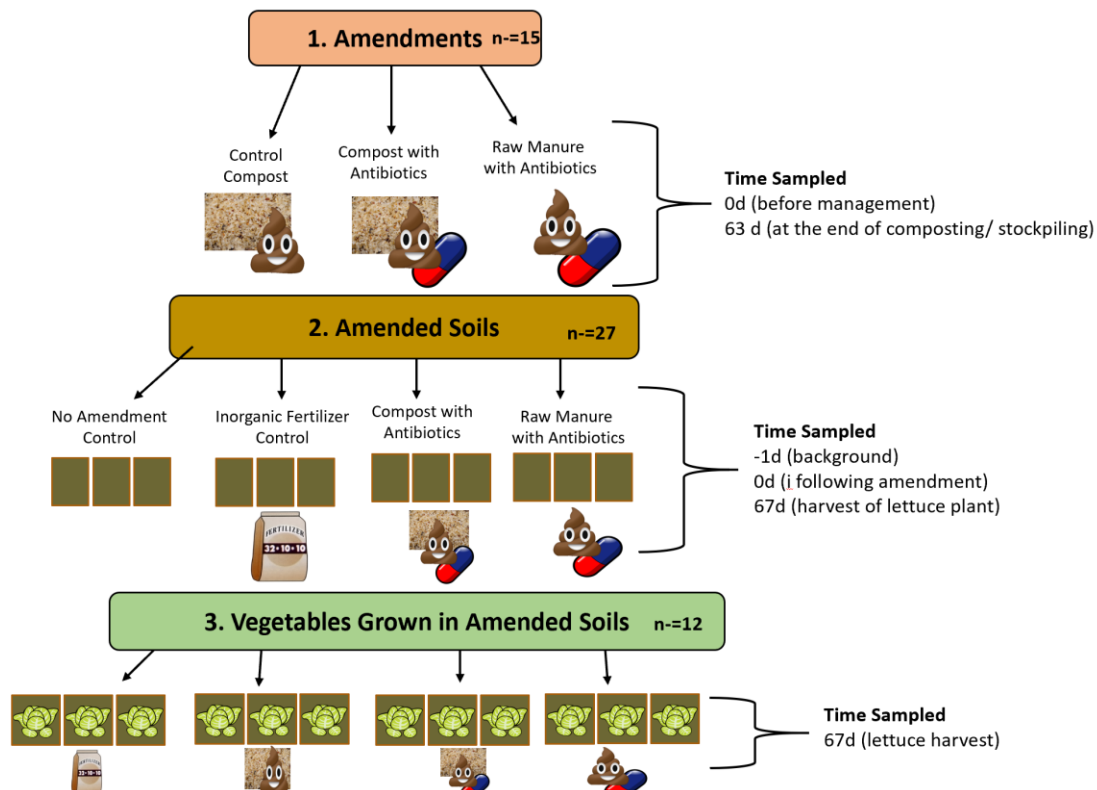
Matrix	Treatment	Time	Replicates	n
Amendment	(1) Control Compost	Before (0d)	1	14
		After (63)	3	
	(2) Compost with Antibiotics	Before (0d)	1	
		After (63)	3	
	(3) Raw Manure with Antibiotics	Before (0d)	3	
		After (63)	3	
Amended Soil	(1) No Amendment Control	Background (-1d)	3	27
		Harvest (67d)	3	
	(2) Inorganic Chemical Fertilizer	Background (-1d)	1	
		Application (0d)	3	
		Harvest (67d)	3	
		Harvest (67d)	3	
	(3) Compost with Antibiotics	Background (-1d)	1	
		Application (0d)	3	
		Harvest (67d)	3	
		Harvest (67d)	3	
	(4) Raw Manure with Antibiotics	Background (-1d)	1	
		Application (0d)	3	
		Harvest (67d)	3	
		Harvest (67d)	3	
Lettuce Surface	(1) Inorganic Chemical Fertilizer	Harvest (67d)	3	12
	(2) Control Compost	Harvest (67d)	3	
	(3) Compost with Antibiotic	Harvest (67d)	3	
	(4) Raw Manure with Antibiotics	Harvest (67d)	3	
		Harvest (67d)	3	

Table S2. Metagenomic raw sequences metadata, including read matching and assembly information.

