# Antimicrobial resistance in soil: Long-term effects on microbial communities, interactions with soil properties, and transport of antimicrobial elements

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**ABSTRACT**

Since penicillin was discovered in 1928, antibiotic usage in human and veterinary medicine and prevalence of antibiotic resistant bacteria (ARB), has been increasing. While antibiotics and antibiotic resistance genes (ARGs) naturally occur in soils, increasing abundances of ARGs correlate with increased antibiotic usage in agricultural settings. When livestock are treated with antibiotics, the antibiotic compounds, ARB, and ARGs can enter soil via manure excreted onto pastures or applied to other fields as fertilizer, thereby spreading antimicrobial resistance (AMR) in the environment. In addition to human health implications, increased AMR has negative impacts on ecosystem services such as carbon and nitrogen cycling. While many studies have researched antibiotic persistence in agricultural systems and their impacts on soil microbial communities, there are still significant knowledge gaps around the long-term effects of antibiotic exposure in soils, how those impacts differ among soils, and how elements of AMR may differentially transport through soil. To address these knowledge gaps, our objectives were to 1) examine the impact of multi-year repeated additions of manure from cattle administered antibiotics on soil microbial communities, 2) determine the interactive effects of soil moisture and type on soil microbial communities exposed to

antibiotics and manure, and 3) differentiate between vertical transport of AMR in the form of viable ARB or ARGs in extracellular plasmids.

Our results demonstrate that soil bacterial community structures were consistently altered by 3-year additions of manure from cattle administered antibiotics compared to soil amended with antibiotic-free manure. Furthermore, ARG abundances were higher in soils with manure additions compared to soil without manure, although this was true regardless of whether the cattle were administered antibiotics, suggesting that manure and antibiotic impacts on soil microbial communities can persist over multi-year of repeated manure applications. Additionally, in microcosms, effects of manure from cattle administered antibiotics on ARG abundances, microbial community structures, respiration, and nitrogen pools in soil were seen across multiple soil types and moisture contents, suggesting environmental conditions can alter how manure and antibiotics impact microbial community structure and nutrient cycling. Finally, ARB flowed readily through saturated soil, but were also detectable in the top 5 cm of soil columns. However, ARGs on extracellular plasmids did not flow through soil columns and were not detected in soil, indicating that extracellular DNA does not persist or transport through the soil to any meaningful degree.

Overall, these results indicate a nuanced approach is required to mitigate the environmental spread of AMR. Soil management strategies for addressing the AMR crisis should consider the broader context of manure management, as high ARG abundances can come from application of manure from antibiotic-free cattle, and soil microbial communities in individual environments may have varied responses to manure

antibiotic exposure. Furthermore, the transport of AMR through soil is complex and dynamic, as elements of AMR may transport differently through soil and require separate consideration in modeling and management. Future AMR management practices that consider diverse factors that affect persistence and spread of AMR in the environment can help protect livestock productivity and maintain the efficacy of antibiotics to protect human and animal health.

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**GENERAL AUDIENCE ABSTRACT**

Antibiotics are an important tool used to fight infections in humans, pets, and livestock. As antibiotics are used more frequently, the bacteria they target are more likely to develop resistance to the antibiotics, leading to increasing cases of infections that are harder to treat and higher risk. Antibiotic resistance can persist and spread in multiple forms, including the antibiotic compounds themselves, as antibiotic resistant bacteria (ARB), or as the genetic material that encodes for antibiotic resistance genes (ARGs). In agricultural systems, when livestock are treated with antibiotics they can excrete the antibiotics, along with ARB and ARGs, in the manure, which is then applied to land as fertilizer. In addition to the associated health risks, the spread of antibiotic resistance impacts microscopic bacteria and fungi in the soil, which are important for recycling nutrients for plants and maintaining ecosystem health. The overall goal of this dissertation was to gain a better understanding of how manure from cattle given antibiotics impacts these bacteria and fungi when manure is applied to the soil. The specific objectives were to 1) look impacts after long-term (multiple years) of manure addition, 2) examine how bacteria and fungi might respond differently to antibiotics in soils of different type or with different amounts of water, and 3) determine if ARGs that

exist as free genetic material outside of living bacteria can be moved through the soil with flowing water in the same way as living bacteria.

Results showed that while the composition of bacterial and fungal communities in the soil vary from year to year, adding manure with and without antibiotics had both caused different and consistent changes on the composition of bacterial communities. There were also higher concentrations of ARGs in soil that had manure added, however antibiotics in the manure did not cause ARGs to increase further, suggesting that even antibiotic-free manure can impact the spread of antibiotic resistance. Experimental work also demonstrated that the soil type and water content of soil can alter how bacteria and fungi respond to antibiotics in manure. The composition of bacterial and fungal communities, their activity rates, and the amount of nitrogen – an important plant nutrient with availability that is strongly affected by microbial activity – all differed with soil type and water content. Thus, while antibiotic resistance antibiotic resistance can cause measurable changes in soil across a range of environmental conditions, it is also likely to persist and spread in different ways in different environments. Finally, when water containing elements of AMR was added to soil, ARB were shown to both move through the soil easily and remain near the top of soil. In contrast, ARGs contained on genetic material outside of living cells did not move through the soil and were broken down within a few days, suggesting that antibiotic resistance likely spreads through living bacteria more than genes outside of cells. Overall, this work highlights the complexity of understanding the role of environmental transmission in the antibiotic resistance crisis

and demonstrates the need for nuanced management approaches that take specific environments and conditions into account.

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# Chapter 1. Introduction

## 1.1. An Emerging Crisis

### *1.1.1 Increasing usage and resistance*

Since antibiotics were discovered, usage has been increasing for both human and veterinary medicine. Currently, most antibiotics (80%) are used in livestock production (Hollis and Ahmed, 2013). Additionally, antibiotic use in livestock is projected to increase by 67% from 2010 to 2030 as the global demand for meat increases (Van Boeckel et al., 2015). With increasing antibiotic usage, increasing resistance to antibiotics has become a major concern for human health, with antimicrobial resistance (AMR) being considered a crisis as early as the 1990's (Neu, 1992). Veterinary use of antibiotics can lead to resistant infections in humans, and infants and young children are particularly at risk of developing serious conditions from AMR bacterial infections (Shea, 2003). In 2013, the Center for Disease Control and Prevention (CDC) released a report to outline the current threats of AMR on human health (Center for Disease Control and Prevention, 2013). In addition to outlining the most common threats, the report also listed several steps to address the crisis. The first step is preventing infections and the spread of resistance, including resistance in meat and dairy. A report from the President's Council of Advisors on Science and Technology (PCAST) recognized that antibiotic use in agriculture can pose risks to human health, although the extent of the risk is unknown (PCAST, 2014). In addition to outlining practical steps to combat the AMR crisis, the PCAST report highlights the possibility of antibiotic resistant bacteria (ARB) transmission from livestock to humans.

Both antibiotic resistance genes (ARGs) and antibiotics are considered environmental contaminants (Pruden et al., 2006; Sanderson et al., 2016). Within a few years of widespread use of penicillin, strains of bacteria resistant to penicillin were identified (Taubes, 2008). Since then, the number of antibiotic-resistant infections has been on the rise. As preferred antibiotics are increasingly ineffective due to bacterial resistance, treating infections is growing more difficult (Lushniak, 2014). Currently, the CDC estimates that AMR infections kill 23,000 people a year (Center for Disease Control and Prevention, 2013). In addition to the ramifications of AMR to human health, lack of effective treatments to infections could cost as much as $3 trillion globally per year (Aslam et al., 2018).

Overcoming the AMR crisis will require multiple approaches. For example, one strategy is to avoid overuse of antibiotics in veterinary feed. New rules from the FDA aim to reduce unnecessary use of antibiotics in livestock feed (FDA, 2022). In addition to overuse of antibiotics, the CDC recognizes the spread of ARB as the second major factor of the growth of AMR (Center for Disease Control and Prevention, 2013). Resistant bacteria and ARGs may be spread directly among humans but also from environmental sources to humans. Because AMR is a complex problem with interactions among people and the environment, it should be addressed with a holistic One Health approach (McEwen and Collignon, 2018), which recognizes that human health is related to animal and environmental health. Environmental reservoirs of AMR, including soil and agricultural settings, are important areas to consider in the One Health perspective of the AMR crisis.

### *1.1.2 Antibiotics and ARGs in soil*

AMR is not a new phenomenon. Antibiotics and ARGs that confer the ability to degrade or deactivate antibiotics have been around for millions of years (Allen et al., 2010). Although, it was not until the 1930's that soil was identified as a source of antibiotic-producing bacteria (Waksman and Woodruff, 1940). As both antibiotic compounds and ARGs are naturally found in soils, soil ecosystems serve as a reservoir of ARGs and possible vector for transmission (Fletcher, 2015; Nesme and Simonet, 2015). Soil isolates have been found to be resistant to multiple antibiotics (Hu et al., 2017). Evidence suggests that multiple ARGs can exist on one plasmid, causing an increase in the resistance to multiple drugs, when organisms are exposed to only one drug (Herrick et al., 2014). Further research has indicated that ARGs can be shared between soil environments and clinical pathogens, suggesting that AMR in soil is directly tied to human health (Forsberg et al., 2012).

Antibiotic compounds are produced by microorganisms for a multitude of reasons. Antibiotics are mainly hypothesized to be a way of competing for nutrients by inhibiting other bacteria (Martinez, 2008; Fletcher, 2015). More isolates of *Streptomyces* are resistant to other *Streptomyces* when soils were amended with soil carbon, indicating that nutrients correlate to production of antibiotics (Dundore-Arias et al., 2019). At low concentrations, antibiotic compounds can also be used to send signals to other bacteria (Allen et al., 2010). These signals can be sent to cells of the same bacterial strain, or they can be signals sent from one microbial species to another (Fajardo and Martínez, 2008). Antibiotic production is a necessary function

for many bacteria, although the full ecological role of antibiotic compounds is not fully understood (Nesme and Simonet, 2015). Antibiotic resistance genes can also exist for multiple reasons. For example, organisms that produce antibiotics would likely have ARGs to protect themselves (Hu et al., 2017). In some cases, the function of an ARG can be resistance to other toxic compounds and metals, or to increase overall fitness (Allen et al., 2010). Additionally, resistance genes can be part of other functions including virulence and signal trafficking (Martinez, 2008).

While antibiotics are naturally found in soil, higher concentrations of naturally occurring and synthetic antibiotics can occur because of human activity. Since the widespread use of antibiotics began in the 1940's, ARG abundance in soils has subsequently increased as well (Knapp et al., 2010). Low selective pressures, such as low concentrations of antibiotics, can increase ARG abundances in soil, especially if the pressure occurs over a long time (Michael et al., 2016). Previous work has shown an increase of ARGs in areas impacted by agriculture (Pruden et al., 2006) and these increases may be attributed to manure and biosolids additions (Gothwal and Shashidhar, 2015).

*1.1.3 AMR is a problem for ecosystem services*

Antibiotics tend to decrease microbial abundance, and the ratio of bacteria to fungi can be decreased by antibiotics such as sulfadiazine that target bacteria but not eukaryotes (Reichel et al., 2014; Lin et al., 2017; Grenni et al., 2018). Differential effects on survival and abundance from antibiotics also impact the structure soil of the microbiome, and the length and severity of the impact can be dependent on the type of antibiotic (Lin et al., 2016). As a result, overall

microbial diversity is also adversely impacted by antibiotics (Lin et al., 2017; Grenni et al., 2018). Although some impacts of antibiotic additions may disappear over time, other impacts can be long-lasting (Lin et al., 2017).

Anthropogenic sources of antibiotics have negative impacts in the soil beyond decreased microbial abundance and diversity. In additional to structural changes, antibiotics can have physiological effects on microbial communities that in turn impact rates of important ecosystem processes. Overall, antibiotics can decrease nitrification, denitrification, and iron reduction, while increasing methanogenesis (Grenni et al., 2018). The effects on nitrogen cycling are thought to be due to the susceptibility of gram-negative bacteria to antibiotics (Ahmad et al., 2014). For example, many common denitrifiers, including *Nitrosomonas* and *Pseudomonas*, are gram negative, and thus are likely to be impacted by antibiotics.

In terms of carbon cycling, an increase in respiration can be tied to increased ARG abundance in soils that have previous manure and antibiotic exposure, due to higher metabolic costs to maintain AMR (Wepking et al., 2017). Antibiotics have also been found to increase methane emissions from cow manure (Hammer et al., 2016). Plants can also be affected as they may uptake antibiotics from soil, and high concentrations of antibiotics can stunt plant growth (Tasho and Cho, 2016). The impacts of antibiotics on ecosystem services and human health are only beginning to be explored, however, and long-term implications are not well known.

### 1.2. Soil microbial communities respond to heterogeneous soils and environmental change

#### *1.2.1 Microbial communities vary with soil properties*

While soils may act as a reservoir for AMR, soils are heterogeneous ecosystems with many other varying physical, chemical, and biological properties that affect the resident microorganisms. Soil texture can influence microbial communities, with greater microbial richness in coarser soils (Chau et al., 2011). However, when a sandy loam and a clay loam were compared, greater biomass was found in the finer soil (Roberts et al., 2011). Additionally, soil texture and land cover had a greater impact on soil microbial community composition than did climate across France (Dequiedt et al., 2009). Soil texture can also impact how microbial communities recover after disturbance, with faster recovery in finer-textured soil (Bach et al., 2010). Perhaps one reason for the influence of texture on microbial communities is the limits it places on the ability of protozoa and nematodes to feed on bacteria (Brussaard, 1998). With finer textures and smaller pores, bacteria are better protected from predation in some microhabitats, contributing to the co-existence of more taxa.

Soil chemistry also has strong documented effects on soil microbial communities. Over large scales, soil pH can describe a large amount of variation in soil microbial community structure (Fierer and Jackson, 2006; Fierer et al., 2009; Lauber et al., 2009), although the effect may not always be the same for fungal communities (Lauber et al., 2008). The importance of soil pH and texture as main drivers of microbial abundance and richness is evident in agricultural landscapes as well (Constancias et al., 2015). In agricultural settings, nutrient inputs such as inorganic nitrogen fertilizer and organic carbon from plant residues can also alter microbial community structure (Chávez-Romero et al., 2016). The ratio of carbon:nitrogen (C:N) is particularly important, with greater fungi:bacteria ratios observed in soils with higher C:N (Fierer et al., 2009). C:N balance can also interact with phosphorus to impact soil fungal

community structure, with greater abundance of Agaricales in soils with higher C:N and lower P, and opposite trends in Sordariomycetes (Lauber et al., 2008). In one study, additions of nitrogen and phosphorus resulted in increases in Actinobacteria, Alphaproteobacteria, and Gammaproteobacteria, and decreases in Acidobacteria, Planctomycetes, and Deltaproteobacteria (Leff et al., 2015).

Soil properties that can vary with climate, such as temperature and moisture, are also important factors affecting soil microbial communities. In one study where texture, precipitation, nutrients and plants all impacted fungi:bacteria ratios and microbial community composition, precipitation was found to have the greatest influence (de Vries et al., 2012). Different microbial groups respond to moisture content in different ways, with greater fungal abundance at higher moisture content, and greater bacterial abundance at lower moisture (Borowik and Wyszkowska, 2016). Another study found that fungi are sensitive to small changes in soil moisture content, while bacteria are not (Kaisermann et al., 2015). In grasslands, moisture impacts soil microbial community structure, while temperature does not, and microbial diversity increases with moisture (Lupatini et al., 2019). However, other studies have found changes in soil microbial communities with changes in temperature. Furthermore, specific bacterial taxa respond differently to changes in soil temperature. For example, Actinobacteria and Chloroflexi have been observed to increase with temperature, while Bacteroidetes tend to decrease (Oliverio et al., 2017). Overall, it remains difficult to make generalizations at a large taxonomic scale, as groups within any one phylum may have both taxa that increase and decrease with temperature. Given these variable responses of microbial communities to environmental factors – and the complex

interactions among them – it is clear that more information is required to understand the role they play in controlling AMR in the environment.

*1.2.2 Microbially-mediated processes depend on soil properties*

The collective activities of soil microorganisms are critical mediators of biogeochemical cycling in terrestrial systems. In addition to altering community structure, soil properties also impact these microbially-driven nutrient cycling processes. For example, microbial activity and nitrogen mineralization rates were found to be most influenced by soil texture and soil organic carbon (SOC), with the greatest rates in fine textured soils with high SOC (Šantrůčková et al., 2003). In another study, denitrification in soils is higher in poorly drained, finer textured soils (Morales et al., 2015). From a metagenomic perspective, the composition of functional genes in soil is related to soil texture as well as pH and organic matter (Paula et al., 2014).

As would be expected, nutrient availability in soils has direct impacts on biogeochemical processes such as the cycling of carbon and nitrogen. For example, in the nitrogen cycle, denitrification is correlated with both nitrate and dissolved organic nitrogen, but also impacted by pH and soil texture (Enwall et al., 2010). Additionally, higher emissions of N from soil is related to both high nitrate and low ammonium and soluble carbon (Morales et al., 2015). Changes in pH can be related to changes in denitrifiers, nitrate reducers, and nitrifying archaea (Bru et al., 2011). Total bacteria abundance and denitrifiers increase with nutrients (carbon, nitrogen, phosphorus), and decrease with pH and coarser soils (Deslippe et al., 2014). Total bacteria, as well as denitrification and nitrogen fixation potential increase with greater moisture content (Morugán-Coronado et al., 2019).

In the carbon cycle, soil moisture generally increases microbial activity and respiration. In particular, increased respiration occurs when a dry soil is rewetted (Bardgett et al., 2008), but microbial activity decreases with further increased moisture stress (Manzoni et al., 2012), while lower respiration occurs with lower moisture (Kaisermann et al., 2015). Another study found increased respiration with increased water content, and with a finer-textured soil (Dijkstra and Cheng, 2007). However, enzyme activity was found to be highest at relatively low moisture content (20% water capacity), corresponding with greater bacterial abundance (Borowik and Wyszkowska, 2016). With the complex interactions of microbial functional responses to environmental factors, more information is required to understand how these processes are impacted by AMR.

### 1.3. Soil properties influence on persistence of antibiotics and ARGS

#### *1.3.1 Moisture*

Given that both antibiotic exposure and soil characteristics can affect microbial communities, many studies that examined both concurrently found that varying physical, chemical, and biological properties of soils affect the persistence of antibiotics. Soil moisture can affect both rates of antibiotic degradation and microbial communities' responses to antibiotic exposure. Generally, increased moisture increases degradation of antibiotics. Braschi et al. (2013) reported that 2 β-lactams degraded more quickly at field capacity than at wilting point in both a forest (loam) and a calcareous soil (clay loam). However, antibiotic degradation was due to both microbial communities and abiotic factors, which may include texture, pH, or mineralogy. Similarly, in a manure application study conducted in corn and grasslands, varying

degradation rates of sulfonamides and their metabolites throughout the experiment appeared to be due to moisture effects on both the sorption of the antibiotics and microbial activity (Rosendahl et al., 2011). Similar effects have been seen for tetracycline and chlortetracycline degradation, where higher moisture content reduced sorption to the soils, allowing microorganisms to degrade them (Bansal, 2012).

The temporal dynamics of moisture changes can also be important. Soil undergoing repeated cycles of wetting and drying was found to have increased concentrations of sulfadiazine over time compared to soil that was held at constant moisture. (Reichel et al., 2014). Additionally, soil microbial abundance was decreased in soils exposed to sulfadiazine, but the effect was more pronounced in soils with fluctuating moisture than in soil at constant moisture. As sulfonamides primarily sorb to organic matter in the soil, drought conditions are thought to impede the sorption and degradation of the antibiotics. Drought can change the conformation of organic matter, which can affect how well antibiotics sorb to organic functional groups (Radl et al., 2015). Alternatively, this phenomenon could also be a result of reduced microbial activity in dry soils that impedes biodegradation.