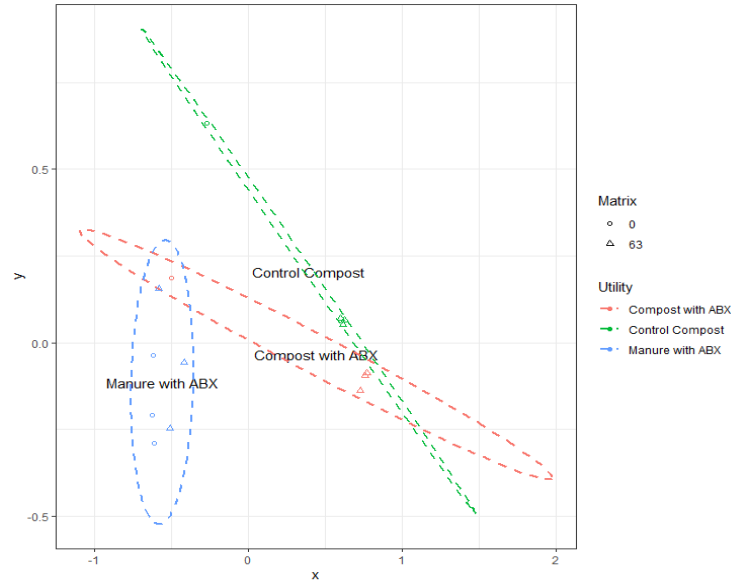
Sample	Sample Type	Treatment	Time (d)	Rep	Raw Reads	Reads QC	Fwd Surviving	Rvs Surviving	Dropped	% Dropped
1	Soil	No Amendment Control	-1	1	20,659,604	19,798,255	596,094	212,265	52,990	0.26
2	Soil	No Amendment Control	67	2	22,401,388	21,689,541	431,972	235,732	44,143	0.20
3	Soil	No Amendment Control	-1	1	22,145,101	21,502,235	360,845	244,871	37,150	0.17
4	Soil	No Amendment Control	-1	1	24,624,173	23,795,355	510,393	263,161	55,264	0.22
5	Soil	No Amendment Control	67	1	24,534,603	23,677,213	569,331	227,493	60,566	0.25
6	Soil	No Amendment Control	67	3	21,973,422	21,260,947	445,737	219,296	47,442	0.22
AVERAGE					22,723,049	21,953,924	485,729	233,803	49,593	0.22
7	Soil	Inorganic Chemical Fertilizer	-1	1	21,657,794	20,986,191	387,606	240,716	43,281	0.20
8	Soil	Inorganic Chemical Fertilizer	67	2	24,718,879	23,976,014	433,429	265,576	43,860	0.18
9	Soil	Inorganic Chemical Fertilizer	67	3	22,456,689	21,767,292	400,543	247,021	41,833	0.19
10	Soil	Inorganic Chemical Fertilizer	0	3	24,885,670	24,133,384	460,402	240,942	50,942	0.20
11	Soil	Inorganic Chemical Fertilizer	0	2	26,418,408	25,610,060	497,634	255,206	55,508	0.21
12	Soil	Inorganic Chemical Fertilizer	0	1	25,505,509	24,775,848	427,968	255,234	46,459	0.18
13	Soil	Inorganic Chemical Fertilizer	67	1	23,756,283	23,052,661	431,877	224,981	46,764	0.20
AVERAGE					24,199,890	23,471,636	434,208	247,097	46,950	0.19
14	Soil	Compost with ABX	0	2	22,683,806	21,759,418	626,929	235,667	61,792	0.27
15	Soil	Compost with ABX	-1	1	25,853,125	25,078,686	450,601	276,035	47,803	0.18
16	Soil	Compost with ABX	67	2	23,787,045	22,997,478	486,710	253,149	49,708	0.21
17	Soil	Compost with ABX	67	1	21,945,957	21,251,568	440,353	205,556	48,480	0.22
18	Soil	Compost with ABX	67	3	23,861,824	23,138,239	441,855	234,759	46,971	0.20
19	Soil	Compost with ABX	0	1	24,962,727	23,962,440	703,404	230,312	66,571	0.27
20	Soil	Compost with ABX	0	3	23,994,068	23,245,501	457,428	241,234	49,905	0.21
AVERAGE					23,869,793	23,061,904	515,326	239,530	53,033	0.22
21	Soil	Manure with ABX	67	2	22,214,289	21,209,646	7,078,016	234,272	62,555	0.28
22	Soil	Manure with ABX	0	3	22,379,951	21,678,139	416,354	241,585	43,873	0.20
23	Soil	Manure with ABX	67	1	21,952,981	21,215,381	445,406	246,748	45,446	0.21
24	Soil	Manure with ABX	-1	1	21,632,223	20,980,799	382,140	229,938	39,346	0.18
25	Soil	Manure with ABX	0	2	24,034,276	23,314,186	419,468	258,290	42,332	0.18
26	Soil	Manure with ABX	0	1	27,354,209	26,495,907	534,701	266,177	57,424	0.21
27	Soil	Manure with ABX	67	3	25,083,431	24,299,077	482,537	250,457	51,360	0.20
AVERAGE					23,521,623	22,741,876	1,394,089	246,781	48,905	0.21