Although the effect of moisture on the degradation of antibiotics in soil is well documented, there is less known about the interactive effects of moisture and antibiotics on resistant organisms and microbial communities. Sulfonamide resistance and integrase (an indicator of horizontal gene transfer potential among bacteria) genes increase with application of sulfadiazine, but gene abundances are reduced in soils with fluctuating moisture (Jechalke et al., 2016). Another study found sulfonamide resistance gene abundances to be negatively correlated with soil temperature and precipitation (Zhou et al., 2017). Responses can be taxon-specific also,

as fluctuations in soil moisture interacting with sulfadiazine were shown to affect some microbial functional groups but not others (*e.g.*, nitrifiers but not denitrifiers; Radl et al., 2015). In this case, as denitrification is an anaerobic process, it is likely that even with fluctuating water content, soils were not sufficiently saturated to promote denitrification. However, nitrification, being an aerobic process, was reduced with sulfadiazine and with moisture stress, likely due to a combination of low moisture inhibiting microbial activity, and low moisture causing sulfadiazine to be more persistent, also inhibiting microbial activity. Changes in nitrification and denitrification rates are critical in the N cycle as they can have major impacts on overall soil nutrient availability and plant growth. Although studies such as these demonstrate that moisture content can affect microbial community composition and reduces the abundance of some ARGs, more information is needed to determine whether the effects are similar for other antibiotics.

### *1.3.2 Texture and Mineralogy*

Antibiotics bound to soil particles can retain antimicrobial properties, and soil texture can affect antibiotic binding and persistence. Soils with higher clay and organic matter content sorb more antibiotics and have a stronger inhibitory effect on bacteria (Chander et al., 2005). Soil texture also influences how long bacteria from manure survive in soil, with longer survival in clay soils (Sharma and Reynnells, 2016). One study found the type of clay mineral as well as the proportion of clay impacts the sorption and availability of oxytetracycline. Increased clay content, along with increased organic matter, iron, and aluminum content are positively correlated with how tightly bound the antibiotic is. The capacity of soil to sorb oxytetracycline increases with increased illite content, but decreases with kaolinite, likely because 2:1 phyllosilicate minerals, such as illite, have more surface area and charge to sorb the

oxytetracycline than 1:1 clay minerals, such as kaolinite (Kong et al., 2012). In another study, dissipation of tetracyclines was more rapid in a silt loam than a sandy loam (Li et al., 2016). The differences were most likely due to higher heavy metal content in the sandy loam, as tetracyclines can complex with divalent metals. However, dissipation rates of sulfamethazine, tylosin, chlortetracycline, and pirlimycin in a sandy loam, silt loam, and silty clay loam are negligible (C. Chen et al., 2018). Only sulfamethazine has varying dissipation rates among soil textures, with faster dissipation in the finer textured soil. β-lactams may also degrade more quickly in finer soils. Braschi et al., (2013) found the β-lactams degraded more quickly in a calcareous clay loam soil than a forest loam soil, which supports the hypothesis that texture influences antibiotic degradation. However, since only 2 soils were compared, it is not possible to say whether texture, pH, or mineralogy was responsible for the difference.

The effect of texture on persistence of different types of antibiotics is not uniform. Where tetracyclines and β-lactams generally degraded faster with a finer textured soil, degradation of sulfadiazine was increased in a loamy sand compared to a silt, likely due to better air and water movement through the soil (Engelhardt et al., 2015). However, sorption of the antibiotic was higher in the silt. Antibiotics in finer textured soils may have a muted effect on microbial communities due to the sorption of compounds to clay particles. This phenomenon may cause additional concerns with the spread of AMR resulting from transport of antibiotics bound to soil particles. One study found that tetracycline bound to soil particles could still be bioavailable, although antibiotics were more available in sandy soil (Chen et al., 2017).

Soil texture can also play an important role on responses of soil microbial communities to additions of antibiotics. One study found that microbial community composition was much more

strongly affected by manure and antibiotics (doxycycline and streptomycin) in a sandy soil than a loam (Blau et al., 2018). Furthermore, the abundance of ARGs was different between the two soils, with increased concentrations of *intI1*, *aadA*, *qacE*, *qacE1* and *sul1* in the sandy soil, and increased *tet*(M) and *tet*(Q) in the loam. Another study of manure from antibiotic-treated dairy and beef cattle found that while differences in dissipation rates of sulfamethazine, tylosin, chlortetracycline, and pirlimycin in a sandy loam, silt loam, and silty clay loam were negligible (C. Chen et al., 2018), the response of both ARGs and microbial community structure to antibiotic additions interacted with texture (Pankow, 2017). Microbial community composition was significantly altered by the addition of antibiotics and manure. In general, the sandy soil had higher ARG abundance than the finer textures, especially for ARGs that convey resistance to sulfonamides. As sulfamethazine was more persistent in a sandy loam (C. Chen et al., 2018), microorganisms in that soil are exposed to more of the antibiotic for a longer period of time. Interestingly, tetracycline resistance genes were also more abundant in the sandy loam soil (Pankow, 2017), although chlortetracycline dissipation was not significantly different among soil textures (C. Chen et al., 2018). This phenomenon could be explained by co-selection of multiple ARGs under the presence of only one antibiotic, as previous evidence has indicated can happen (Herrick et al., 2014). Most studies agree that finer textured soils generally sorb more antibiotic than coarse soils, however the effect of soil texture and antibiotics on ARG abundance in soils is not agreed upon. Further research is required to tease apart the complex interactions of soil texture, antibiotics, and microbial communities.

### *1.3.3 Manure*

The application of manure to soils can directly be a vector for transmission of AMR to soil. However, the manure itself can also have indirect effects by altering soil properties such as nutrients and organic matter content that then in turn affect AMR. For example, manure may contain ARGs and resistant bacteria, even from animals without a history of antibiotic exposure (Heuer et al., 2011). ARGs in cattle manure are found in diverse microbial taxa and many are flanked by mobile genetic elements, suggesting horizontal gene transfer is taking place (Wichmann et al., 2014). However, exposure of animals to antibiotics can further alter AMR in the manure. For example, cows treated with a β-lactam have increased β-lactam resistance genes, although total ARG abundance does not increase (Chambers et al., 2015). Additionally, ARG abundance in manure is correlated with antibiotic abundance as well as heavy metal abundance (Ji et al., 2012).

When AMR in manure is altered after cattle that have been administered antibiotics elements of AMR can enter soils via application of manure or excretion of feces from grazing animals (Tang et al., 2015; Tasho and Cho, 2016). In some cases, long-term additions of manure can lead to a persistent increase of ARGs in soil (Fang et al., 2015; Graham et al., 2016; Wepking et al., 2017). However, another study found ARG abundance generally decreases over time following manure and antibiotic additions (Zhang et al., 2017). It is also important to note, however, that increases some ARGs can be found in soils after application of manure from cattle that have not been administered antibiotics (Udikovic-Kolic et al., 2014). Persistence of AMR following manure additions depends on many of the factors previously described, including soil texture, light, temperature, and pH (Tasho and Cho, 2016). Although we know that manure with or without antibiotics may contain ARGs, and additions of manure over a long period of time

increases ARG abundance, there are still significant knowledge gaps around the differences between the impact of manure alone and manure with antibiotics.

### 1.4. Antibiotic transport in soil

When considering the human health risks of transmission of AMR from soil environments to humans, movement of AMR elements – as well as persistence – are critical components. As water moves through soil, soil particles, chemicals, and bacteria move with it. Water movement can facilitate the transport of antibiotics, ARB, and ARGs. While some antibiotics are highly mobile in soil, others are not. One study found that lincomycin and sulfamethazine leach through soil, but monensin does not (D'Alessio et al., 2019). However, Davis et al. (2006) found that of seven antibiotics tested, including sulfamethazine, monensin has the greatest loss in surface runoff. Such differences in sorption of various antibiotics can often be explained by interactions with changing soil properties, such as pH and cation exchange capacity (Chee-Sanford et al., 2009).

In addition to antibiotic compounds themselves, DNA molecules such as chromosomes and plasmids that contain ARGs can also move through soil via water movement. Interestingly, extracellular DNA has been shown to move up in soil via capillary action (Ceccherini et al., 2007). Extracellular DNA, specifically a plasmid containing the ARG *aadA*, was able to leach through a soil column and be taken up by *Acinetobacter* sp. (Poté et al., 2010). In saturated columns of quartz sand, plasmids of varying sizes (2.7-33.4 kbp) with ARGs were applied to the soil surface and allowed to move through the columns (Chen et al., 2015). Results showed plasmids readily moved through silica columns, but transport was impacted by plasmid size and

ionic strength of soil solution. Generally, greater adsorption to soil particles occurred with smaller plasmids and higher ionic strength. Additionally, another quartz sand study compared the flowthrough of ARGs to plasmids comparing ARB and found that while plasmids moved much more readily than bacteria, both elements of AMR were able to move through the columns. Thus, it is possible that AMR deposited at the surface may be transported through soil and have impacts elsewhere.

However, as quartz sand is not a fully realistic model of a naturally occurring soil, and properties of more complex soils can affect transport. In a follow-up study by Chen et al. (2018), they examined the transport of ARG-containing plasmids through both quartz sand and a naturally occurring soil and found that the transport of the plasmid was comparable between the two substrates (P. Chen et al., 2018). However, the movement of antibiotics and antibiotic resistance through soil can also depend on soil physical structure. Antibiotics and ARGs tend to accumulate in soil near preferential water flow paths (Lüneberg et al., 2018), and Kay et al. (2005) found that more sulphachloropyridazine leached through soil that had not been tilled, compared to tilled soil. Furthermore, in the latter study the antibiotic degraded more thoroughly in the tilled soil, likely due to slower leaching and greater contact with the soil.

Coupled transport and persistence dynamics illustrate the complexities of how agricultural activities such as manure applications can lead to the spread of elements of AMR. For example, simulated rainfall after application of antibiotics resulted in increased concentrations of tetracycline, chlortetracycline, sulfathiazole, erythromycin, tylosin, and monensin below the soil surface (Kim et al., 2010). The tetracyclines, along with sulfathiazole, have the greatest concentrations deeper in the soil (20-30 cm). The authors hypothesized this is

due to colloid-facilitated transport, as tetracyclines can bind strongly to soil particles. Similarly, increases in ARG concentrations have been observed in drainage water from soils that had manure applied compared to soil without manure (Luby et al., 2016). Likewise, reported correlations in ARG abundances between agricultural feedlot wastewater and nearby soils irrigated with wastewater indicate that ARGs can spread also with wastewater irrigation (Li et al., 2012).

However, transport processes are complex with reported differences in mobility across antibiotic types, different elements of AMR, and environments. When soils were irrigated with wastewater spiked with sulfamethoxazole and ciprofloxacin, increased sulfonamide ARGs were found in the water flow paths (Lüneberg et al., 2018), but increased ciprofloxacin ARGs were not, likely due to the limited mobility of the antibiotic compound itself. Other work has demonstrated that while manure applications increased ARG abundance in surface soils, there was no increase in ARGs below 5 cm of soil depth, nor any increase of ARGs down the slope of a field (Fahrenfeld et al., 2014). Even the method of manure application can matter. When manure was either spread on the soil surface or injected into the subsurface and then subjected to simulated rainfall , higher abundances of ARB were found in runoff from surface applications compared to subsurface and abundances decreased after 7 days (Hilaire et al., 2022). However, a similar study comparing broadcast, incorporation, and subsurface injection methods only saw differences in ARG abundances in runoff among the 3 manure application methods for 2 of the 4 ARGs tested (Joy et al., 2013).

### 1.5 Research objectives

The overall goal of this dissertation was to gain a better understanding of the factors that contribute to the spread of AMR in soil. Much work has been done on persistence and transport of antibiotics and ARGs over relatively short time frames and or in specific scenarios, but there are many aspects of how environmental variability – and particularly with regard to interactions among environmental variables – impacts AMR that are not yet fully understood. Addressing these gaps will help inform management policies surrounding the handling and application of manure from cattle treated with antibiotics to mitigate the spread of AMR across multiple environmental conditions. More detailed explanations of knowledge gaps and objectives for each chapter are listed below.

**Chapter 2: Application of manure from cattle administered antibiotics has sustained multi-year impacts on soil resistome and microbial community structure**

Knowledge Gaps: Many studies have looked at direct short-term impacts of antibiotics or manure and antibiotics, but few have looked at the impacts of long-term controlled and repeated additions of manure and antibiotics on soil microbial communities. Many studies also add antibiotics to manure, as opposed to administering antibiotics to livestock and then collecting manure. Thus, there is a critical knowledge gap on the impacts of long-term additions of representative manure from livestock treated with antibiotics on both microbial community structure and ARG abundance. This is an important area of research as it is critical to understand how AMR may be spreading in livestock grazing fields.

Specific Objectives:

1. Identify long-term impacts of repeated manure additions from cattle under different antibiotic treatments on soil microbial community structure and ARG abundances.
2. Identify potential co-occurrences among ARGs.

**Chapter 3: Environmental conditions alter soil microbial responses to antibiotic and manure exposure**

Knowledge Gap: While soil properties are known to affect the persistence of antibiotics in the environment, less is known about how those soil properties might mediate the persistence of ARGS or the responses of soil microbial community structure and function to additions of manure from cattle treated with antibiotics. Furthermore, the studies that do look at the influence of soil properties and antibiotics on soil microbial communities have focused on one property, such as texture or moisture. Thus, little is known about the interactive effects of soil properties on soil microbial communities in response to exposure to manure from cattle treated with antibiotics. In order to effectively manage the environmental spread of AMR, it is critical to understand how microbial communities in varying soils will respond across multiple axes of environmental variability.

Specific Objectives:

1. Examine the impact of controlled changes in soil moisture and texture on the responses of ARG abundances and microbial community structure to the addition of manure and manure from cattle treated with antibiotics.
2. Identify changes in microbial activity among these same treatments.

**Chapter 4: Genes or bacteria: disentangling differential transport of antibiotic resistance through saturated soil columns**

Knowledge Gap: The vertical transport of antibiotics has been relatively well studied, but fewer studies have focused on the transport of ARGs and ARB, which can both also spread AMR to novel soil microbes. Furthermore, most previous studies have used silica sand rather than naturally occurring soils to examine transport of AMR. Therefore, the pathways of ARG transport through natural soils in the form of extracellular DNA versus inside viable bacteria is still not well understood. Yet, it is crucial to disentangle the transport of ARB and ARGs to understand their respective contributions to the proliferation of AMR.

Specific Objectives:

1. Determine whether resistant bacteria and extracellular plasmids containing resistance genes were transported at similar rates through a saturated soil column.
2. Determine concentrations of resistant bacteria and extracellular plasmids that accumulated in the soil matrix after surface addition.

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**Chapter 2. Application of manure from cattle administered antibiotics has sustained multi-year impacts on soil resistome and microbial community structure**

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## 2.1 Abstract

In agroecosystems, application of manure from livestock treated with antibiotics has the potential to spread antibiotic compounds, resistant bacteria, and antibiotic resistance genes (ARGs) to soil. Although environmental transmission of antibiotic resistance is a major human health concern, few studies have looked at long-term effects on soil microbial communities from applying manure from livestock administered antibiotics. We examined the impacts of three years of repeated manure additions from cattle under different antibiotic treatments on microbial community structure and ARG abundances. While manure additions altered both soil bacterial and fungal communities, manure from cattle administered antibiotics further altered soil bacterial communities, but not fungal, compared to manure from antibiotic-free cattle. Furthermore, addition of manure from antibiotic-free cattle resulted in increased abundances of several ARGs compared to soil with no manure inputs, but manure from cattle administered antibiotics did not change overall profiles of ARG abundances compared to manure from antibiotic-free cattle. Finally, although bacterial and fungal community structure and ARG abundances varied among years, manure treatment effects on each were persistent during the full three-year period. Taken together, our results suggest that manure and antibiotic impacts on soil microbial communities can persist for long periods of repeated manure application. Furthermore, soil management strategies for addressing the antibiotic resistance crisis should consider the broader context of manure management.

## 2.2 Introduction

Currently, 80% of total antibiotic usage in the US is in livestock production (Hollis and Ahmed, 2013), and global antibiotic use for livestock is projected to increase 67% from 2010 to 2030 as the demand for meat increases (Van Boeckel et al., 2015). When livestock are treated, antibiotic compounds, antibiotic resistance genes (ARGs), and resistant microorganisms can enter soils via land application of manure or direct excretion from grazing animals (Tasho and Cho, 2016). Anthropogenic inputs of antibiotics and ARGs are considered environmental contaminants that can contribute to the ongoing antimicrobial resistance (AMR) crisis (Pruden et al., 2006; Sanderson et al., 2016). Long term manure additions can increase detectable concentrations of both antibiotic compounds and ARGs in soil, including tetracycline (Fang et al., 2015; Tang et al., 2015) and cephalosporin (Wepking et al., 2017). In fact, a recent examination of archived soils from 1894-2010 suggests that the impacts of manure on soil AMR began after the 1940's, when widespread antibiotic usage began (Graham et al., 2016).

Beyond the risk of AMR transmission, manure inputs from cattle administered antibiotics can also alter the structure and function of soil microbial communities broadly. Exposure to antibiotics can increase microbial diversity (Grenni et al., 2018; Reichel et al., 2014), as well as alter overall community structure (Lin et al., 2016; Wepking et al., 2017). Additionally, antibiotics can impact the function of microbial communities, including impacts on nitrogen cycling in terms of changes in pools of N or in genes associated with N cycling (Ahmad et al., 2014; Grenni et al., 2018; Radl et al., 2015; Wepking et al., 2019) and respiration and carbon cycling (Hammer et al., 2016; Wepking et al., 2019, 2017).

In light of concerns about environmental transmission of AMR, there are several factors that complicate our understanding of interactions among antibiotics, soil microbial communities, and AMR. Firstly, antibiotics are a well-known natural mechanism for competition among microorganisms (Waksman and Woodruff, 1940), and both antibiotic compounds and ARGs are naturally present in soils. Likewise, resistant bacteria and ARGs can be present even in manure from animals without a history of antibiotic exposure (Heuer et al., 2011). Nutrient availability can have a strong impact on ARG distributions in soil (Fierer et al., 2012; Zhao et al., 2017), and abundances of antibiotic resistant bacteria and ARGs can increase in soil after application of manure from antibiotic-free cattle (Udikovic-Kolic et al., 2014). Furthermore, multiple ARGs often exist on one plasmid (Herrick et al., 2014) and soil bacterial isolates have been found to be resistant to multiple antibiotics (Hu et al., 2017). Thus, multiple classes of ARGs can be co-selected following exposure to only one drug (Pal et al., 2015), complicating the factors that control AMR across the landscape. Given that previous studies sometimes add antibiotics directly to soil (Čermák et al., 2008; Lin et al., 2016) or artificially spike antibiotic compounds into manure (Blau et al., 2018; Jechalke et al., 2016; Zhang et al., 2017), rather than administering the antibiotic to the animal, such approaches may miss important interactions that occur in real-world scenarios.

A second challenge in understanding the role of agricultural soils in AMR transmission is that most studies focus on relatively short-term impacts, while only a few have spanned multiple years. For example, Zhang et al. (2017) found an initial increase in ARGs in soil one day after exposure to manure spiked with antibiotics, but the abundance of ARGs decreased over the next 130 days. Lin et al. (2016) found that the impacts of different antibiotics occurred at different

times following exposure, but observations were limited to 20 days. However, it is not known if these impacts will persist in soil over long-term, repeated exposure. While a few studies have been conducted in fields that had manure applied over long periods, the antibiotics in the manure applied to the sites were not tightly controlled (Tang et al., 2015; Wepking et al., 2017). As a result, while previous work has been highly valuable in showing the potential for manure additions to impact AMR, it remains difficult to disentangle which factors related to soil, manure, or antibiotic exposure can be critical management controls or how such controls should be used within the context of temporal variability.