Table S3. Average Resistome Risk Scores at each point along the vegetable production chain determined via MetaCompare.

Sample Type	Treatment	Time	Contigs	ARG	MGE	PAT	Q (ARG)	Q (ARG_MGE)	Q (ARG_MGE_PAT)	Risk_Score
Lettuce Surface	Compost with ABX	Harvest	63564.0	510.0	1229.3	1767.0	0.009847	0.001772	0.000697	97.44
	Control Compost	Harvest	78890.7	455.3	1194.3	1121.3	0.005770	0.001035	0.000376	52.65
	Inorganic Chem Fertilizer	Harvest	44712.0	242.0	338.3	807.0	0.006084	0.000207	0.000037	37.59
	Manure with ABX	Harvest	93396.3	580.0	1276.7	1986.3	0.006238	0.000945	0.000266	52.73
Soil	Compost with ABX	Background	15151.0	39.0	162.0	11.0	0.002574	0.000000	0.000000	24.17
	Inorganic Chem Fertilizer	Background	5731.0	5.0	120.0	1.0	0.000872	0.000000	0.000000	19.56
	Manure with ABX No Amendment	Background	4266.0	5.0	69.0	2.0	0.001172	0.000000	0.000000	20.30
	Control	Background	7396.3	9.0	96.7	2.3	0.001194	0.000000	0.000000	20.36
	Compost with ABX	Time of Application	5672.3	8.0	108.7	2.0	0.001310	0.000000	0.000000	20.71
	Inorganic Chem Fertilizer	Time of Application	8145.0	11.7	169.0	1.3	0.001437	0.000000	0.000000	21.00
	Manure with ABX	Time of Application	14693.3	70.0	253.0	95.7	0.004441	0.000517	0.000129	37.00
	Compost with ABX	Harvest	6142.3	6.7	99.0	1.0	0.001060	0.000000	0.000000	20.02
	Inorganic Chem Fertilizer	Harvest	8797.7	14.7	146.0	4.7	0.001562	0.000000	0.000000	21.37
	Manure with ABX No Amendment	Harvest	5761.0	9.3	105.7	1.7	0.001668	0.000000	0.000000	21.63
	Control	Harvest	5169.3	6.3	105.3	0.7	0.001113	0.000000	0.000000	20.16
	Compost with ABX	Before	110814.0	326.0	1477.0	467.0	0.002942	0.000334	0.000108	27.38
Amendment	Control Compost	Before	128508.0	621.0	2123.0	959.0	0.004832	0.000521	0.000210	37.38
	Manure with ABX	Before	153790.5	365.0	1386.5	511.5	0.002374	0.000326	0.000120	25.41
	Compost with ABX	After	108475.3	291.7	1260.7	84.7	0.002690	0.000289	0.000021	25.87
	Control Compost	After	102768.0	295.7	1123.3	168.3	0.002909	0.000240	0.000039	26.49
	Manure with ABX	After	173146.3	442.3	1638.0	554.3	0.002553	0.000296	0.000117	25.87



S1 Figure. Study design flowchart of the field-scale vegetable production chain employed in this study. Point 1: Compost mixtures of raw manure without antibiotics (control) and with antibiotics was examined before and after composting and compared to raw manure with antibiotics before and after stockpiling during the same period. Point 2: four triplicate soil plots were amended with the compost with antibiotics and raw manure with antibiotic amendments from point 1 and compared to an inorganic chemical fertilizer control and to a no amendment control. Point 3: Lettuce were grown in and harvested from the soil plots for the three amendments examined in Point 2 + plots receiving a fourth amendment of no antibiotic control compost. Surficial microbiota on the lettuce phyllosphere were examined. Note: no lettuce was grown in the no amendment control soils because they were nutritionally-lacking.



S2 Figure. Non-metric multidimensional (NMDS) scaling of the Bray-Curtis dissimilarity distances highlight the distinct ARG profiles among the amendment types, control compost, compost with antibiotics, and manure with antibiotics. (ANOSIM, $p < 0.001$). ARGs were annotated against the CARD v2.0.1 database.

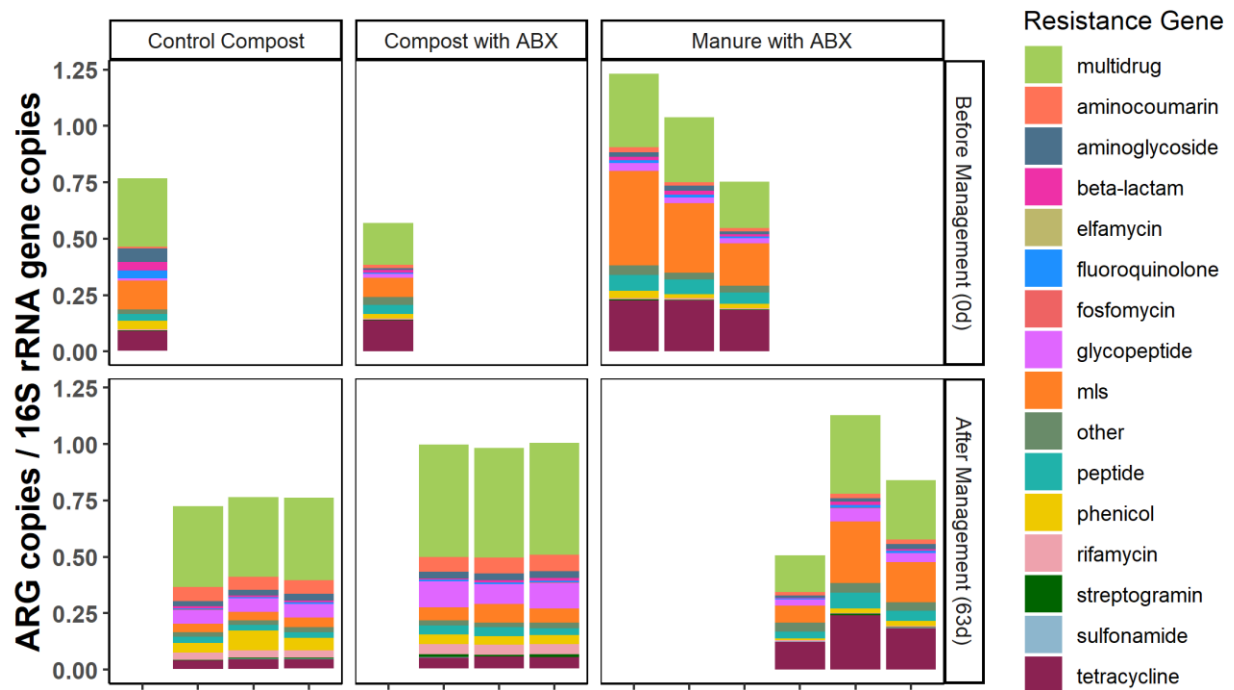