To address these knowledge gaps, we analyzed bacterial and fungal community structure and abundances of multiple ARGs during a three-year grassland manure-addition experiment. The work is an extension of early results from these same experiments previously reported by Wepking et al. (2019). The goal for this component of the study was to describe multi-year impacts to soil microbial communities of repeated and controlled additions of manure alone and manure from animals administered different antibiotics. The specific objectives of this work were to 1) identify long-term impacts of repeated manure additions from cattle under different antibiotic treatments on soil microbial community structure and ARG abundances and 2) identify potential co-occurrences among ARGs, as previous studies have suggested (Hammer et al., 2016; Herrick et al., 2014).

## 2.3 Methods

### *2.3.1 Study Design*

The samples and data collected for this study were from long-term experimental manure additions to grassland plots conducted between 2014-17 at the Virginia Tech Kentland Research Farm in Blacksburg, VA (N37.199490, W-80.584659). The plots were situated on grasslands previously managed by occasional mowing. The location was specifically selected to have no known history of grazing or application of manure or other inputs. The soils in experimental plots were composed of Unison and Braddock cobbly soil series. Experimental details, including manure collection methods, and soil nutrient data from the first year of the experiment, were first described by Wepking et al. (2019). For this study, which aims to detail long-term changes in soil microbiota and ARG abundances, monthly manure amendments were continued for a full three-year period. The experimental site was divided into six replicate blocks with four treatment plots in each block. The four treatments included a control treatment with no manure (NM), a control treatment with manure from cattle administered no antibiotics (CON), manure from cattle administered cephapirin benzathine (CEPH), and manure from cattle administered pirlimycin hydrochloride (PIR). Both cephapirin benzathine and pirlimycin hydrochloride were chosen because they are commonly used to treat mastitis in dairy cattle. However, they differ in mode of action; cephapirin is a first-generation cephalosporin that causes cell lysis, and pirlimycin hydrochloride is a lincosamide that inhibits protein synthesis.

Manure for spreading on the treatment plots was collected once per year from cattle two to three days after administration of antibiotics, homogenized, and preserved at -20º C until use.

Frozen manure was then thawed as needed immediately prior to monthly application (see Wepking et al., 2019 for details). The concentrations of antibiotics, C, and N in manure from the first year is reported in Wepking et al. (2019). Briefly, cephapirin was below detection (<0.36 ng g manure$^{-1}$) in manure collected from cattle administered cephapirin. Pirlimycin was present at 149 ± 3.4 ng g manure$^{-1}$ in manure collected from cattle administered pirlimycin. The carbon content in CEPH manure was slightly lower (49.0%) than CON (49.9%) and PIR (49.6%), while the nitrogen content was not statistically different among CON, CEPH, and PIR manures (3.3%, 3.4%, and 3.5%, respectively). Collecting manure from cattle after administration of antibiotics, rather than spiking manure with a known concentration of antibiotic was chosen to mimic realistic inputs that also include resistant bacteria and ARGs that may be in manure as a result of antibiotic treatment. While the concentrations of antibiotics in manure could not be controlled through this approach, as there is variability in the amount excreted by animals, the doses of antibiotics the cattle received were controlled. Thus, although there were low and variable concentrations of antibiotics in the applied manure, the experimental design better captures the real-world effects that active antibiotics, degraded metabolites, resistant bacteria, and ARGS can have on soil microbial communities.

Manure was applied to the treatments monthly at 648 g/m$^2$ for three years, beginning in Nov. 2014. To identify long-term changes in soil microbial communities, soil samples from each plot were first collected eight months after the first manure application (May 2015) and then one and two years later in May (2016-17), for a total of 72 samples. Soil samples were collected to a depth of 10 cm in a 0.05 m$^2$ monolith cut from each plot. After plant roots were removed, the soil

was sieved through 4.75-mm mesh and homogenized. A soil subsample (~2-3 g) was collected into a Whirl-Pack bag and stored at -80ºC for microbial analysis.

*2.3.2 Microbial Community Structure.*

The structure of soil microbial communities were analyzed via marker gene amplicon sequencing. Total DNA was extracted from soil samples using Qiagen PowerSoil DNA extraction kits (Qiagen, Hilden, Germany). The V4 region of the 16S rRNA gene and the internal transcribed spacer region between the 18S and the 5.8S rRNA gene (ITS1) were amplified to characterize the structure of bacterial/archaeal and fungal components of the community, respectively, following Earth Microbiome protocols (Caporaso et al., 2012). Each sample was amplified in triplicate via polymerase chain reaction (PCR) on conventional thermal cyclers (Bio-Rad, Hercules, CA, U.S.A.). Amplification primers were 515F (5'-GGA CTA CNV GGG TWT CTA AT-3') (Parada et al., 2016) and 806RB (5'-GTG YCA GCM GCC GCG GTA A-3') (Apprill et al., 2015) for bacteria and ITS1-F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') (Gardes and Bruns, 1993) and ITS2 (5'-GCT GCG TTC TTC ATC GAT GC-3') (White et al., 1990). After amplification, PCR triplicates were pooled and visualized on agarose gels with negative controls for each sample to verify no contamination was present. PCR products were purified with QIAquick PCR purification kits. Amplicons were sequenced on the Illumina MiSeq platform with 300-bp single reads at the Biocomplexity Institute at Virginia Tech. Raw sequence reads are archived under BioProject ID PRJNA632833.

Raw sequence reads were processed with USEARCH (Edgar, 2010) and QIIME version 1.8 (Caporaso et al., 2010). Sequences less than 240 bp or greater than 1 expected error were

discarded. Sequences passing quality filtering were then clustered into operational taxonomic units (OTUs) at 97% similarity. Chimeric sequences were identified and removed using UCHIME (Edgar et al., 2011). Taxonomy was assigned using the RDP classifier (Wang et al., 2007) with GreenGenes 13.8 (DeSantis et al., 2006) and UNITE 6.97 (Kõljalg et al., 2013) reference databases for bacteria and fungi, respectively.

Quantitative PCR (qPCR) was used to obtain absolute abundances of total bacteria in each sample using the same DNA extracts as described above. Total abundances of bacteria were quantified using a standard curve of plasmids containing a known copy number as described previously (Fierer et al., 2005). Bacterial primers for qPCR were EUB518 (5'-ATT ACC GCG GCT GCT GG-3') and EUB338 (5'-ACT CCT ACG GGA GGC AGC AG-3'). Samples were amplified in triplicate, and the mean value for each sample was used for statistical analysis. To ensure data quality, values were only accepted when the standard curve from the same plate had an $R^2 > 0.95$, and an efficiency of 0.9 - 1.1.

*2.3.3 Antibiotic Resistance Gene Abundances*

To quantify absolute abundances of ARGs, we used microfluidic qPCR (mfqPCR) to simultaneously quantify the abundances of 47 different genes in each sample. The gene targets are summarized in Table S1, with details and protocols outlined in Ahmed et al. (2018) and Sandberg et al. (2018). The targeted genes include tetracycline, sulfonamide, β-lactamase, and lincosamide resistance genes, along with metal resistance and integrase genes. By using this broad panel of genes, we could identify patterns among genes related to the antibiotics applied in this study, but also to other ARGs that may be present in the soil or manure. Furthermore, we

could better examine co-occurrences among multiple genes related to AMR to address our third objective.

*2.3.4 Statistical Analysis*

To identify significant changes in bacterial and fungal communities, the unrarefied matrices of OTU relative abundances by sample were analyzed in R (version 3.5.1; R Core Team, 2018) using *vegan* (version 2.5-6; Oksanen et al., 2019), *phyloseq* (version 3.9; McMurdie and Holmes, 2013) and *DESeq2* (version 3.9; Love et al., 2014). Variance stabilizing transformations ("varianceStabilizingTransformation" in the package *DESeq2*) were used to achieve homoscedastic variance among samples before further analysis (McMurdie and Holmes, 2014). Patterns in microbial communities among treatments and years were visualized with Principle Coordinates Analysis (PCoA) of Bray-Curtis distance matrices. Differences in overall microbial community structure were tested using PERMANOVA with the function "adonis2" in the package *vegan*. The main effect was treatment with year as an uncontrolled covariate. Post-hoc pairwise comparisons were analyzed with "pairwise.adonis" in the package *pairwiseAdonis* (version 0.01; Arbizu, 2017). Taxa that were differentially abundant, or responsive, to treatment or year were determined using the function "DESeq" in the package *DESeq2*. Taxa were deemed responsive if the *P*-value was $< 0.05$ and the $\log_2$ fold change was greater than 1.

To compare ARG abundances across samples, PCoA on Bray-Curtis distance matrices were used to visualize patterns in ARG profiles. Differences among profiles (i.e., abundances of all measured ARGs considered together) were analyzed similarly to methods described above using "adonis2" and "pairwise.adonis". Differences in abundances of individual genes among

treatments and years were analyzed with Mann-Whitney U tests with false discovery rate (FDR)-adjusted p-values to correct for multiple comparisons. Jaccard's similarity matrix was used to test for co-occurrence of ARGs. Only genes that were detected in at least 20% of all samples were examined for correlations. For all analyses, $p < 0.05$ was considered significant; $p < 0.1$ was considered marginally significant for some important results.

**2.4. Results**

The overarching goal of this study was to determine how multiple aspects of the soil microbial community responded to manure from cattle under different antibiotic regimes over a three-year period. Thus, for the purpose of analysis, the main effect was manure (NM, CON, CEPH, and PIR), with year as an uncontrolled covariate. Primary response variables included bacterial community structure, fungal community structure, and ARG abundance profiles. Interactions between time and year were included in additional statistical models for bacterial and fungal community structures as well as ARGs. However, none of the interactions were significant ($p > 0.05$).

*2.4.1 Impacts of manure and antibiotics on microbial community structure*

Both bacterial ($F_{3,68} = 1.81$, $P = 0.001$; Figure 2.1A) and fungal ($F_{3,71} = 2.90$, $P = 0.001$; Figure 2.1B) community structures were impacted by manure treatments across all three years. The addition of manure from antibiotic-free cattle (CON) resulted in significantly altered community structure for both bacteria ($F_{1,44} = 1.58$, $P = 0.02$) and fungi ($F_{1,44} = 2.87$, $P = 0.01$) compared to no manure controls (NM). Sixteen bacterial OTUs were differentially abundant between the NM and CON treatments (Figure 2.1C). Apart from one OTU in the phylum

Latescibacteria (identified as WS3 in GreenGenes, but since renamed; Lin and Pan, 2015) that were relatively more abundant in CON, all other responsive OTUs decreased in the CON samples. Most responsive OTUs belonged to Proteobacteria or Bacteroidetes. Those that could be identified to genus included *Flavobacterium* in the phylum Bacteroidetes, *Cellvibrio*, *Devosia*, and *Methylotenera* in the phylum Proteobacteria, and *Luteolibacter* in the phylum Verrucomicrobia. For fungi, there were 44 responsive OTUs between NM and CON, mostly from Ascomycota (Figure 2.1D). Among those that could be identified at the genus level, *Preussia*, *Phialophora*, *Scedosporium*, and *Chaetomium*, in the phylum Ascomycota, and *Agrocybe* and *Panaeolus* in the phylum Basidiomycota were all relatively more abundant in CON. In contrast, *Paraglomus* in the phylum Glomeromycota and *Psilocybe* in the phylum Basidiomycota were relatively more abundant in NM.

Manure from cattle treated with antibiotics also caused significant shifts in bacterial community structure compared to manure from antibiotic-free cattle across all three years. Specifically, bacterial community structure in both the CEPH and PIR treatments were different from those in CON ($F_{1,44}$ = 2.29, $P$ = 0.003 and $F_{1,44}$ = 3.05, $P$ = 0.003 for CEPH and PIR, respectively). Importantly, the soil bacterial communities of CEPH and PIR shifted in the opposite direction from NM treatments in ordination space compared to those in the CON treatment (Figure 2.1A). Similar to changes seen between NM and CON, most responsive OTUs that were differentially abundant between CON and CEPH and PIR belonged to the phyla Proteobacteria or Bacteroidetes. Additionally, most responsive OTUs among the treatments were increased in the antibiotic treatments compared to CON, although there were a few exceptions (Figure 2.1C). For example, *Nitrospira* was relatively more abundant in CON than PIR.

Although CEPH and PIR did not have significantly different bacterial communities overall ($F_{1,44}$ = 1.31, $P$ = 0.08), individual OTU differences from CON were not the same in each of these treatments. More bacterial OTUs were different between PIR and CON (100) than CEPH and CON (39). Of the 39 OTUs that were different between CEPH and CON, 23 were also different between PIR and CON.

In addition to the main treatment effects, bacterial community structure also varied among years ($F_{2,68}$ = 2.20, $P$ = 0.001), with different communities in each year (all $P$ <0.05; Figure 2.1A). Year explained a similar amount of variance as treatment ($R^2$ = 0.060 and 0.075, respectively), but there were fewer responsive bacterial OTUs among years compared to manure treatments (Appx A, Figure A.1). Most responsive bacterial OTUs belonged to Proteobacteria, and most differential abundance changes were relatively small.

In contrast to bacteria, there were no differences across all three years in fungal community structure among treatments using control manure or manure from cattle administered antibiotics. Although CEPH and PIR had different fungal communities compared to NM ($P$ < 0.05), CEPH and PIR fungal communities were not different from CON communities ($P$ = 0.35 and 0.44 for CEPH and PIR, respectively), indicating that changes were primarily a result of manure additions, regardless of antibiotics. Fungal communities also varied among years ($F_{3,71}$ = 15.76, $P$ = 0.001) and, again, all years were different from one another (all $P$ <0.05; Figure 2.1B). In contrast to bacteria, year explained a much larger portion of variance than treatment ($R^2$ = 0.297 and 0.082, respectively). Most of the responsive fungal OTUs differed in 2015 and years primarily belonged to the phyla Ascomycota and Basidiomycota (Appx A, Figure A.2).

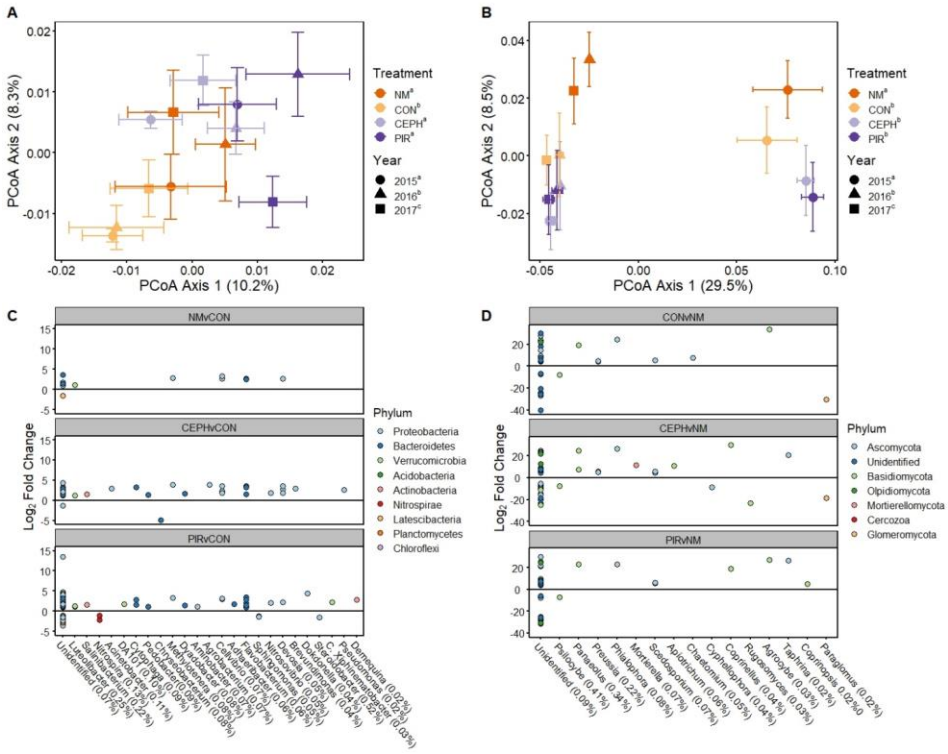


**Figure 2.1.** Upper plots are principal coordinate analyses visualizing similarities among treatments and years of soil bacterial/16S (A) and fungal/ITS (B) communities. Points represent centroids for each treatment and year combination. Error bars represent standard error. Superscript letters in the legend indicate significant differences based on PERMANOVA among treatments and years. Lower plots represent fold changes between treatment pairs that significantly varied between treatment pairs for bacteria (C) and fungi (D). Percent values in brackets show the mean relative abundance of each genus across all samples. Each point is one OTU, colored by phylum and grouped by genus.

*2.4.2 Impacts of manure treatments on ARGs*

Of the 47 ARGs in the mfqPCR array, 33 were detected in at least one sample, and 18 were detected in at least 20% of all samples. The most ubiquitous genes were *intI1*, $bla_{SHV}$,

*tet*(W), and *floR*, found in 97%, 96%, 81%, and 80% of samples, respectively. The ARGs with the greatest abundance across all samples were $bla_{SHV}$, *intI1*, *floR*, and *mexB*, with 7.6±1.7, 6.2±1.2, 4.4±2.4, and 4.3±3.3 log copies/g soil, respectively (mean ± standard deviation). Because the use of mfq-PCR provided data on many ARGs, we used two approaches for evaluating changes in ARG abundances. Firstly, we compared ARG profiles (*i.e.*, relative abundance changes among all ARGs present in each sample) among treatments and years using PCoA and PERMANOVA. However, a lack of change in overall profiles of ARG abundances using this approach does not mean that no biologically important changes in individual ARGs occurred. Thus, we then followed up this first approach by determining changes in abundances of all ARGs individually using Mann-Whitney U tests.

When considering absolute ARG abundances (copies $g^{-1}$ soil), overall profiles of ARG abundance varied among treatments ($R^2 = 0.53$, $F_{3,71} = 26.6$, $P = 0.001$, Figure 2.2A), but differences among years were only marginally significant ($R^2 = 0.02$, $F_{2,71} = 2.80$, $P = 0.069$, Figure 2.2A). Among treatments, the only pairwise significant differences were between NM and the other three treatments (CON, CEPH, and PIR; all $P < 0.05$). Generally, individual ARGs were more abundant in plots with any manure applied, regardless of antibiotic exposure (Figure 2.2B). Raw mean ARG abundances (*i.e.*, not corrected for fold change) and *P*-values from Mann-Whitney U tests are shown in Appx A, Table A.2. Eleven genes – including integrase genes and tetracycline, sulfonamide, and aminoglycoside resistance genes – had greater abundances in all three manure treatments (CON, CEPH, and PIR) compared to NM (all $P < 0.05$) based on Mann-Whitney U tests. Additionally, the tetracycline resistance gene *tet*(A) had greater abundance in CON than NM, *aadD* and *tet*(L) had greater abundance in both CEPH and

PIR compared to NM, and PIR had a greater abundance of *merA*, but lower abundance of $bla_{SHV}$ compared to NM. The lack of significant change in total ARG profile does not preclude biologically important changes in individual genes. However, when individual genes were analyzed among CON, CEPH, and PIR, the only additional significant difference detected was a higher abundance of *tet*(L) in PIR compared to CON.

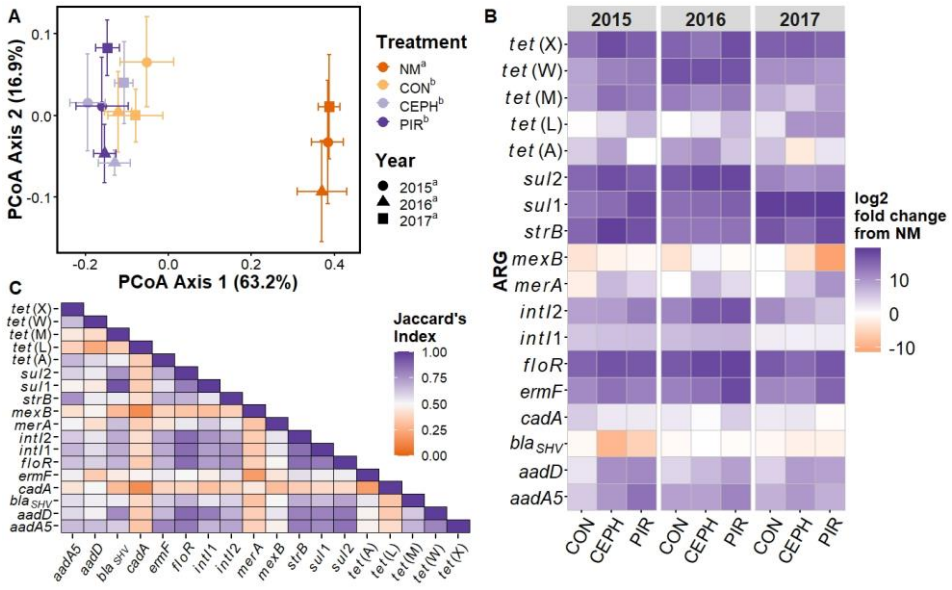


**Figure 2.2.** Principle coordinate analysis visualizing similarities among treatments and years in ARGs quantified via mfqPCR (A). Points represent centroids for each treatment and year combination. Error bars represent standard error. Superscript letters in the legend indicate significant differences based on PERMANOVA among treatments and years. $Log_2$ fold change in absolute abundance (copies $g^{-1}$ soil) of CON, CEPH, and PIR to NM in genes that were observed in >20% of all samples in each treatment and year (B), and co-occurrence of genes with Jaccard's Index across all samples (C). P-values from all pairwise comparisons by treatment are shown in Table S2.

Total 16S rRNA gene abundances did not differ among years ($F_{2,71}$ = 1.68, $P$ = 0.19) or treatments ($F_{3,71}$ = 0.65, $P$ = 0.58. However, when we normalized ARG abundances to 16S rRNA gene abundances, there were some changes in the importance of factors affecting ARGs. The effect of manure treatments on normalized ARG profiles was marginally significant ($R^2$ = 0.07, $F_{3,71}$ = 1.83, $P$ = 0.078; Figure 2.3), but no pairwise comparisons of treatments yielded FDR-adjusted $P$-values < 0.05. Conversely, year explained a greater portion of the variance in normalized ARG profiles ($R^2$ = 0.10, $F_{2,71}$ = 8.44, $P$ = 0.001, Figure 2.3), where ARG profiles each year were statistically different ($P$ <0.05). When differences in individual genes were analyzed among treatments, the results were nearly the same as absolute ARG abundances, with the exception that *bla*$_{SHV}$ was not significantly different between PIR and NM, and *tet*(L) was not significantly different between PIR and CON. (Figure 2.3B; Appx A, Table A.3).

Finally, because microfluidic qPCR estimates abundances of several genes simultaneously, we were also able to examine co-occurrence among ARGs. We quantified co-occurrence of all possible ARG pairs based on the presence or absence of genes across all samples, using Jaccard's Index. Several genes, including *strB*, *sul1*, *sul2*, *florR*, and *tet*(W) tended to occur in the same samples (Jaccard's Index ≥ 0.80, Figure 2.2C). Additionally, *intI1* tended to co-occur with *floR* and *tet*(W). Meanwhile, several genes, including *cadA*, *merA*, *tet*(A), and *tet*(L), had consistently low pairwise Jaccard values, suggesting they do not tend to co-occur with other ARGs in soil.

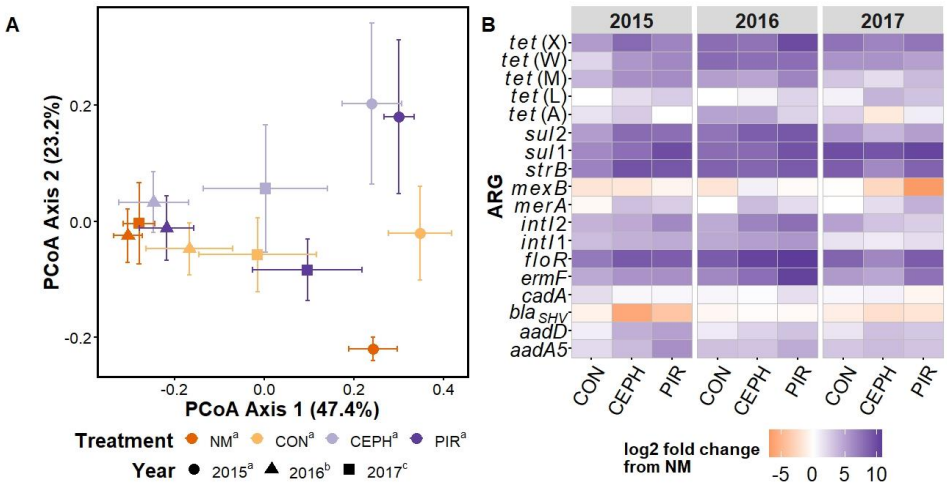


**Figure 2.3.** Principle coordinate analysis visualizing similarities among treatments and years in overall ARG profiles quantified via mfqPCR, normalized to 16S (bacterial) abundance (A). Points represent centroids for each treatment and year combination. Error bars represent standard error. Superscript letters in the legend indicate significant differences based on PERMANOVA among years and treatment. $Log_2$ fold change of ARG copies per $1x10^7$ 16S copies of CON, CEPH, and PIR to NM in genes that were observed in >20% of all samples in each treatment and year (B). P-values from all pairwise comparisons by treatment are shown in Appx A, Table A.3.

## 2.5 Discussion

Two key aspects of this study were 1) the controlled, continuous addition of manure from cattle under multiple antibiotic regimes for three years, and 2) the amount of data simultaneously collected describing different components of the soil microbiome, including the structure of bacterial and fungal communities and numerous ARG abundances. As a result of this design, we were able to control for multiple critical components of the AMR puzzle, including the effects of applying manure from cattle with no antibiotics administered, the effects of applying manure

from cattle administered different antibiotic types, and the temporal variability that occurs in key response variables over multiple years.

### *2.5.1 Impacts of manure and antibiotics on microbial community structure*

A first key outcome of this work is that relative impacts of manure from cattle under different antibiotic regimes are important in structuring the soil microbiome, but the responses vary among components of the microbial community. Similar to previous studies, manure additions altered bacterial community structure (Blau et al., 2018; Han et al., 2018; Udikovic-Kolic et al., 2014). Nutrient and organic matter inputs were likely an important factor in manure effects on both soil bacteria and fungi. For example, bacterial OTUs that increased in CON plots were mostly in Proteobacteria and Bacteroidetes, which are typically considered copiotrophic (Fierer et al., 2007; Sauvadet et al., 2019). Likewise, soil fungal community composition is known to be sensitive to C:N ratios (Lauber et al., 2008) and organic inputs (Sauvadet et al., 2019). Fungal genera that increased in manure treatments included *Mortierella* and *Taphrinia*, which are also copiotrophic (Sauvadet et al., 2019), and *Panaeolus* and *Preussia*, which are commonly associated with manure (Doveri, 2010; Sarrocco, 2016; Wepking et al., 2017).

In contrast to manure from antibiotic-free cattle, manure from cattle under two specific antibiotic treatments (CEPH and PIR) shifted soil bacterial communities in the opposite direction from NM than did manure from cattle without antibiotics (Figure 2.1A). This suggests that the effect of antibiotic-free manure and manure from cattle administered antibiotics on bacterial community structure are distinctly different. Previous studies have also found shifts in microbial community composition between soils amended with manure or manure and antibiotics (Blau et

al., 2018; Pankow, 2017). Among responsive taxa, two bacterial genera that had higher relative abundances in both antibiotic treatments matched those previously shown to have increased relative abundance following antibiotic exposure, including *Leuteolibacter* (Wepking et al., 2019) and *Devosia* (Hassan et al., 2015). Other genera that increased in both antibiotic treatments have been shown to be resistant to other various chemical disturbances, including *Salinibacterium* (Igun et al., 2019; Otlewska et al., 2017) and *Cellvibrio* (Filimon et al., 2014). Additionally, we identified several bacterial genera that were relatively more abundant in PIR compared to CON, including uncultured *Verrucomicrobia* Da101, *Adhaeribacter*, *Dokdonella*, *Candidatus Xiphinematobacter*, and *Demequina*, which have not been previously documented to respond to antibiotic exposure.

Underlying manure and antibiotic treatment effects, it is important to note that we observed significant temporal variation in both bacterial and fungal community structure from year to year across all treatments. Although this study was not designed to identify environmental drivers of temporal variability in microbial communities, weather conditions are one obvious possibility. The local weather station recorded lower total rainfall in the week prior to sampling in 2015 (3 mm) than in 2016 (24 mm) and 2017 (34 mm), coinciding with a large shift in fungal community structure in 2015, and soil moisture is known to impact soil microbial community structure (Kaisermann et al., 2015; Lupatini et al., 2019).

*2.5.2 Impacts of manure and antibiotics on ARGs*

Another important finding of this work is that the overall ARG profiles observed in soil differed primarily with the application of manure, regardless of whether cattle were administered

antibiotics. This was unexpected, given that previous work has reported higher ARG abundances in soils amended with manure and antibiotics, compared to manure without antibiotics (Zhang et al., 2017). However, increased ARG abundances have also been observed in manure from animals not treated with antibiotics (Heuer et al., 2011; Heuer and Smalla, 2007; Udikovic-Kolic et al., 2014). It is also important to note that this result may be specific to our study conditions and additional experimental investigation is required to determine if this is a common effect. If manure from antibiotic-free cattle consistently has large impacts on ARG abundances, this is a critical point to consider in managing AMR. It is also important to note that although ARG abundances were typically lower in the no-manure control soils, there were still detectable quantities of ARGs. Antibiotics and resistance genes are commonly found naturally in soils (Fletcher, 2015; Nesme and Simonet, 2015), thus it is important to remember that we cannot expect ARGs to be absent from any given soil, even in undisturbed ecosystems.

The use of mfqPCR allowed simultaneous examination of a wide range of ARGs and many genes varied in ways that were unexpected based on the antibiotic treatments applied. Of the 15 genes that increased in abundance in at least one of the manure treatments compared to NM, only two (*bla*$_{SHV}$ and *ermF*) are known to confer resistance to the antibiotics given to cattle in our study. Furthermore, *ermF* confers resistance to lincosamides such as pirlimycin, but was actually more abundant in all three manure treatments compared to NM. The response of *bla*$_{SHV}$, which confers resistance to β-lactams such as cephapirin, was also counterintuitive; it was more abundant in NM compared to PIR, but the abundance was not different between NM and CEPH. One possible explanation for increases in unexpected classes of ARGs is co-selection, which can occur when multiple ARGs are found on a single plasmid (Herrick et al., 2014). In such a case,

exposure to one antibiotic can increase resistance to multiple classes of antibiotics (Hammer et al., 2016; Herrick et al., 2014). This would also help explain our observation that several genes tended to occur together. For example, the Jaccard similarity values from our data suggested groupings of *strB*, *sul1*, *sul2*, and *tet*(W), as well as *intI1* with *floR* and *tet*(W). Some of these relationships agree with previously published evidence for co-occurrence of *sul1*, *sul2*, and *intI1* (Pal et al, 2015).

While absolute abundance of ARGs in soil can be indicators of the overall amount of AMR, such changes can be difficult to interpret. ARG abundances can be problematic because they can change due to an increase in total bacteria or because of selection for individual bacteria that are more likely to carry one or more copies of a particular ARG (Udikovic-Kolic et al., 2014). In the latter case, analyzing ARG abundance normalized to total 16S rRNA gene abundance can be a better indicator of the relative number of bacteria that may carry a resistance gene. For example, we found that treatment explained a large portion of the variance in ARG profiles $g^{-1}$ soil but explained little variance for overall ARG profiles normalized to 16S rRNA gene abundance. Thus, the patterns among ARG profiles appear to be related to changes in overall bacterial abundance, but this was further complicated in our study as we did not detect significant changes in 16S rRNA gene abundances among treatments.

Despite no significant differences in ARG profiles normalized to 16S rRNA abundance among treatments, examining the normalized abundance changes in individual ARGs showed many significant results. In fact, most of the pairwise comparisons that were statistically significant when comparing absolute ARG abundances were also significant when comparing normalized ARG abundances. When coupled with the fact that 16S copy numbers were not

significantly different among treatments, these results suggest that the relative increases in ARG abundances with manure application were the result of selection for AMR and not simply due to increased bacterial abundance. Regardless, increased bacterial numbers and selection for more resistant bacteria can both clearly be important pieces of ARG abundance and the AMR puzzle. Additional research establishing whether human health or transmission risks are more clearly associated with one change or the other will be necessary to determine how best to monitor and manage AMR in soil systems.

### 2.6. Conclusions

Adapting soil and manure management practices to address the AMR crisis will require a nuanced understanding and approach. Responses of the soil microbiome to manure from cattle treated with antibiotics clearly vary by microbial taxa and over time. We found marked differences in the responses of fungal and bacterial communities in soil following application of manure from under different antibiotic regimes. Specifically, manure from cattle treated with antibiotics affected bacterial community structure compared to both manure from antibiotic-free cattle and no manure additions. In contrast, fungal community structure was affected only by addition of any manure, regardless of whether cattle were given antibiotics. Critically, we also found that manure inputs from antibiotic-free cattle increased ARG abundances compared to soil without manure amendments, yet there were almost no differences between manure inputs from cattle that received antibiotics and those that did not. And finally, we observed that these changes are detectable over multiple years with repeated manure additions, despite variation in microbial community structure over time. Clearly, a better understanding is required of how factors other

than antibiotic usage alone influence soil AMR over longer time scales. In particular, future work should 1) further examine the potential influence of microbial-C substrate availability, nutrient status, and environmental factors on microbial community responses to antibiotics and manure, and 2) allow for more rigorous examination of possible interactions among multiple controlling factors.

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**Chapter 3. Soil type and moisture content alter soil microbial responses to antibiotic and manure exposure**

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### 3.1 Abstract

Manure from livestock treated with antibiotics may spread antibiotic compounds, resistant bacteria, and antibiotic resistance genes to soil. With growing concerns about the global antimicrobial resistance crisis, it is critical to understand the factors that affect persistence of antibiotic resistance in soil, and how soil microbial communities are influenced by antibiotic exposure. In agroecosystems, however, these responses are complex because environmental factors may influence how soil microbial communities respond to manure and antibiotic exposure. The objective of this study was to determine how experimentally controlled changes in soil moisture and type alter responses of microbial communities to additions of manure from cattle treated with antibiotics. We conducted a soil microcosm experiment with 2 soil textures – sandy loam (SL) and sandy clay loam (SCL) – at 15, 30, or 45% gravimetric water content. Microcosms received biweekly additions for 10 weeks of manure from cattle given either cephapirin or pirlimycin, manure from cattle not given antibiotics, or no manure.

Although soil type and moisture had the largest effects on soil microbial communities, detectable impacts on microbial community structure and activity were observed by manure treatment from cattle treated with antibiotics across a wide range of environmental conditions. For example, manure from cattle treated with cephapirin increased respiration, while pirlimycin decreased respiration compared to the control manure. And while manure treated with antibiotics had increased ammonium and decreased nitrate availability in some scenarios, dissolved nitrogen pools in soil, the effect was heavily influenced by soil type and moisture. In contrast, there was no effect of antibiotic presence in manure on overall profiles of concentrations of antibiotic resistance genes in soil. Overall, this work demonstrates that exposure of manure from cattle

treated with antibiotics can alter microbial community structure, soil respiration, and nitrogen concentrations across a range of soil environmental conditions. However, environmental conditions can also alter how manure and antibiotics impact microbial community structure and nutrient cycling. These results suggest a nuanced approach that considers individual environments, rather than a one-size-fits-all approach, is required when managing antibiotic resistance from manure applications.

### 3.2 Introduction

Antimicrobial resistance (AMR) is a growing concern for both human and animal health (Nesme and Simonet, 2015; Sanderson et al., 2016). Currently, 79% of antibiotics are used in livestock production (Hollis and Ahmed, 2013) and manure from treated cattle may contain antibiotic residues, antibiotic resistance genes (ARGs) and viable antibiotic-resistant bacteria (ARB) (Unc and Goss, 2003; Chee-Sanford et al., 2009; Gothwal and Shashidhar, 2015; Aslam et al., 2018). ARGs in cattle manure can be carried by diverse microbial taxa and many are often flanked by mobile genetic elements, suggesting horizontal gene transfer is taking place (Wichmann et al., 2014). Although resistant organisms and ARGs can be present in animals without a history of antibiotic exposure (Heuer et al., 2011), exposure to antibiotics can further increase ARG concentrations in animals. For example, Chambers et al. (2015) found that cows treated with a β-lactam antibiotics have increased β-lactam resistance genes, and other authors reported that ARG abundance in manure was correlated with antibiotic abundance as well as heavy metal abundance (Ji et al., 2012).

Antibiotic compounds, ARB, and ARGs are naturally found in soils, and soil ecosystems can serve as a reservoir and possible vector for transmission of AMR (Fletcher, 2015; Nesme and Simonet, 2015). Application of manure from livestock, whether or not the animal has been administered antibiotics, can result in resistance genes and resistant organisms entering soils (Udikovic-Kolic et al., 2014; Tasho and Cho, 2016). Long term additions of manure can lead to an increase of ARGs in soil (Fang et al., 2015; Graham et al., 2016; Wepking et al., 2017). ARG abundance generally decreases over time after manure and antibiotic additions (Zhang et al.,

2017), but persistence of antibiotics, and thus selective pressure for ARGs, in soil depends on environmental factors including soil texture, light, temperature, and pH (Tasho and Cho, 2016).

In addition to concern about soil acting as a reservoir of AMR persistence, antibiotics can also alter soil microbial communities. Antibiotic additions can decrease microbial abundance, the ratio of bacteria to fungi (Reichel et al., 2014; Lin et al., 2017; Grenni et al., 2018), and reduce overall microbial diversity (Lin et al., 2017; Grenni et al., 2018). Differential effects on survival and abundance of microbial taxa following exposure to antibiotics can also alter the structure of the soil microbiome (Wepking et al., 2017, 2019; Shawver et al., 2021). However, the length and severity of the change can be dependent on the type of antibiotic (Lin et al., 2016). So, while additions of manure from cattle treated with antibiotics can have a variety of effects on soil microbiota, the interacting dynamics among manure addition, antibiotic usage, and environmental change are still poorly understood.

When considering soil as a reservoir for AMR, soils must be considered as heterogeneous ecosystems with many other varying physical, chemical, and biological properties that affect the resident microorganisms. For example, soil texture can influence microbial biomass and community structure (Dequiedt et al., 2009; Chau et al., 2011; Roberts et al., 2011) and even how microbial communities recover after disturbance (Bach et al., 2010). Similarly, soil microbial community structure and diversity are heavily impacted by soil moisture (Lupatini et al., 2019). Different microbial groups respond to soil moisture content in different ways, as fungi have been shown to be more sensitive to small changes in moisture than bacteria (Kaisermann et al., 2015), with greater fungal abundance at higher moisture, with greater bacterial abundance at lower moisture (Borowik and Wyszkowska, 2016). Of course, these factors can also interact. For

example, de Vries et al., (2012) demonstrated that texture, precipitation, nutrients and plants all had interactive impacts on soil fungi:bacteria ratios and microbial community composition, with precipitation having the greatest influence.

Soil nutrients are also major drivers of microbial community change, especially in agricultural settings. Inorganic nitrogen and phosphorous amendments as well as organic carbon from plant residues can all alter microbial community structure (Leff et al., 2015; Chávez-Romero et al., 2016). Carbon cycling process such as microbial respiration are also impacted by soil moisture (Manzoni et al., 2012; Kaisermann et al., 2015) and soil texture (Dijkstra and Cheng, 2007). Likewise, soil properties such as texture, pH, and organic carbon (Šantrůčková et al., 2003; Enwall et al., 2010; Deslippe et al., 2014; Morales et al., 2015) and moisture content (Morugán-Coronado et al., 2019) can impact microbially-driven nitrogen cycling processes. With the complex interactions of microbial functional responses to environmental factors, more information is required to understand how these processes are impacted by AMR.