Figure S3. Amendment resistome profiles demonstrated as the relative abundance of ARGs before (0d) and after (63d) stockpiling and composting processes represented according to class of antibiotic to which they encode resistance. ARGs were annotated using CARD v2.0.1 via shotgun metagenomic sequencing.

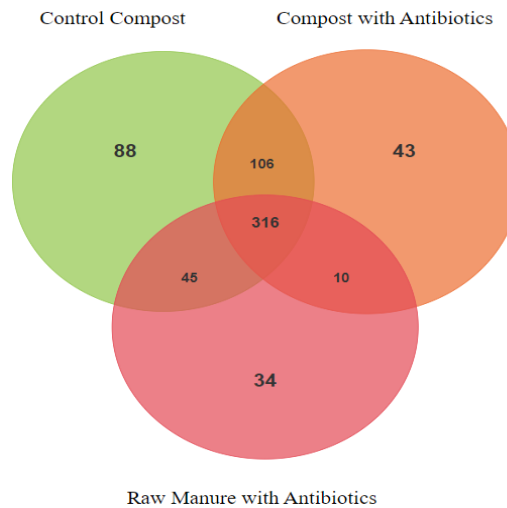


Figure S4. Total number of ARGs annotated via the CARD v2.0.1 database within the three amendment types. Of the 642 ARGs annotated, 316 ARGs were detected within each point (i.e. the amendments, soils, and vegetables).

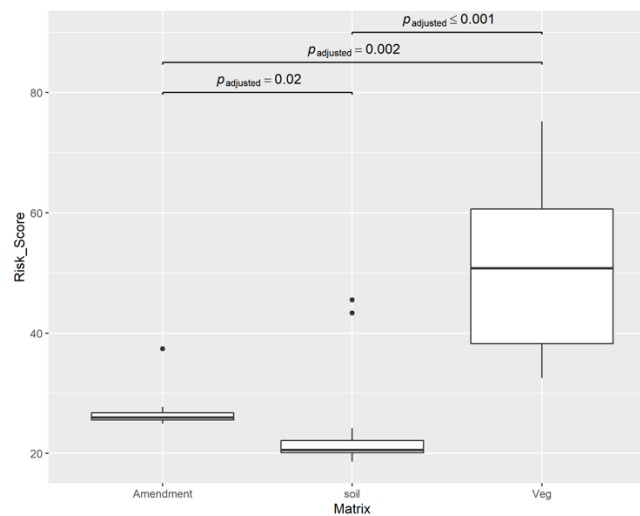


Figure S5. Agricultural resistome risk scores analyzed through Kruskal-Wallis non-parametric test. Amendment comprises of control compost, compost with antibiotics and raw manure with antibiotic conditions (n=14). Soil comprises of the no amendment control, inorganic fertilizer control, compost with antibiotics, and raw manure with antibiotics samples at the times of background, application, and harvest (n=27). Vegetables comprises of lettuce samples grown in the amended soils at the time of harvest (n=11*), there was out lettuce sample with a risk score of 166 which was considered an outlier for the visualization; further details can be found in Table S2).