Given the dominant importance of environmental factors in broadly mediating microbial processes, it is critical to better understand how they specifically mediate interactions among antibiotics, resistant organisms, and microbial communities. Soil moisture can play an important role, as sulfonamide resistance gene abundances have been shown to be negatively correlated with temperature and precipitation (Zhou et al., 2017) and fluctuations in soil moisture in combination with sulfadiazine can differentially affect some microbial functional groups (*e.g.*, nitrifiers but not denitrifiers; Radl et al., 2015). Likewise, soil texture can mediate antibiotic impacts on soil communities by affecting binding and persistence of antibiotic compounds. Soil texture can also influence how long pathogens from manure, which are potentially ARB, survive

in soil, with longer survival in clay soils (Sharma and Reynnells, 2016). However, evidence of the effect of texture is inconsistent, with some studies suggesting that finer textured soils will bind to antibiotics more strongly and have a greater impact on soil microbial communities (Chander et al., 2005), while others suggest that antibiotics in coarse soils will cause greater shifts in microbial community structure and abundance of ARGs (Chen et al., 2017, 2018; Pankow, 2017; Blau et al., 2018). These results may differ because soils usually vary in more than just texture. They may also differ in the mineral composition, pH, organic matter content, and nutrient concentrations, which could all impact results.

A common characteristic of early work studying the influence of antibiotics, manure, and soil properties on soil microbial communities is that only one soil property is studied at a time, such as texture (Chander et al., 2005; Chambers et al., 2015; Sharma and Reynnells, 2016; Pankow, 2017; Blau et al., 2018; Chen et al., 2018), moisture (Reichel et al., 2014; Radl et al., 2015; Jechalke et al., 2016), or temperature (Lin et al., 2017). Thus, little information exists on the interactive effects of soil properties on soil microbial structure, functional response, and ARG abundances impacted by manure additions. The purpose of this study was to (1) examine the impact of controlled changes in soil moisture and type on the responses of ARG abundances and microbial community structure to the addition of manure and manure from cattle treated with antibiotics and (2) identify changes in microbial activity among these same treatments.

## 3.3. Methods

### *3.3.1 Study Design*

This study was conducted as a microcosm experiment with 3 factors: type, moisture, and manure. Soil was collected from Virginia Tech's Eastern Shore Agricultural Research and Extension Center (SL; 37.585602, -75.823327) and the Reynolds Homestead Forestry Research Center (SCL; 36.644596, -80.148816). The SL soil was mapped as Bojac sandy loam, and the SCL soil was mapped as Fairview sandy clay loam (Soil Survey Staff, 2019). It is important to note that while the soils used in the study were chosen for their textures, they had different soil properties (Table 3.1; Appx B, Table B.1) and in this paper, the term "type" refers to the different soils. Soils were air dried, ground, sieved to 2 mm, and thoroughly homogenized. The gravimetric moisture treatments were 15, 30, and 45% wt/wt. These values were chosen to cover a range of dry to moist conditions for both soils. The manure treatments were a control manure from cattle that did not receive antibiotics (CO), manure from cattle administered cephapirin benzanthine, a bactericidal cephalosporin (CE), manure from cattle treated with pirlimycin hydrochloride, a bacteriostatic lincosamide (PI), and a no manure control (NM). Manure was collected as described in Wepking et al. (2019). Five replicate microcosms of each type, moisture, and manure combination were used in this experiment. Subsamples of soil and manure before the start of the experiment were frozen at -80ºC for analysis.

For each mesocosm, 15 g of soil (dry weight) was added to a 50 ml conical centrifuge tube. Autoclaved deionized water was added to each tube to achieve the correct moisture content. Tubes were loosely capped to allow for gas exchange and incubated in the dark at room

temperature (~22ºC). Moisture content was maintained by replacing water based on mass loss twice per week. Soils were incubated for 5 days at the designated moisture content for each treatment prior to starting the experiment to allow initial adjustment of soil microbial communities. Manure was applied at 10g/kg soil every other week for 10 weeks for a total of 5 applications. The application rate was based on average density of stocking dairy cattle as used in previous studies (Wepking et al., 2019; Shawver et al., 2021). Soils were mixed thoroughly after manure applications to ensure homogenization. NM samples were also mixed to avoid differences in aeration affecting results.

**Table 3.1.** Soil data from the 2 soil samples used in the study. Map unit and texture data were obtained from the Soil Survey (Soil Survey Staff, 2019), while organic matter (OM), pH, and cation exchange capacity (CEC) were measured from soil samples.

| Soil | Map unit | Texture | Sand % | Clay % | OM % | pH | CEC (meq $100g^{-1}$) |
|---|---|---|---|---|---|---|---|
| SL | Bojac sandy loam | Sandy loam | 65 | 10 | 0.8 | 5.8 | 3.1 |
| SCL | Fairview sandy clay loam | Sandy clay loam | 58 | 26 | 3.8 | 6.3 | 5.2 |

*3.3.3 Microbial Respiration and Soil Nitrogen*

During the experiment, soil respiration was measured weekly. Microcosm tubes were flushed with $CO_2$-free air, capped tightly, and incubated at room temperature for 24 hr. At the end of the 24 hr incubation, accumulated $CO_2$ in the headspace was measured using a bench-top infrared gas analyzer (IRGA, LI-7000 CO2 H2O Analyzer, Li-Cor, Lincoln, NE). Soil respiration incubations were conducted immediately following manure applications and at the same time the following week to capture both the responses to manure and antibiotic additions and the background respiration throughout the experiment, respectively. At the end of the 10-

week incubation, substrate induced respiration (SIR) was measured as an estimate of active microbial biomass. A 2 g aliquot of soil from each mesocosm was incubated in a 50 ml conical centrifuge tube at 20ºC overnight. Four ml of autolyzed yeast (99 g/ml) was then added to each tube. Tubes were then shaken for 1 hour, tightly capped, flushed with $CO_2$-free air and incubated for 5 hours. After 5 hours, $CO_2$ concentrations were then measured using an IRGA as described above.

Following the 10-week incubation, aliquots of soil were set aside and stored at -80ºC for N extractions. Soils and manure from the start of the experiment were also measured to estimate N at the start of the experiment and quantify how much N was added with manure. Soils were thawed and 2.5 g (dry weight) was placed in 50 ml conical centrifuge tubes along with 25 ml of 2M KCl and shaken for 1 hr. Samples were then centrifuged and the supernatant was decanted and filtered. The filtrate was stored at 4ºC until analyzed (less than 24 hours). The filtrate was analyzed for $NH_4$ and $NO_3$ on a Lachat Instruments QuickChem 8500 auto analyzer (Lachat Instruments, Hatch Company, Loveland, CO) using QuickChem methods 12-107-06-2-A for $NO_3$ and 12-107-04-1-B for $NH_4$ (Hofer, 2003; Knepel, 2003). Percent changes in $NO_3$ and $NH_4$ concentrations were calculated by comparing the amount of $NH_4$ or $NO_3$ in the samples at end of the incubation from the amount that was added to the tubes from the soil and the manure.

*3.3.4 Microbial Analysis*

Aliquots of frozen soil collected at the end of the 10-week experiment were analyzed for microbial community structure and ARG abundances. Total DNA was extracted from soil samples using Qiagen PowerSoil DNA extraction kits (Qiagen, Hilden, Germany.). The V4

region of the 16S rRNA gene and the ITS1 region were amplified in triplicate for bacterial and fungal community composition, respectively, following Earth Microbiome protocols (Caporaso et al., 2012). Bacterial primers were 515F (5'-GGA CTA CNV GGG TWT CTA AT-3') (Parada et al., 2016) and 806RB (5'-GTG YCA GCM GCC GCG GTA A-3') (Apprill et al., 2015). Fungal primers were ITS1-F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') (Gardes and Bruns, 1993) and ITS2 (5'-GCT GCG TTC TTC ATC GAT GC-3') (White et al., 1990). Triplicate amplifications for each sample were pooled and visualized on agarose gel with no template controls for each tagged primer to verify no contamination was present. PCR products were purified with QIAquick PCR purification kits and cleaned amplicons were sequenced on the Illumina MiSeq platform with 300 bp single reads.

Raw sequence reads were processed with QIIME version 2 (Bolyen et al., 2019). Sequences were filtered using DADA2 (Callahan et al., 2016). Conditions for DADA2 included trimming the first 13 bp due to low quality, truncation at 250 bp, and a max expected error of 2.0. Amplicon sequence variants (ASVs) were then identified with a trained naïve Bayes classifier (Pedregosa et al., 2011) and GreenGenes 13.8 (DeSantis et al., 2006) and UNITE 6.97 (Kõljalg et al., 2013) reference databases for bacteria and fungi, respectively.

*3.3.5 Antibiotic Resistance Genes*

Microfluidic quantitative PCR (mfqPCR) was used to quantify the absolute abundances of a wide variety of genes including integrase, metal-resistant, and several classes of antibiotic resistance genes. A full list of genes can be found in Table S1. Protocols were done following the methods in Ahmed et al. (2018) and Sandberg et al. (2018).

*3.3.6 Statistical Analysis*

Differences in respiration, SIR, $NO_3^-$, and $NH_4$ were tested using 3-way ANOVAs with Tukey's HSD tests for pairwise comparisons. The 16S and ITS datasets were analyzed in R (version 3.5.1 (R Core Team, 2018)) using *vegan* (Oksanen et al., 2019) and *phyloseq* (McMurdie and Holmes, 2013). Differences in microbial community structure among treatments, soil type, and moisture were visualized with principal coordinates analysis (PCoA) of Bray-Curtis distance matrices. Differences in overall microbial community structure were tested using PERMANOVA with the function "adonis" in the package *vegan* (Oksanen et al., 2019). Post-hoc pairwise comparisons were made with "pairwise.adonis" in the package *pairwiseAdonis* (Arbizu, 2017). Taxa that were re sponsible for changes in community structure were determined with indicator species analysis, using "multipatt" in the package *indicspecies* (De Cáceres et al., 2020). P-values were adjusted for multiple comparisons using FDR adjusted p-values.

Overall patterns in ARG abundances were also analyzed using PCoA and tested using "adonis" and pairwise.adonis". To determine which ARG abundances changed among samples, nonparametric factorial ANOVAs using the function "art" in the package *ARTool* (Kay et al., 2021) were used with FDR-adjusted p-values for each gene. For genes that were significantly different for one or more of the main effects (manure treatment, soil type, or moisture content), post-hoc analyses were conducted using the function "art.con" in the package ARTool.

### 3.4. Results

*3.4.1 ARGs*

Multivariate profiles of all ARG abundances varied by moisture ($F_{2,71}$= 8.51, P = 0.001) and type ($F_{1,71}$=17.41, P = 0.001), but not among manure treatments ($F_{3,71}$=1.71, P = 0.07; Figure 3.1). The interactions of manure treatments and moisture ($F_{6,71}$=1.67, P = 0.03) and moisture and type ($F_{2,71}$=9.72, P = 0.001) were significant but the interaction of all three main effects was not ($F_{6,71}$=1.38, P = 0.101). Of the main effects and interactions that were significant, the interaction of moisture and type explained the most variation ($R^2$ = 0.15), followed by type ($R^2$ = 0.13), moisture ($R^2$ = 0.13), and the interaction of manure treatments and moisture ($R^2$ = 0.08).

Post-hoc analysis showed that SL and SCL soils had different ARG profiles ($F_1$ = 9.83, P = 0.001). Additionally, the 30 and 45% moisture treatments had different ARG profiles compared to 15% (P < 0.005), but ARG profiles at 30 and 45% moisture were not different ($F_1$ = 2.34, P = 0.060). Within the interactions of moisture and type, all pairwise comparisons were significant (P < 0.05), except SCL samples at 15% moisture were not different from SCL samples at 30% moisture ($F_1$ = 1.89, P = 0.089) or 45% ($F_1$ = 1.30, P = 0.228). Pairwise comparisons of the interactions of manure treatments and moisture showed no significant differences once P-values were adjusted for multiple comparisons.

Changes in specific ARG concentrations can be important regardless of whether the overall profile of ARG concentrations changes, so we also evaluated individual ARGs to provide a more complete picture of changes in the soil. Of the 47 genes tested, 38 varied with moisture content (Appx B, Table B.2). All genes that had significantly different abundances between 15%

and 45% moisture had higher abundances at 45%. Twenty-seven genes varied with manure treatment (Appx B, Table B.3). Generally, genes that varied in abundance with manure treatment tended to have greater abundance in the no manure and pirlimycin treatments compared to the control manure and cephapirin treatments. Thirty-seven genes varied with soil type (Appx B, Table B.4); 21 of those had a higher abundance in SL, while 16 had a higher abundance in SCL.

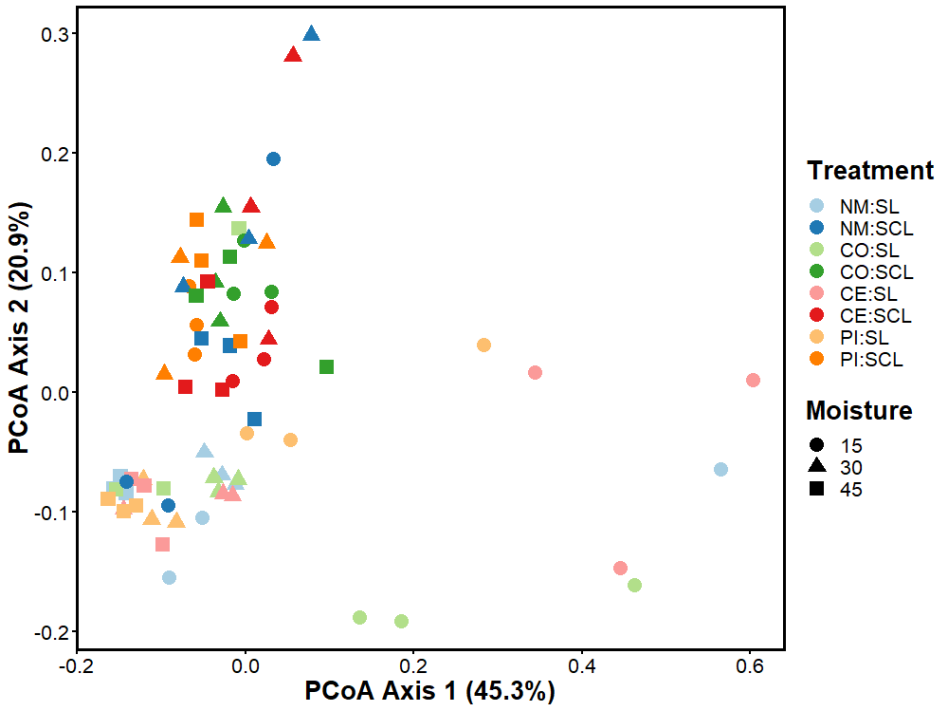


**Figure 3.1.** PCoA of ARG abundance profiles after 10 weeks in a Sandy clay loam (SCL) or sandy loam (SL) soil. Soils were kept at 15, 30, or 45% moisture content, and treatments of no manure (NM), antibiotic-free manure (CO), or manure from cattle administered either cephapirin (CE) or pirlimycin (PI) was added every other week. Shapes represent the moisture content, while colors indicate the treatment and type, Light colors show SCL samples, and darker colors show SL.

The 10 genes with the highest average abundance across all samples were *bla*$_{SHV}$ (3.16 ± 0.15 log copies g$^{-1}$), *tetA* (3.14 ± 0.16 log copies g$^{-1}$), *intI1* (3.10 ± 0.58 log copies g$^{-1}$), *sul1* (2.99 ± 0.60 log copies g$^{-1}$), *merA* (2.93 ± 0.74 log copies g$^{-1}$), *acrD* (2.87 ± 0.83 log copies g$^{-1}$), *nikA* (2.85 ± 0.84 log copies g$^{-1}$), *tetM* (2.83 ± 0.98 log copies g$^{-1}$), *cadA* (2.82 ± 0.51 log copies g$^{-1}$), and *ampC* (2.77 ± 0.96 log copies g$^{-1}$). Eight of the 10 (not *intI1* or *tetA*) most abundant genes varied with moisture (Appx B, Table B.2). Of those that did vary, *bla*$_{SHV}$, *acrD*, *nikA*, *cadA*, and *ampC* had higher abundances in 30 and 45% moisture than 15% moisture, *su1* and *merA* had higher abundances in 45% moisture than 30 or 15%, and *tetM* was higher at 45% moisture than 15%, but 30% moisture was not different from either 15 or 45%. All of the most abundant genes except for *tetA* varied with manure treatment (Appx B, Table B.3). Four genes (*intI1*, *acrD*, *nikA*, and *ampC*) were approximately twice as abundant in no manure and pirlimycin than control manure and cephapirin treatments, while *bla*$_{SHV}$ and *tetM* had lower abundances in the cephapirin treatments than all other manure treatments, and *sul1* was more abundant in cephapirin than all other manure treatments. The genes *cadA* and *merA* were most abundant in pirlimycin. All the most abundant genes except for *merA* varied with type. The genes *bla*$_{SHV}$, *acrD*, *nikA*, *tetM*, *cadA*, and *ampC* were more abundant in SCL, while *tetA*, *intI1*, and *sul1* were more abundant in SL.

### *3.3.2 Microbial communities*

Changes in soil microbial community structure were detected as changes in ASVs identified via 16S for bacteria or ITS amplicon sequencing, for fungi. Bacterial community

structure varied across all main effects and interactions (Appx B, Table B.5, P = 0.001). all post-hoc pairwise comparisons were significantly different ($P < 0.05$), except for the comparison of no manure to control manure in SL at 45% moisture. Given the number of significant pairwise comparisons, it is important to note that not all main effects had an equal impact on bacterial community structure. Type explained the most variation ($R^2 = 0.25$), followed by moisture ($R^2 = 0.10$), and manure treatment ($R^2 = 0.07$). Likewise, sample clustering in PCoA was clearly strongest across SCL and SL samples, followed by smaller cluster differences in moisture, and finally manure treatment (Figure 3.2). Within each soil type, no manure samples of all moisture contents clustered together, while the other three treatments clustered by moisture content.

Changes in the bacterial communities were evident at the phylum level (Figure 3.3), with large changes across treatments in relative abundances of even the most common phyla such as Acidobacteria, Actinobacteria, Bacteroidetes, and Proteobacteria. However, to get a more detailed look at finer resolution at the role of antibiotic exposure, we analyzed changes in relative abundances of genera among the manure treatments (Table 3.2). Indicators for specific treatments included 18 genera for the no manure treatment, 9 genera for the control manure, 4 genera for cephapirin, and 7 genera for pirlimycin. Genera that were indicators for multiple treatments included 3 genera for control manure and cephapirin, 1 genus for cephapirin and pirlimycin, 8 genera for control manure and pirlimycin, and 28 genera for the presence of manure (ie, control manure, cephapirin, and pirlimycin combined). Of the 18 genera associated with no manure, 3 were from the class Alphaproteobacteria, 3 were from Clostridia, and 3 were from Bacilli. Among the 28 genera associated with manure broadly (control, cephapirin, and pirlimycin) 10 were from Proteobacteria, with 4 in Alphaproteobacteria, 2 in Betaproteobacteria,

2 in Deltaproteobacteria, and 2 in Gammaproteobacteria. Five of the 28 genera were in the class Clostridia, 3 were in Flavobacteria, and 3 were in Saprospirae.

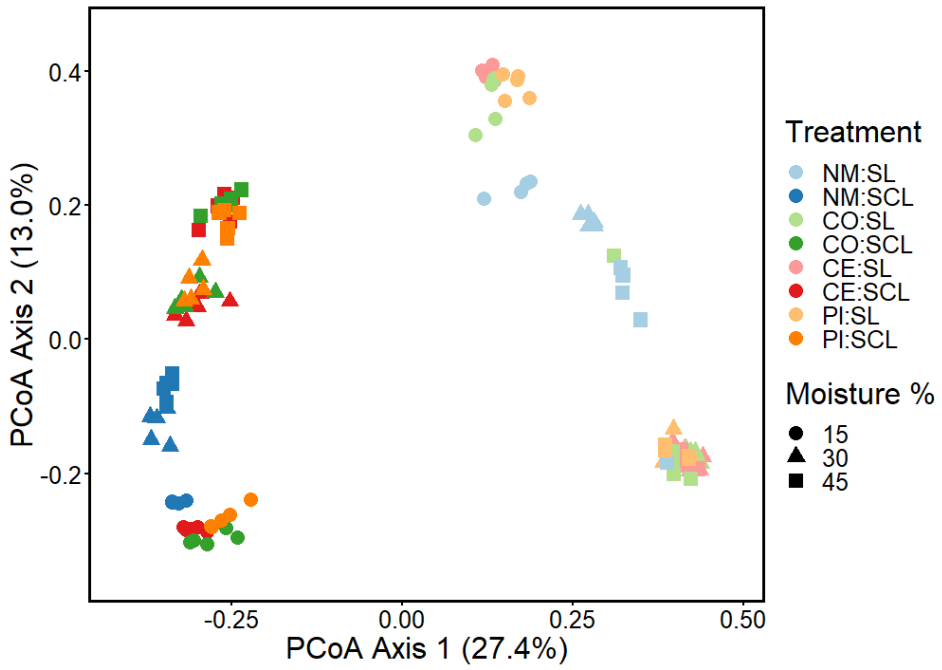


**Figure 3.2.** Soil bacterial communities after 10 weeks in a sandy clay loam (SCL) or sandy loam (SL) soil. Soils were kept at 15, 30, or 45% moisture content, treatments of no manure (NM), antibiotic-free manure (CO), or manure from cattle administered either cephapirin (CE) or pirlimycin (PI) was added every other week. Shapes represent the moisture content, light colors indicate manure treatment in SCL, and dark colors indicate manure treatments in SL.

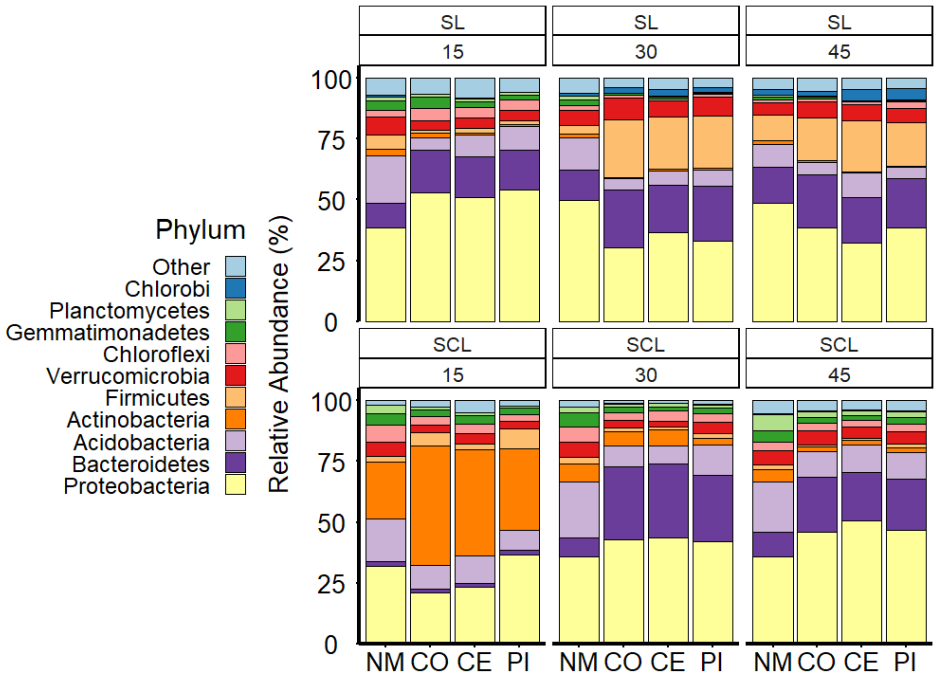


**Figure 3.3.** Average relative abundance (%) of the 10 most abundant phyla in soil bacterial communities after 10 weeks in a sandy clay loam (SCL) or sandy loam (SL) soil. Soils were kept at 15, 30, or 45% moisture content, treatments of no manure (NM), antibiotic-free manure (CO), or manure from cattle administered either cephapirin (CE) or pirlimycin (PI) in was added every other week.

Fungal communities showed similar patterns to bacterial community structure (Figure 3.4). Similar to bacteria, in fungal communities, all main effects and interactions of the PERMANOVA were significant (Appx B, Table B.6, $P = 0.001$). Post-hoc analyses also showed all pairwise comparisons were significantly different ($P < 0.05$). Type explained the most variation ($R^2 = 0.06$), followed by treatment ($R^2 = 0.05$), and moisture ($R^2 = 0.03$). Large-scale changes were seen in fungal communities at the phylum level (Appx B, Figure B.6), particularly

among Ascomycota, Basidiomycota, and Mortierellomycota. At the genus level, 20 genera were indicators for no manure, two genera were indicators for control manure, four genera were indicators for cephapirin, and eight genera were indicators pirlimycin. Furthermore, one genus (*Piromyces*) was an indicator for both control manure and cephapirin, and two genera were indicators for control manure, cephapirin, and pirlimycin. Of the 20 genera associated with no manure, 17 were in the phylum Ascomycota. Eight of the genera were in the class Sordariomycetes, five were in Dothideomycetes, and three were in Eurotiomycetes. The two genera associated with all three manure amendments were both in the family Ascobolaceae, and one was identified as the genus *Ascobolus*, while the other genus was unidentified.

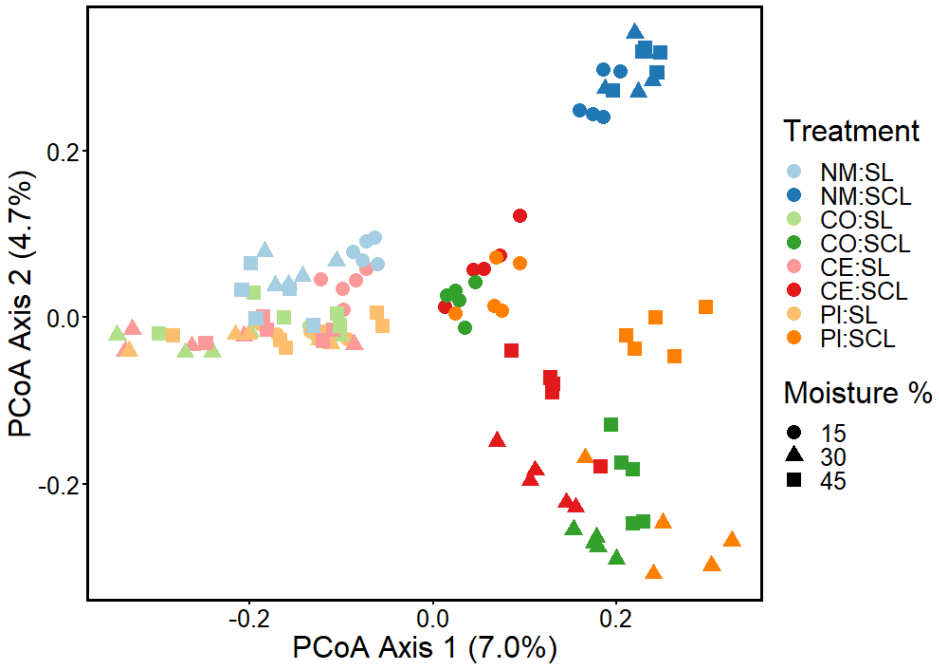

**Figure 3.4.** Soil fungal communities after 10 weeks in a sandy clay loam (SCL) or sandy loam (SL) soil. Soils were kept at 15, 30, or 45% moisture content, and treatments of no manure (NM), antibiotic-free manure (CO), or manure from cattle administered either cephapirin (CE) or pirlimycin (PI) was added every other week. Shapes represent the moisture content, light colors indicate manure treatment in SCL, and dark colors indicate manure treatments in SL.

*3.3.3 Respiration*

Based on a three-way ANOVA, all main effects and interactions had significant effects on total respiration over the 10-week incubation (Appx B, Table B.7, $P < 0.01$). Post-hoc analysis showed that that soils with manure had on average 5-fold higher respiration compared to the no manure treatments (Figure 3.5). At low moisture content (15%) in both SL and SCL soils, respiration was higher in the cephapirin treatment and lower in the pirlimycin treatment compared to the manure controls ($P < 0.05$). However, those differences disappeared as the moisture content increased. Notably, at 30% moisture in SCL, pirlimycin was 17% lower than the control while cephapirin was not different from the control. In contrast, in SL at 30%, cephapirin had 20% higher respiration than the control, while pirlimycin was not different. There were no differences in the no manure samples among moisture or type ($P > 0.05$). In SCL samples, manure treatments had highest respiration at 30% moisture, while the highest respiration in the manure treatments was at 15% moisture.

Similar to respiration, all main effects and interactions of three-way ANOVA for active microbial biomass – measured as SIR – were significant at the end of the experiment (Appx B, Table B.8, $P < 0.001$). No manure treatment SIR rates were approximately half of the SIR rates of soils with manure (Figure 3.6). Among the manure treatments, patterns were variable with moisture and type. While there were differences among the manure treatments in SCL, there were no differences among the control, cephapirin, and pirlimycin manure treatments in SL

samples at any moisture content (P > 0.05). In SCL, at 15% moisture in SCL samples, the cephapirin treatment generally had the highest SIR rate (5.05 ± 1.07 µg C $g^{-1}$ $hr^{-1}$), although this difference was clearly strongest at 15% moisture, it was not always significant at higher moisture contents. At 30% moisture, there were no significant differences among cephapirin, pirlimycin, and the control manure (P > 0.05).

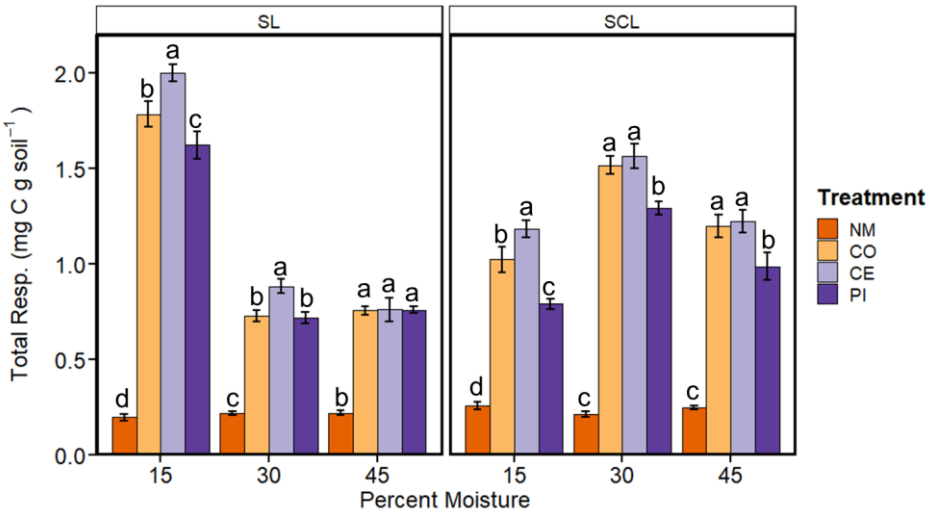


Figure 3.5. Average ± SE (n=5) cumulative respiration over 10 weeks in a sandy clay loam (SCL) or sandy loam (SL) soil. Soils were kept at 15, 30, or 45% moisture content by mass, and treatments of no manure (NM), antibiotic-free manure (CO), or manure from cattle administered either cephapirin (CE) or pirlimycin (PI) was added every other week. Lowercase letters indicate statistical significance of treatments within moisture and soil type groups.

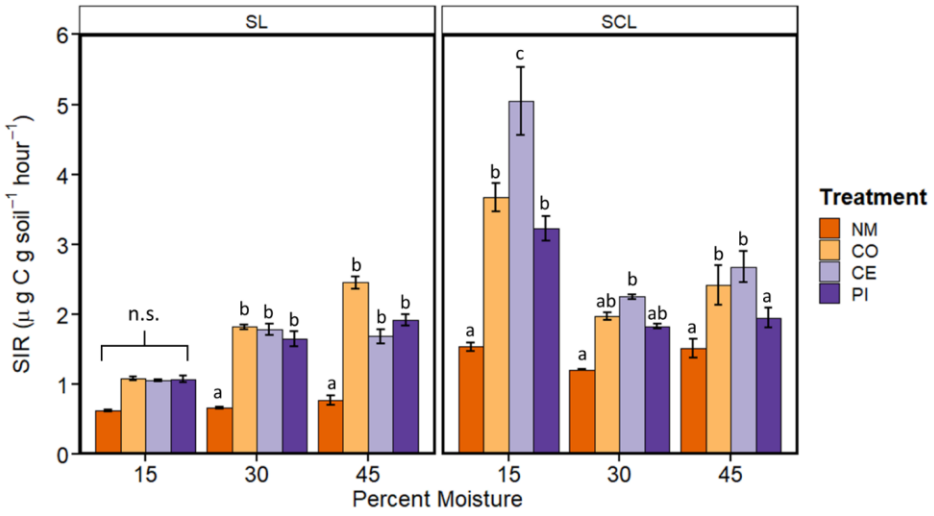


**Figure 3.6.** Average ± SE (n=5) SIR in a sandy clay loam (SCL) or sandy loam (SL) soil kept at 15, 30, or 45% moisture content by mass, and treatments of no manure (NM), antibiotic-free manure (CO), or manure from cattle administered either cephapirin (CE) or pirlimycin (PI) was added every other week for 10 weeks prior to SIR analysis. Lowercase letters indicate statistical significance of treatments within moisture and soil type groups. N.s. = no statistical significance.

*3.3.4 Inorganic soil nitrogen pools*

In the SCL soil, $NH_4$ was at 8.05 ± 0.39 ppm, while SL was at 3.44 ± 0.28 ppm before any manure additions. Changes in $NH_4$ varied based on manure treatment ($F_{3, 96}$ = 65.44, P < $2x10^{-16}$) and type ($F_{i,96}$ = 93.83, P = $7x10^{-16}$), but not moisture ($F_{2,96}$ = 2.58, P = 0.081). Additionally, all interactions of the 3-way ANOVA were significant (P < 0.01). At 15% moisture in SL and 30 and 45% moisture in SCL, $NH_4$ decreased and there were no differences among treatments (Figure 3.7). In contrast, there were differences among treatments at 15% moisture in SCL and 30 and 45% moisture in SL. When there were differences in $NH_4$ concentrations among treatments, the no manure treatment either decreased or had little change, while the three manure

treatments all showed increases. The cephapirin treatment tended to have the largest increase followed by pirlimycin, then the manure control, although in SL at 45% moisture, the control manure was not different from cephapirin or pirlimycin.

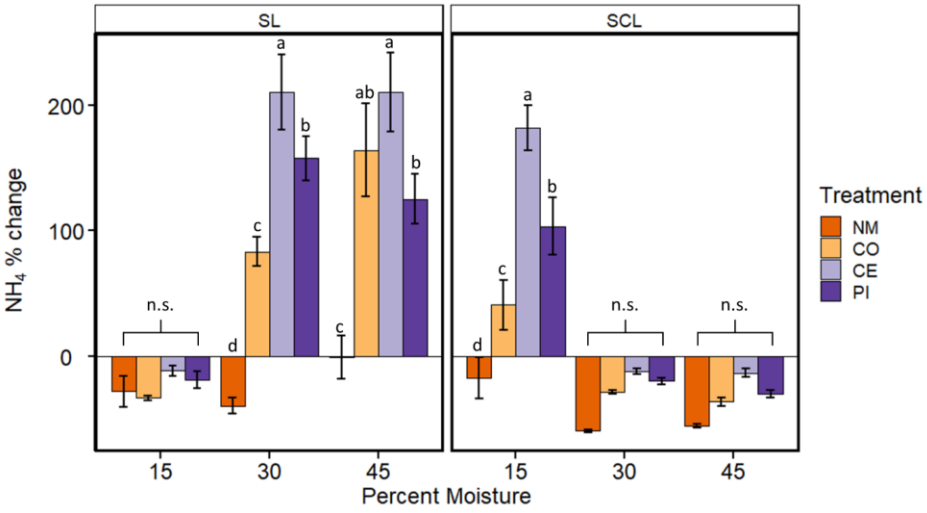


**Figure 3.7.** Average ± SE (n=5) percent change in $NH_4$ before and after experimental incubations of sandy clay loam (SCL) or sandy loam (SL) soils kept at 15, 30, or 45% moisture content (by mass). Treatments of no manure (NM), antibiotic-free manure (CO), or manure from cattle administered either cephapirin (CE) or pirlimycin (PI) were added every other week for 10 weeks. Lowercase letters indicate statistical significance of treatments within moisture and soil type groups. "n.s." = no significant differences.

$NO_3^-$ concentrations started at 1.64 ± 0.04 ppm in SCL and 3.96 ± 0.14 in SL, before any manure additions. Based on the 3-way ANOVA, all main effects and interactions were significant ($P < 0.001$), but changes in $NO_3^-$ pools throughout the experiment did not show consistent patterns. In the SL soil, changes in $NO_3^-$ concentration over time were relatively small, and there were no significant differences among treatments at any moisture content (Figure 3.8).

In the SCL soil, treatments showed large increases from initial concentrations in $NO_3^-$ except for at 45% moisture, where the control manure, cephapirin, and pirlimycin treatments showed little to no change from initial concentrations. In contrast to $NH_4$, the increase in $NO_3^-$ was highest in the no manure samples, although the no manure treatment was not different compared to any other treatment at 15% moisture.

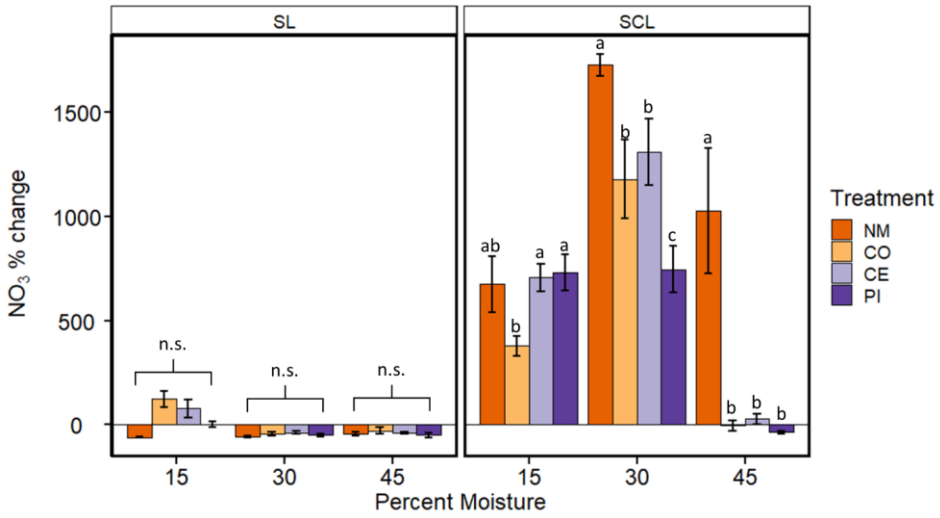


**Figure 3.8.** Average ± SE (n=5) percent change in $NO_3^-$ before and after sandy clay loam (SCL) or sandy loam (SL) soils were kept at 15, 30, or 45% moisture content with treatments of no manure (NM), antibiotic-free manure (CO), or manure from cattle administered either cephapirin (CE) or pirlimycin (PI) added every other week for 10 weeks. Lowercase letters indicate statistical significance of treatments within moisture and soil type groups. N.s. – no significant differences.