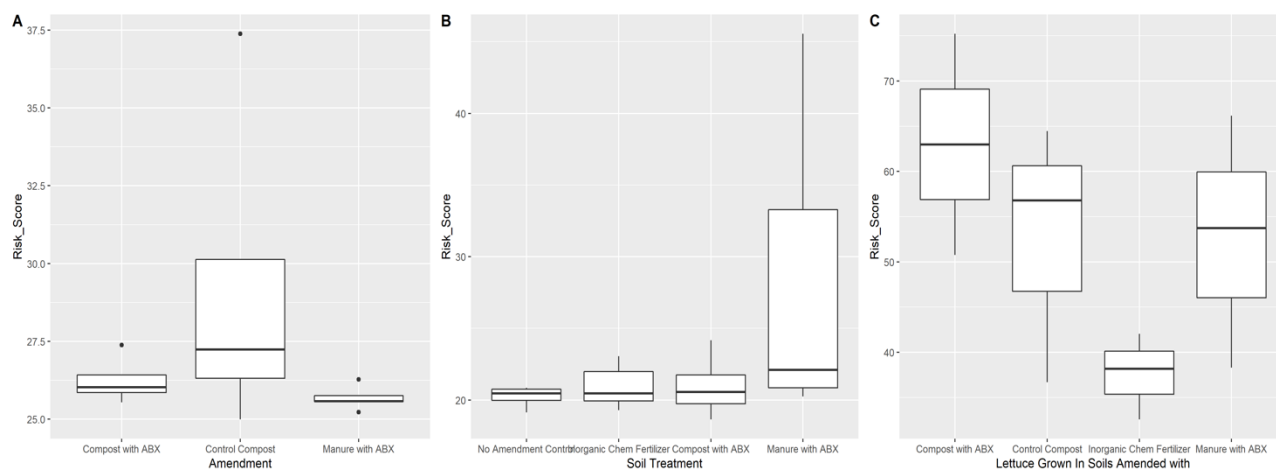


Figure S6. Agricultural resistome risk scores analyzed through Kruskal-Wallis non-parametric test at the (A) Amendment, (B) Soil, and (C) Vegetable points along the production chain. Amendment comprises of control compost, compost with antibiotics and raw manure with antibiotic conditions (n=14). Soil comprises of the no amendment control, inorganic fertilizer control, compost with antibiotics, and raw manure with antibiotics samples at the times of background, application, and harvest (n=27). Vegetables comprises of lettuce samples grown in the amended soils at the time of harvest (n=11*), there was out lettuce sample with a risk score of 166 which was considered an outlier for the visualization; further details can be found in Table S3).

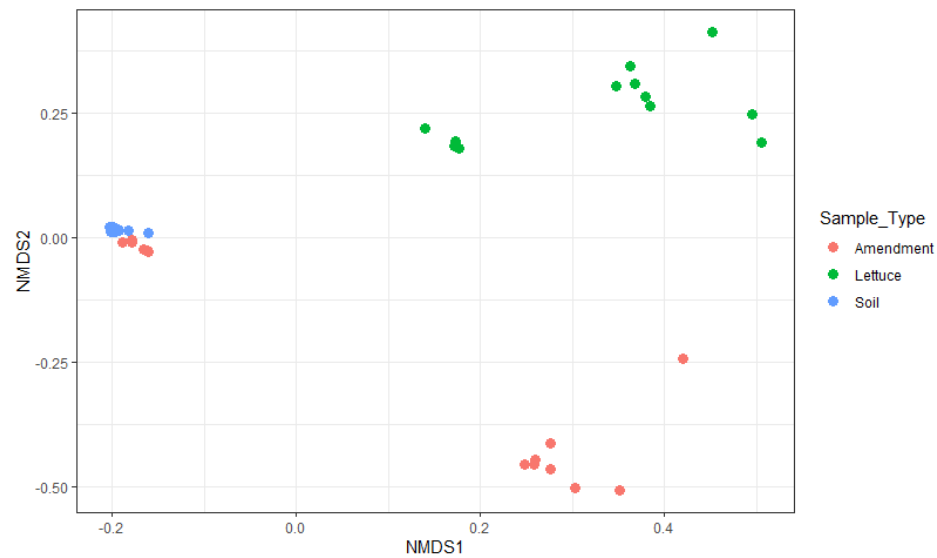


Figure S7. NMDS comparing the taxonomic profiles across the vegetable production chain.