**Table 3.2.** Bacterial indicator genera for manure treatments. Groups indicates the manure treatments associated with the genera. Treatments were no manure (NM), antibiotic-free manure (CO), or manure from cattle administered either cephapirin (CE) or pirlimycin (PI). Stat refers to the association of the genus, with higher values corresponding to stronger associations. P-values are FDR adjusted for multiple comparisons.

| Groups | Phylum | Class | Order | Family | Genus | Stat | P-value |
|---|---|---|---|---|---|---|---|
| CE | Firmicutes | Bacilli | Bacillales | Planococcaceae | *Rummeliibacillus* | 0.491 | 0.00887 |
| | Proteobacteria | Gammaproteobacteria | Xanthomonadales | Xanthomonadaceae | *Luteibacter* | 0.467 | 0.00537 |
| | Proteobacteria | Betaproteobacteria | Neisseriales | Neisseriaceae | *Formivibrio* | 0.415 | 0.00887 |
| | Proteobacteria | Alphaproteobacteria | Rhizobiales | Phyllobacteriaceae | *Phyllobacterium* | 0.397 | 0.01432 |
| CO | Actinobacteria | Actinobacteria | Actinomycetales | Corynebacteriaceae | *Corynebacterium* | 0.726 | 0.00537 |
| | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | *Hydrogenophaga* | 0.497 | 0.02436 |
| | Euryarchaeota | Methanobacteria | Methanobacteriales | Methanobacteriaceae | *Methanosphaera* | 0.487 | 0.00537 |
| | Bacteroidetes | Bacteroidia | Bacteroidales | Porphyromonadaceae | *Paludibacter* | 0.483 | 0.00887 |
| | Chloroflexi | Anaerolineae | Anaerolineales | Anaerolinaceae | *T78* | 0.447 | 0.00887 |
| | Proteobacteria | Alphaproteobacteria | Rhizobiales | Phyllobacteriaceae | *Aminobacter* | 0.437 | 0.01432 |
| | Firmicutes | Clostridia | Clostridiales | Peptococcaceae | *Sporotomaculum* | 0.408 | 0.02164 |
| | Firmicutes | Clostridia | Clostridiales | [Mogibacteriaceae] | *Mogibacterium* | 0.406 | 0.02833 |
| | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Paracoccus* | 0.365 | 0.04026 |
| NM | Bacteroidetes | Cytophagia | Cytophagales | Cytophagaceae | *Sporocytophaga* | 0.778 | 0.00537 |
| | Nitrospirae | Nitrospira | Nitrospirales | FW | *29-Apr* | 0.694 | 0.00537 |
| | Bacteroidetes | [Saprospirae] | [Saprospirales] | Chitinophagaceae | *Segetibacter* | 0.621 | 0.00537 |
| | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | *Variovorax* | 0.6 | 0.04845 |
| | Proteobacteria | Alphaproteobacteria | Rhizobiales | Methylobacteriaceae | *Methylobacterium* | 0.597 | 0.01672 |
| | Proteobacteria | Deltaproteobacteria | Syntrophobacterales | Syntrophaceae | *Smithella* | 0.595 | 0.00537 |
| | Bacteroidetes | Cytophagia | Cytophagales | Cytophagaceae | *Hymenobacter* | 0.57 | 0.00537 |
| | Proteobacteria | Alphaproteobacteria | Rhizobiales | Hyphomicrobiaceae | *Parvibaculum* | 0.563 | 0.00887 |
| | Cyanobacteria | Oscillatoriophycideae | Oscillatoriales | Phormidiaceae | *Phormidium* | 0.535 | 0.00537 |
| | Firmicutes | Bacilli | Bacillales | Paenibacillaceae | *Paenibacillus* | 0.531 | 0.00537 |
| | Firmicutes | Clostridia | Clostridiales | Peptococcaceae | *Desulfosporosinus* | 0.525 | 0.00887 |

| Groups | Phylum | Class | Order | Family | Genus | Stat | P-value |
|---|---|---|---|---|---|---|---|
| | Firmicutes | Bacilli | Bacillales | Paenibacillaceae | *Ammoniphilus* | 0.522 | 0.00887 |
| | Cyanobacteria | Nostocophycideae | Nostocales | Nostocaceae | *Nostoc* | 0.499 | 0.00537 |
| | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | *Telmatospirillum* | 0.49 | 0.01432 |
| | Firmicutes | Clostridia | Clostridiales | Peptococcaceae | *Desulfurispora* | 0.475 | 0.02164 |
| | Firmicutes | Clostridia | Clostridiales | Peptococcaceae | *Pelotomaculum* | 0.471 | 0.00887 |
| | Cyanobacteria | Synechococcophycideae | Pseudanabaenales | Pseudanabaenaceae | *Leptolyngbya* | 0.454 | 0.01432 |
| | Firmicutes | Bacilli | Bacillales | Paenibacillaceae | *Brevibacillus* | 0.424 | 0.01200 |
| PI | Firmicutes | Bacilli | Bacillales | Planococcaceae | *Planomicrobium* | 0.99 | 0.00537 |
| | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Psychrobacter* | 0.943 | 0.00537 |
| | Actinobacteria | Actinobacteria | Actinomycetales | Nocardiaceae | *Rhodococcus* | 0.79 | 0.00537 |
| | Firmicutes | Clostridia | Clostridiales | Peptostreptococcaceae | *Clostridium* | 0.58 | 0.00537 |
| | Firmicutes | Bacilli | Lactobacillales | Carnobacteriaceae | *Desemzia* | 0.552 | 0.00537 |
| | Proteobacteria | Gammaproteobacteria | Xanthomonadales | Xanthomonadaceae | *Stenotrophomonas* | 0.537 | 0.00537 |
| | Spirochaetes | Spirochaetes | Spirochaetales | Spirochaetaceae | *Treponema* | 0.371 | 0.02164 |
| CE+CO | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | *Ruminococcus* | 0.507 | 0.01200 |
| | Tenericutes | Mollicutes | Anaeroplasmatales | Anaeroplasmataceae | *Asteroleplasma* | 0.504 | 0.00537 |
| | Actinobacteria | Actinobacteria | Actinomycetales | Promicromonosporaceae | *Cellulosimicrobium* | 0.391 | 0.04648 |
| CE+NM | Proteobacteria | Deltaproteobacteria | Myxococcales | Polyangiaceae | *Aetherobacter* | 0.476 | 0.04650 |
| CE+PI | Euryarchaeota | Thermoplasmata | E2 | [Methanomassiliicoccaceae] | *Methanomassiliicoccus* | 0.512 | 0.01200 |
| CO+PI | Firmicutes | Bacilli | Turicibacterales | Turicibacteraceae | *Turicibacter* | 0.985 | 0.00537 |
| | Proteobacteria | Alphaproteobacteria | Caulobacterales | Caulobacteraceae | *Mycoplana* | 0.713 | 0.00537 |
| | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | *Rubrivivax* | 0.702 | 0.00537 |
| | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | *Epulopiscium* | 0.638 | 0.00537 |
| | Proteobacteria | Deltaproteobacteria | Myxococcales | Polyangiaceae | *Chondromyces* | 0.498 | 0.01672 |
| | Actinobacteria | Actinobacteria | Actinomycetales | Dietziaceae | *Dietzia* | 0.432 | 0.02164 |
| | Firmicutes | Bacilli | Bacillales | Staphylococcaceae | *Jeotgalicoccus* | 0.412 | 0.01974 |
| | Firmicutes | Bacilli | Lactobacillales | Aerococcaceae | *Facklamia* | 0.412 | 0.03074 |
| CE+CO | Proteobacteria | Alphaproteobacteria | Rhodospirillales | Rhodospirillaceae | *Skermanella* | 0.706 | 0.01430 |

| Groups | Phylum | Class | Order | Family | Genus | Stat | P-value |
|---|---|---|---|---|---|---|---|
| +NM | | | | | | | |
| CE+CO +PI | Proteobacteria | Gammaproteobacteria | Pseudomonadales | Moraxellaceae | *Acinetobacter* | 0.959 | 0.00537 |
| | Firmicutes | Clostridia | Clostridiales | Clostridiaceae | *SMB53* | 0.959 | 0.00537 |
| | Proteobacteria | Deltaproteobacteria | Myxococcales | Polyangiaceae | *Sorangium* | 0.881 | 0.00537 |
| | Bacteroidetes | Flavobacteriia | Flavobacteriales | Flavobacteriaceae | *Flavobacterium* | 0.858 | 0.00537 |
| | Proteobacteria | Alphaproteobacteria | Sphingomonadales | Sphingomonadaceae | *Sphingobium* | 0.854 | 0.00537 |
| | Gemmatimonadetes | Gemmatimonadetes | Gemmatimonadales | Gemmatimonadaceae | *Gemmatimonas* | 0.83 | 0.00537 |
| | Euryarchaeota | Methanobacteria | Methanobacteriales | Methanobacteriaceae | *Methanobrevibacter* | 0.822 | 0.00537 |
| | Bacteroidetes | Sphingobacteriia | Sphingobacteriales | Sphingobacteriaceae | *Pedobacter* | 0.813 | 0.00537 |
| | Bacteroidetes | Cytophagia | Cytophagales | Cytophagaceae | *Dyadobacter* | 0.805 | 0.00537 |
| | Proteobacteria | Alphaproteobacteria | Caulobacterales | Caulobacteraceae | *Caulobacter* | 0.767 | 0.00537 |
| | Bacteroidetes | [Saprospirae] | [Saprospirales] | Chitinophagaceae | *Chitinophaga* | 0.759 | 0.02623 |
| | Proteobacteria | Deltaproteobacteria | Myxococcales | Nannocystaceae | *Nannocystis* | 0.73 | 0.00537 |
| | Proteobacteria | Betaproteobacteria | Methylophilales | Methylophilaceae | *Methylotenera* | 0.676 | 0.03859 |
| | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | *Coprococcus* | 0.662 | 0.02623 |
| | Proteobacteria | Alphaproteobacteria | Rhodobacterales | Rhodobacteraceae | *Rhodobacter* | 0.651 | 0.00537 |
| | Proteobacteria | Alphaproteobacteria | Sphingomonadales | Sphingomonadaceae | *Sphingopyxis* | 0.648 | 0.00537 |
| | Bacteroidetes | [Saprospirae] | [Saprospirales] | Chitinophagaceae | *Flavihumibacter* | 0.622 | 0.00537 |
| | Bacteroidetes | [Saprospirae] | [Saprospirales] | Chitinophagaceae | *Lacibacter* | 0.603 | 0.00537 |
| | Firmicutes | Clostridia | Clostridiales | Ruminococcaceae | *Clostridium* | 0.602 | 0.01672 |
| | Bacteroidetes | Flavobacteriia | Flavobacteriales | [Weeksellaceae] | *Chryseobacterium* | 0.594 | 0.01200 |
| | Bacteroidetes | Flavobacteriia | Flavobacteriales | Cryomorphaceae | *Crocinitomix* | 0.584 | 0.00537 |
| | Firmicutes | Clostridia | Clostridiales | Veillonellaceae | *Sporomusa* | 0.58 | 0.04239 |
| | Euryarchaeota | Methanobacteria | Methanobacteriales | Methanobacteriaceae | *Methanobacterium* | 0.577 | 0.01432 |
| | Proteobacteria | Gammaproteobacteria | Xanthomonadales | Xanthomonadaceae | *Pseudoxanthomonas* | 0.553 | 0.04026 |
| | Proteobacteria | Betaproteobacteria | Burkholderiales | Comamonadaceae | *Acidovorax* | 0.544 | 0.01672 |
| | Verrucomicrobia | Verrucomicrobiae | Verrucomicrobiales | Verrucomicrobiaceae | *Luteolibacter* | 0.544 | 0.01200 |
| | Firmicutes | Clostridia | Clostridiales | [Tissierellaceae] | *Sedimentibacter* | 0.533 | 0.02833 |

| Groups | Phylum | Class | Order | Family | Genus | Stat | P-value |
|---|---|---|---|---|---|---|---|
| | Spirochaetes | Spirochaetes | Spirochaetales | Spirochaetaceae | *Spirochaeta* | 0.532 | 0.02623 |

**Table 3.3.** Fungal indicator genera for manure treatments. Groups indicates the manure treatments associated with the genera. Treatments were no manure (NM), antibiotic-free manure (CO), or manure from cattle administered either cephapirin (CE) or pirlimycin (PI). Stat refers to the association of the genus, with higher values corresponding to stronger associations. P-values are FDR adjusted for multiple comparisons.

| Group | Phylum | Class | Order | Family | Genus | Stat | P-value |
|---|---|---|---|---|---|---|---|
| CE | Basidiomycota | Tremellomycetes | Cystofilobasidiales | Mrakiaceae | *Mrakia* | 0.944 | 0.0102 |
| | Mucoromycota | Mucoromycetes | Mucorales | Mucoraceae | *Mucor* | 0.828 | 0.0102 |
| | Basidiomycota | Tremellomycetes | Trichosporonales | Trichosporonaceae | *Trichosporon* | 0.732 | 0.0251 |
| | Mucoromycota | Mucoromycetes | Mucorales | Mucoraceae | *Actinomucor* | 0.43 | 0.0331 |
| CO | Ascomycota | Sordariomycetes | Sordariales | Chaetomiaceae | *Botryotrichum* | 0.545 | 0.0102 |
| | Basidiomycota | Wallemiomycetes | Wallemiales | Wallemiaceae | *Wallemia* | 0.483 | 0.0184 |
| NM | Ascomycota | Sordariomycetes | Sordariales | fam_Incertae_sedis | *Conlarium* | 0.814 | 0.0292 |
| | Ascomycota | Sordariomycetes | Xylariales | fam_Incertae_sedis | *Robillarda* | 0.687 | 0.0102 |
| | Ascomycota | Leotiomycetes | Helotiales | Dermateaceae | *Mollisia* | 0.676 | 0.0102 |
| | Ascomycota | Eurotiomycetes | Chaetothyriales | Trichomeriaceae | *Knufia* | 0.669 | 0.0102 |
| | Ascomycota | Sordariomycetes | Hypocreales | Nectriaceae | *Cylindrocarpon* | 0.641 | 0.0102 |
| | Ascomycota | Sordariomycetes | Xylariales | Sporocadaceae | *Neopestalotiopsis* | 0.624 | 0.0102 |
| | Ascomycota | Sordariomycetes | Hypocreales | Nectriaceae | *Ilyonectria* | 0.623 | 0.0102 |
| | Ascomycota | Dothideomycetes | Pleosporales | Didymosphaeriaceae | *Paraconiothyrium* | 0.601 | 0.0102 |
| | Ascomycota | Sordariomycetes | Hypocreales | Bionectriaceae | *Bionectria* | 0.577 | 0.0292 |
| | Ascomycota | Sordariomycetes | Hypocreales | Nectriaceae | *Dactylonectria* | 0.573 | 0.0102 |
| | Basidiomycota | Microbotryomycetes | Sporidiobolales | Sporidiobolaceae | *Rhodosporidiobolus* | 0.563 | 0.0443 |
| | Ascomycota | Dothideomycetes | Venturiales | Sympoventuriaceae | *Troposporella* | 0.555 | 0.0292 |
| | Ascomycota | Dothideomycetes | Minutisphaerales | Minutisphaeraceae | *Minutisphaera* | 0.521 | 0.0102 |
| | Ascomycota | Dothideomycetes | Pleosporales | Didymosphaeriaceae | *Bimuria* | 0.501 | 0.0292 |
| | Ascomycota | Sordariomycetes | Chaetosphaeriales | Chaetosphaeriaceae | *Chaetosphaeria* | 0.494 | 0.0102 |

| Group | Phylum | Class | Order | Family | Genus | Stat | P-value |
|---|---|---|---|---|---|---|---|
| | Basidiomycota | Agaricomycetes | Auriculariales | unidentified | *unidentified* | 0.483 | 0.0102 |
| | Basidiomycota | Agaricomycetes | Polyporales | Ganodermataceae | *Ganoderma* | 0.48 | 0.0184 |
| | Ascomycota | Eurotiomycetes | Chaetothyriales | Chaetothyriaceae | *unidentified* | 0.461 | 0.0102 |
| | Ascomycota | Dothideomycetes | Pleosporales | Phaeosphaeriaceae | *unidentified* | 0.447 | 0.0102 |
| | Ascomycota | Eurotiomycetes | Chaetothyriales | unidentified | *unidentified* | 0.44 | 0.0331 |
| PI | Ascomycota | Eurotiomycetes | Eurotiales | Aspergillaceae | *Penicillium* | 0.835 | 0.0102 |
| | Ascomycota | Saccharomycetes | Saccharomycetales | fam_Incertae_sedis | *Candida* | 0.753 | 0.0102 |
| | Ascomycota | Leotiomycetes | Thelebolales | unidentified | *unidentified* | 0.704 | 0.0102 |
| | Basidiomycota | Tremellomycetes | Filobasidiales | Filobasidiaceae | *Naganishia* | 0.696 | 0.0102 |
| | Ascomycota | Eurotiomycetes | Eurotiales | Aspergillaceae | *Xeromyces* | 0.696 | 0.0102 |
| | Basidiomycota | Tremellomycetes | Trichosporonales | Trichosporonaceae | *Cutaneotrichosporon* | 0.645 | 0.0102 |
| | Ascomycota | Leotiomycetes | Thelebolales | Thelebolaceae | *Cleistothelebolus* | 0.632 | 0.0102 |
| | Neocallimastigomycota | Neocallimastigomycetes | Neocallimastigales | Neocallimastigaceae | *Neocallimastix* | 0.555 | 0.0102 |
| CE+NM | Ascomycota | Dothideomycetes | Pleosporales | Didymellaceae | *Peyronellaea* | 0.548 | 0.0331 |
| CO+PI | Neocallimastigomycota | Neocallimastigomycetes | Neocallimastigales | Neocallimastigaceae | *Piromyces* | 0.541 | 0.0184 |
| NM+PI | Basidiomycota | Agaricomycetes | Agaricales | Clavariaceae | *unidentified* | 0.654 | 0.0251 |
| | Basidiomycota | Agaricomycetes | Agaricales | Entolomataceae | *Entoloma* | 0.627 | 0.0102 |
| | Ascomycota | Sordariomycetes | Sordariales | Sordariaceae | *Neurospora* | 0.619 | 0.0251 |
| | Ascomycota | Sordariomycetes | Magnaporthales | Magnaporthaceae | *Pseudophialophora* | 0.592 | 0.0102 |
| | Ascomycota | Sordariomycetes | Hypocreales | fam_Incertae_sedis | *Stilbella* | 0.586 | 0.0102 |
| | Ascomycota | Geoglossomycetes | Geoglossales | Geoglossaceae | *Hemileucoglossum* | 0.562 | 0.0331 |
| | Ascomycota | Leotiomycetes | Helotiales | Hyaloscyphaceae | *Cistella* | 0.524 | 0.0485 |
| | Ascomycota | Dothideomycetes | Pleosporales | Montagnulaceae | *Staurosphaeria* | 0.428 | 0.0443 |
| | Chytridiomycota | Rhizophydiomycetes | Rhizophydiales | fam_Incertae_sedis | *Operculomyces* | 0.408 | 0.0485 |
| CE+CO+PI | Ascomycota | Pezizomycetes | Pezizales | Ascobolaceae | *Ascobolus* | 0.871 | 0.0102 |
| | Ascomycota | Pezizomycetes | Pezizales | Ascobolaceae | *unidentified* | 0.581 | 0.0292 |

## 3.5 Discussion

The purpose of this study was to examine how soil properties, such as moisture and type, influence the response of soil microbial communities to antibiotics and manure. As expected, we saw strong overall effects of soil moisture and type on microbial communities and activity, as expected from previously published work (Dequiedt et al., 2009; Chau et al., 2011; de Vries et al., 2012; Lupatini et al., 2019). A key result of this work, however, was that manure from cattle treated with antibiotics had an impact on microbial community structures, microbial biomass, respiration, and soil N pools that was detectable across a wide range of soil moisture and type conditions. We also saw interactive effects of moisture and type with manure treatments in ARG abundances, suggesting that microbial responses to antibiotic inputs via manure are complex and varied across environmental gradients.