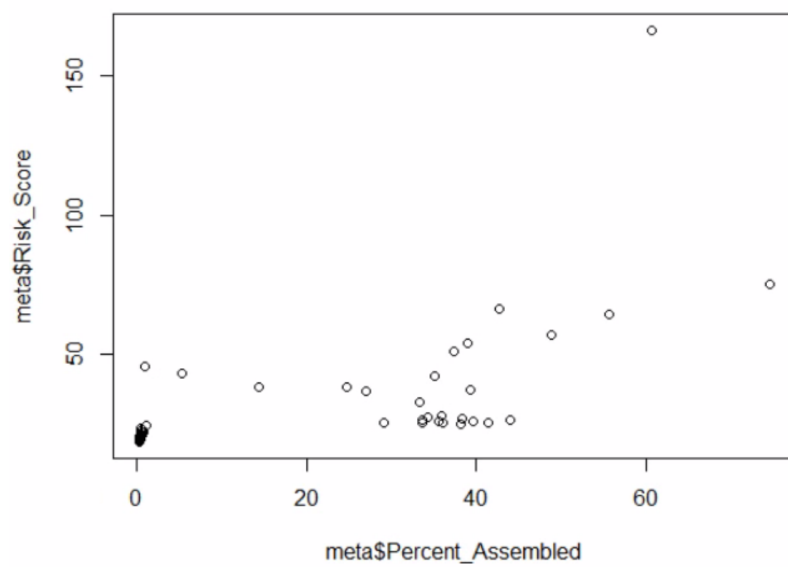


Figure S8. Risk score to % Assembled contigs of the metagenomic samples.

Chapter 6. Conclusions

Agroecosystems can serve as a source of antibiotic resistance, particularly following soil amendment with manures or manure-derived composts, without proper mitigation strategies. The implementation of agricultural best management practices at multiple control points is critical to providing a multi-barrier approach in preventing the spread of ARGs. In this study ARG abundance was less in composted manure compared to raw manure, suggesting that composting is a useful best management practice to prevent the environmental dissemination of resistance markers before soil application. There is evidence that once organic amendments are applied for nutrient and organic matter addition, regardless of manure management/treatment soils may inherently act as a natural ecological buffer for ARG proliferation, i.e. the natural soil resistome and microbial community is indistinguishable from untreated soils. Unfortunately, it appears that ARGs are detectable on crop surfaces at harvest when grown in amended soils. Therefore, it is critical to treat manure prior to application to control for ARG spread to the future crop surfaces.

Antibiotic resistance is inherently a natural survival mechanism of bacteria, so the observation of resistance markers is expected in environments as rich and complex as agricultural soils. This work agrees with past examinations of agricultural soils that suggest that one growing season may not be enough time to alter the native soil bacteria community long-term despite initial shifts immediately following application.

Detection method matters when evaluating resistance. Employing one detection method may lead to different conclusions about ARG presence in agricultural soils. For example, qPCR indicated that there are significant differences in anthropogenic targeted ARGs over time but metagenomics did not. Although metagenomics offers a much broader perspective on resistance, the complexity of the soil matrix substantially reduced sequencing depth. Shotgun metagenomic sequencing depth and coverage was not enough to assemble contigs for ARG-MGE co-occurrence analysis. Successful application in the analysis of agricultural soils will require substantial improvements in DNA extraction and purification to ensure sufficient sequencing depth.

Chapter 7. Recommendations & Future Work

There are multiple valid ways to quantify and characterize antibiotic resistance dependent on the question one is looking to answer, and each researcher and/or laboratory has their own protocol in doing so. In order to look at the bigger picture of antibiotic resistance through a One Health lens, there needs to be standardization of quantification methods for environmental AMR. Similar to the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) the US has for tracking resistance in human (CDC), retail meats (FDA), and livestock (USDA), environmental surveillance could be incorporated and follow the same isolate and resistance quantification standards to ultimately be comparable across the domains. NARMS is the leading antibiotic resistant surveillance system in the US, and has been collecting data since 1996.

One way to move forward, is to use known culturable pathogens as the target organism for a contamination proxy for antibiotic resistance in environmental systems. The advantages of culture include known viability, pathogenicity, the capacity to incorporate methods in diagnostic laboratories, and the ability to pinpoint targeted mitigation strategies. Recent risk assessment studies have indicated *Escherichia coli* (*E. coli*) and other pathogenic bacteria are potentially the most efficient way to determine if there is immediate antibiotic resistance risk to human and animals (Zhang et al., 2020; Pelley, 2020). Another way forward is to align antibiotic resistance methods with other standardized protocols for microbial contaminants, i.e., Microbial Source Tracking (MST). MST is a platform where researchers employ varying analytic tools, mainly culture-based and molecular-based qPCR, to detect and trace the origin of fecal pollution in waters. Current antibiotic resistance research is already employing these methods, i.e., qPCR for ARGs (Baquero et al., 2008). In developing a standardized MST-type protocol for antibiotic resistance detection, the benefit of having an efficient, economic, and simple method (i.e., culture-based techniques) that can be applied by stakeholders in any domain will be extremely useful for the field.

Researchers should continue to explore qPCR and NGS to better understand the potential taxonomic and functional characteristics of the system and discover novel ARGs and relationships that could further mechanistic resistance understanding. Additionally, there is much promise in

simultaneously moving forward with NGS techniques, specifically employing Oxford Nanopore (ONP) long-read sequences to characterize whole genomes and potentially identify novel antibiotic resistance genomic regions of interest for future targeted detection. However, in real world systems like agroecosystems, it is most important to know what will harm humans and animals immediately and then dig deeper into the potential risks. I suggest using an MST framework to develop standardization of antibiotic resistance quantification in environmental systems.

NGS is a very powerful tool for novel ARG and resistance mechanism presence and discovery but its limitations are clear when it comes to reproducibility, depth, and overall standardization. As the field rapidly moves forward, and independent laboratories advance their efforts finding remarkable results collaboration and reproducibility are essential. AgroSeek is a computational tool that can compare metagenomic sequences from multiple studies based on known metadata (Liang et al., in review). AgroSeek is a way the field can collaborate and explore the depth of metagenomics with respect to antibiotic resistance, and ultimately provide useful information to researchers and policy makers about the antibiotic resistome.

Globally, when it comes to environmental antibiotic resistance, acute and chronic concerns vary dependent on region, accessibility, and policy. Food security and water scarcity must become a permanent ideal when addressing agricultural related antibiotic resistance. Promising work has been done with wastewater reuse in regions of water scarcity, and shifts in manure management and crop production practices are allowing for pinpointed control of potential antibiotic resistance dissemination. However, as these issues tend to affect some regions much greater than others the current recommendations and frameworks for addressing antibiotic resistance globally must shift in accountability. Antibiotic resistance frameworks should be aligned with each of the United Nations sustainable development goals. This will provide conceptual frameworks that focus on the growing areas of concern we face globally, and will address food security, water scarcity, environmental justice etc. and ultimately helps produce policy to address antibiotic resistance globally.

While there is no shortage of investigations into the origins, transfer, and fate of antibiotic resistance in the environment, these efforts are often limited by a lack of shared language and methodologies (Wind et al., in review). The most effective strategies to mitigate resistance spread while meeting agro-economic goals demands a consolidated “One Health” approach. Antibiotic resistance research is still in its silo of human versus animal versus environmental scales, and that must be addressed to truly advance the field, research, and create necessary policy at local, regional, national, and global scales.

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Appendix A

Relevant data processing (metagenomics, 16S rRNA amplicon sequencing) and visualization code can be accessed at <https://github.com/laurenwind/DissertationCode> upon request.

Appendix B

All shotgun metagenomic and 16S rRNA amplicon sequences and relevant metadata is publicly available in the NCBI BioProject PRJNA506850.