### *3.5.1 Impacts on ARG abundances*

Manure treatment alone did not impact the overall makeup of ARGs in soil, which is contrary to previous work documenting a shift in ARG concentrations in soils compared to soil with no manure (Shawver et al., 2021). ARG concentrations varied primarily due to the interaction of moisture and manure treatments, with higher concentrations at higher moisture and in the no manure and pirlimycin manure treatments. However, in a previous study, we observed higher ARG concentrations in soils with manure compared to no manure controls, and no differences between antibiotic-free manure and manure from cattle administered antibiotics (Shawver et al., 2021). Several other studies have shown that manure additions increase ARG concentrations in soil (Fang et al., 2015; Graham et al., 2016; Wepking et al., 2017) and it is

unclear why this pattern was not observed here. However, the use of antibiotics can be a competitive adaptation among oligotrophs and increased antibiotic resistance can be found in soils with lower organic carbon stocks (Dundore-Arias et al., 2019). The relationship between antibiotics and manure in affecting microbial communities and antibiotic resistance in soil is complex and requires additional study.

Among the antibiotics that varied with manure treatment were some of particular relevance to manure and livestock management. In particular, we looked more closely at beta-lactamase genes, which can convey resistance to cephapirin, macrolide-lincosamide-streptogramin B (MSLb) resistance genes, which can convey resistance to pirlimycin, and integrase genes, which indicate potential for horizontal gene transfer. Two beta-lactamase genes (*ampC* and $bla_{NPS}$) as well as an integrase gene (*intI1*) had higher abundances in pirlimycin and no manure than control manure and cephapirin. While it would be expected that manure from cattle treated with cephapirin would lead to an increase of beta-lactamase genes compared to control manure, we observed the opposite. Furthermore, the higher abundance of *intI1* indicates a greater potential for the spread of AMR in soils exposed to manure from cattle treated with pirlimycin and in soils with no manure. Previous work has shown higher abundances of *intI1* in soils with control, pirlimycin, and cephapirin manure compared to no manure (Shawver et al., 2021). Differences in these findings may be due to the experiment time and conditions. Shawver et al. (2021) applied manure monthly over 3 years in a field experiment, whereas this study applied manure biweekly over 10 weeks in a microcosm experiment. It is possible that patterns in ARG abundances take a long time to establish in the soil.

Few studies have tested the effect of environmental conditions on ARG abundances in soil, but we observed that soil moisture and type were just as, if not more, important than manure treatment in affecting overall ARG abundances (eg, Figure 3.1). Five beta-lactamase genes (*ampC, bla*$_{NPS,}$ *bla*$_{OXA}$, *bla*$_{SHV}$, and *bla*$_{VIM}$), 1 MSLb gene (*ermB*), and 2 integrase genes (*intl2* and *intl3*) had higher abundances at 45% moisture compared to 15% moisture, indicating that higher moisture contents in soils may promote increased AMR in soils. Furthermore, 6 beta-lactamase genes (*ampC*, *bla*$_{CTX\text{-}M\text{-}32}$, *bla*$_{imp13}$, *bla*$_{NPS}$, *bla*$_{SHV}$, and *bla*$_{VIM}$), 1 MSLb gene (*ermF*), and 2 integrase genes (*intI1* and *intl2*) varied with type. Among the genes that varied with type, 3 (*ampC, bla*$_{SHV}$, and *bla*$_{VIM}$) had higher abundances in SCL, while the rest had higher abundances in SL. Previous work has shown soil texture can influence ARG abundance after exposure to antibiotics, but the results have been equivocal, with some studies finding larger impacts of ARGs in soils with finer textures (Chander et al., 2005) and others finding larger impacts of ARGs in soils with coarser textures (Pankow, 2017; Blau et al., 2018; Chen et al., 2018). The findings from this study may explain the discrepancies from previous works, as we found that type impacts ARG abundances in response to antibiotic exposure, however the impact varies with individual genes. Regardless, it is clear that environmental conditions, and the interactions of those conditions with antibiotic and manure management, is likely to play an important role in mitigating environmental persistence and transmission of ARGs.

### *3.5.2 Impacts on community structure*

Both fungal and bacterial community structures varied with soil moisture, type, and manure treatments, as well as their interactions. Soil type explained the most variation in bacterial and fungal communities, which was unsurprising as texture is well known to influence microbial

community structure (Dequiedt et al., 2009; Chau et al., 2011; Roberts et al., 2011; de Vries et al., 2012). It is also important to note that in this experiment the two soils came from different locations in different climates and in other properties besides texture, including mineralogy, nutrients, and background microbial community composition. Furthermore, it was clear in these results that the two soils contained microbial communities in the SCL and SL soils were different at the beginning (Appx B, Figures B.1 and B.2).

Moisture had the next largest impact on bacterial communities, but not on fungal communities. Bacterial communities have been previously shown to be responsive to changes in moisture (de Vries et al., 2012; Lupatini et al., 2019) Overall, the main effects and interaction in fungal community structure explained less variation compared to bacterial. Previous studies have shown that fungi are more sensitive to small changes in moisture content than bacteria (Kaisermann et al., 2015). However, our results do not support these findings, possibly because the changes in moisture for our experiment were relatively large.

While soil type and moisture were previously known to alter soil microbial community structure, an important result in this work was that manure treatment had detectable effects across these ranges of soil conditions. To be clear, manure treatment had less impact on community structure than type for both bacteria and fungi, and less impact than moisture for bacteria. However, both the application of manure and whether manure was from cattle given antibiotics resulted in measurable changes in microbial community structure. The biggest changes resulted from manure addition (ie, between the microcosms with no manure and all 3 of the microcosms with manure additions). This is likely due to the influx of organic carbon and other nutrients present in manure, and many studies have shown that nutrients influence

microbial community structure (Lauber et al., 2008; de Vries et al., 2012; Chávez-Romero et al., 2016). Of the 18 bacterial genera associated with no manure, some are common soil bacteria, including *Variovorax* and *Paenibacillus* (Delgado-Baquerizo et al., 2018), while others have known important ecological functions, such as the N-fixers *Phormidium* (Berrendero et al., 2016), *Nostoc* (Lindberg et al., 2004), *Leptolyngbya* (Tsujimoto et al., 2016), *Brevibacillus* (Nehra et al., 2016), and *Desulfosporosinus*, which can also reduce sulfur (Thajudeen et al., 2017). These associated genera were negatively impacted by additions of manure and manure with antibiotics.

Among the genera associated with soils amended with manure, several are common gut bacteria, including *Ruminococcus*, associated with cephapirin and control manure treatments, *Turicibacter*, associated with pirlimycin and control manure treatments, and *Coprococcus*, associated with cephapirin, pirlimycin, and control manure treatments. There were also several pathogen-containing genera associated with soils amended with manure, including *Corynebacterium*, associated with control manure treatments, *Clostridium* and *Treponema*, associated with pirlimycin manure treatments, *Cellulosimicrobium*, associated with cephapirin and control manure treatments, *Dietzia* and *Facklamia*, associated with pirlimycin and control manure treatments, and *Acinetobacter*, associated with control, cephapirin, and pirlimycin treatments. High abundance of *Acinetobacter* have been found previously in soils with a long history of manure applications (Wepking et al., 2017) and the genus is of particular importance as a human pathogen that is increasingly antibiotic resistant (Manchanda et al., 2010). Furthermore, several genera associated with manure from cattle treated with antibiotics have been found to be hosts for ARGS, including *Formivibrio* (Jiang et al., 2021), associated with

cephapirin manure treatments, *Stenotrophomonas* (Ryan et al., 2009), associated with pirlimycin manure treatments, *Facklamia* (Rahmati et al., 2017), associated with control and pirlimycin, and *Pedobacter* (Bjerketorp et al., 2021), associated with control, cephapirin, and pirlimycin manure treatments. Associations of potential pathogens and ARG hosts reinforces the potential that manure additions, particularly manure from cattle treated with antibiotics, can pose risks to human health across a range of soil conditions.

### *3.5.3 Impacts on microbial function*

While human and animal health risks are, understandably, a major area of focus in managing agricultural antibiotic usage, recent evidence suggests that changes to soil microbial communities can also impact biogeochemistry and ecosystem fluxes (Wepking et al., 2019). We observed additional evidence for this, with cephapirin treatments having higher respiration and SIR compared to other treatments. One possible explanation is that the maintenance of AMR has a higher metabolic cost (Wepking et al., 2017). However, if that were the case, we would also expect to see an increased respiration rate in the pirlimycin treatment. Pirlimycin had overall lower respiration rates than cephapirin and the control manure. It is possible that pirlimycin, as a bacteriostatic antibiotic, was inhibiting respiration, although soils amended with pirlimycin manure have previously had higher respiration rates compared to control manure (Wepking et al., 2019). Another explanation for the increased respiration in cephapirin is an increase in fungal biomass in the absence of bacterial competition, which was supported by visual observations of periodic fungal growth on the surface of some of the cephapirin microcosms. Additionally, we saw increased respiration and SIR in soils with manure added. This is not surprising as organic carbon is known to have a positive impact on microbial activity (Šantrůčková et al., 2003).

Although total respiration and SIR had similar patterns among manure treatments, they had different patterns with type and moisture. In SCL samples, the highest total respiration was at 30% moisture, while in SL the highest moisture was at 15% moisture. Respiration typically increases with moisture (Dijkstra and Cheng, 2007; Kaisermann et al., 2015), but decreases with moisture stress (Manzoni et al., 2012). It is likely that the SCL samples at 15% moisture were dry enough to cause moisture stress, while in the SL samples the higher moisture contents were limiting the availability of oxygen in the soil microcosms. However, at 15% moisture, SIR was highest in SCL, while lowest in SL. Previous work has also shown lower microbial biomass in soils with higher sand contents (Roberts et al., 2011), although our study shows different in biomass decrease with increasing moisture content.

To look beyond microbial impacts on carbon cycling, we also quantified dissolved soil inorganic N pools to get a sense of how these changed across microcosms. $NH_4$ concentrations generally increased in manure treatments where respiration was lower (SCL at 15%, SL at 30 and 45%) and decreased where respiration was higher. Among manure treatments, the largest increases were in cephapirin, followed by pirlimycin. Soils amended with pirlimycin manure have been previously shown to have higher N cycling, although soils with cephapirin manure were not different from the control (Wepking et al., 2019). The changes in $NO_3^-$ were less clear. There was a strong influence of soil type, but mixed effects of treatment within the SCL soils. Previous studies have found N cycling processes can be influenced by soil texture (Šantrůčková et al., 2003; Enwall et al., 2010; Morales et al., 2015), moisture content (Morugán-Coronado et al., 2019), and available nutrients such as carbon (Šantrůčková et al., 2003; Deslippe et al., 2014). However, the treatment differences observed here are an important result as, again, they

suggest that antibiotic usage in cattle may actually alter how that manure impacts soil biogeochemistry and the availability of important plant nutrients. Antibiotics are known to inhibit nitrification (Tomlinson et al., 1966; Klaver and Matthews, 1994; Kotzerke et al., 2008; Toth et al., 2011) and denitrification (Hou et al., 2015), although other studies have suggested higher concentrations of antibiotics can stimulate $NO_3^-$ reduction (Ahmad et al., 2014; D'Alessio et al., 2019). As cephapirin and pirlimycin caused large increases in $NH_4$ at low moisture in SCL and high moisture in SL, it is possible in this study that the antibiotics in the manure were inhibiting nitrification. There was also an important interaction with soil type in that that manure from cattle administered antibiotics impacted changes in $NO_3^-$ concentrations in SCL but not in SL. As the percent change in $NO_3^-$ was smaller in cephapirin and pirlimycin treatments compared to no manure at higher SCL contents, it is possible that denitrification was stimulated by low doses of antibiotics. Although this experiment was not designed to parse the many possible biogeochemical fluxes that impact N pools in soil ecosystems, it provides important evidence that manure from cattle administered antibiotics can influence N pools, and that effect is mediated by the soil environment.

### *3.5.4 Conclusions*

Overall, soil conditions played an important role on the impact of manure from cattle administered antibiotics on soil microbial communities. Specifically, type, followed by moisture, had the greatest impacts on bacterial community structure, while the addition of manure had a smaller but still significant impact. Additionally, moisture and type had the greatest impacts on ARG abundances. While adding manure greatly increased respiration, the moisture content

strongly influenced the impact of the manure on respiration. Changes in inorganic N pools were most heavily impacted by the interaction of moisture and type.

While an important result of this work is demonstrating effects of antibiotics in manure across a range of soil conditions, this work also showed that the class of antibiotic can influence the impact of antibiotic exposure. With manure from cattle treated with cephapirin, we saw higher respiration, greater accumulation of $NH_4$, and lower ARG abundances than in soil that was treated with manure from cattle administered pirlimycin. Pirlimycin and cephapirin have different modes of action (bacteriostatic and bactericidal), which likely explain the differences in responses. Thus, it is clear that aspects of the soil environment as well as the type of antibiotic affect how microbial communities respond to exposure of manure and antibiotics. Notably, higher moisture content corresponds with higher ARG abundances, thus management strategies should aim to avoid manure applications in wet areas. Further work is needed to fully understand the relationships and interactions among the environment, manure and antibiotic management, and antimicrobial resistance in soil.

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**Chapter 4. Viable bacteria or extracellular DNA: disentangling differential transport of antibiotic resistance through saturated soil columns**

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## 4. 1 Abstract

Livestock manure can spread antibiotic compounds, resistant bacteria, and antibiotic resistance genes (ARGs) to soil. With growing concerns about antimicrobial resistance (AMR), it is critical to understand how movement of these elements in the environment may contribute to the proliferation of AMR. While considerable work has focused on mobility of antibiotic compounds themselves, the spread of AMR also occurs via transport of other elements such as ARGs and antibiotic resistant bacteria (ARB), which are less commonly considered. The overarching goal of this work was to examine independent transport of ARGs and ARB through soil. Ampicillin resistant *E. coli* (Bacteria) or plasmids containing the $bla_{TEM}$ ampicillin resistance gene (Plasmid) were applied to saturated soil columns. ARB and ARGs were quantified in soil and leachate via spread-plating and qPCR. Results show 100-fold higher ARB concentrations in leachate and 2x higher ARB in soil from Bacteria-applied columns than control. However, there were no differences in ARG abundances in soil or leachate from Plasmid-applied columns compared to controls. Overall, results suggest that bacteria readily transport through soil columns, while plasmids do not. These results highlight how multiple elements of AMR may be transported differently through soil and require separate consideration in modeling and management.

## 4.2 Introduction

Antimicrobial resistance (AMR) is a growing concern in both human and veterinary medicine. Antibiotics are often used to treat livestock and are excreted in manure, resulting in risk of increased ARGs persisting in agricultural soils (Allen et al., 2010). Such persistence has led to the increased concentrations of a wide range of antibiotics in agroecosystems across the globe (Williams-Nguyen et al., 2016). Ultimately, spread of AMR through the environment is a key concern, with both antibiotic resistance genes (ARGs) and antibiotic compounds considered environmental contaminants (Pruden et al., 2006; Sanderson et al., 2016). Thus, in order to understand and mitigate risks of AMR transmission in agricultural systems, we must also understand how it is transported through the soil environment.

As water moves through the soil matrix, soil particles, chemicals, and bacteria move with it, which can facilitate the transport of antibiotic compounds, ARB, and ARGs. The mobility of antibiotic compounds themselves can affect the spread AMR by selecting for resistance among native soil bacteria. While some antibiotics are highly mobile in soil, others are not, depending on chemical characteristics. For example, some antibiotics such as erythromycin and tylosin have a high affinity for soil, and would be expected to be bound to soil particles and immobilized, while others such as sulfamethazine and sulfadimethazine have a low affinity for soil binding (Radolinski et al., 2022). Another study found sulfamethoxazole is highly mobile in saturated silica media at a slightly acidic pH (5.7), but ciprofloxacin is not (Chen et al., 2011). Additionally, some antibiotics are hydrophilic and can leach through soil, but many are also protonated at a typical soil pH and can bind to soil particles and organic matter, limiting movement (Chee-Sanford et al., 2009). Even for the same antibiotic, results can differ between

modes of transport. For example, Davis et al. (2006) found high amounts of monensin in surface runoff, but D'Alessio et al. (2019) did not find monensin leached through soil. While chemical characteristics of antibiotic compounds can clearly affect their motility through soil matrices, the antibiotics themselves represent only one way in which AMR transmission can occur.

In addition to antibiotic transport, AMR can also spread by movement of viable bacteria or extracellular DNA containing ARGs through soil, which could potentially be taken up or transferred to native soil bacteria. Extracellular DNA can move through soils (Ceccherini et al., 2007) and a plasmid containing the *aadA* gene for aminoglycoside resistance was shown to leach through a soil column and then be taken up by *Acinetobacter* sp. (Poté et al., 2010), confirming that AMR deposited at the surface may be transported through soil and have impacts elsewhere. Chen et al. (2015) examined the transport of 8 plasmids ranging in size from 2.7-33.4 kb through columns packed with quartz sand. They showed that plasmids have the potential to transport through a saturated porous media and that transport was affected by the ionic strength of the solution, with less adsorption to the sand at lower ionic strengths. More recently, similar breakthrough curves in both quartz sand and soil were observed (Chen et al., 2018), providing further evidence that ARGs on extracellular plasmids can move through soil.

While harder to control than soil columns, evidence from field soils also suggest that additions of manure in agricultural landscapes can lead to transport of multiple AMR elements. Multiple studies provide evidence that antibiotics and ARGs can move through soil (Poté et al., 2010; Luby et al., 2016), and detectable levels of ARB have been observed in runoff following surface manure applications (Hilaire et al., 2022). Reported correlations in ARG abundances between agricultural feedlot wastewater and nearby soils irrigated with the same wastewater

indicate that ARGs can spread also with wastewater irrigation (Li et al., 2012). Antibiotics and ARGS were also detected in runoff from a field following application of manure with antibiotics, and ARGs were detected in the top 5 cm of soil, indicating that both surface runoff and vertical transport of antibiotics and ARGs can occur (Joy et al., 2013). However, vastly lower concentrations of ARGs were detected deeper in the soil (20-25cm), indicating there may be limitations to how well various elements of antibiotic resistance can move vertically thorough soil. However, like antibiotic compounds, transport of biological AMR elements is not always detected (Fahrenfeld et al., 2014). Modeling predicts that the movement of ARGs may be dependent on several factors such as antibiotic concentration, organic matter, and the rate of horizontal gene transfer in soil, which may explain differences in research findings (Gothwal and Thatikonda, 2018).

While the potential for multiple AMR elements to be transported through soil has been clearly demonstrated, varying rates of mobility among the different elements are common. Generally, resistance genes, either in ARB or as free DNA, are likely to have very different mobilities than the antibiotic compounds for which they confer resistance. When soils were irrigated with wastewater spiked with sulfamethoxazole and ciprofloxacin, increased sulfonamide ARGs were found in the water flow paths (Lüneberg et al., 2018). However, ciprofloxacin ARGs were not detected, likely due to the limited mobility of the antibiotic. Similarly, an increase in ARGs but not ARB was found in the drainage water from soils that had manure applied compared to soil without manure, indicating that plasmids may be more mobile than bacteria (Luby et al., 2016). In controlled experimental systems, ARB in addition to plasmids containing ARGs were also able to move through silica sand, although plasmids moved

more freely than bacteria (Rysz and Alvarez, 2006). Thus, these additional potential transport dynamics are a critical piece of understanding how AMR may spread through soil systems.

Previous studies examining the movement of AMR have relied on correlative field data, focused mainly on antibiotics or ARGs, and/or used artificial soil media. However, with increasing reliance on PCR and metagenomic-based methods for tracking the spread of AMR, it is important to understand differential transport between genes moving through soil inside bacteria or as extracellular DNA. This study aims to address this knowledge gap by examining both ARGs and ARB transport through a natural soil. Culturable ARB and ARG abundances were quantified concurrently in soil and in leachate after adding either ARB or a plasmid containing the matching ARG to saturated soil columns. The objectives were to determine 1) whether resistant bacteria and extracellular plasmids containing resistance genes were transported at similar rates through a saturated soil column and 2) concentrations of each that accumulated in the soil matrix after surface addition. Based on the existing literature, ARB were expected to be detected in the leachate, but also be retained in the soil. ARGs were also expected to have high concentrations in the leachate, but low retention in the soil.

## 4.3 Methods

### *4.3.1 Study design*

Saturated soil columns were used to compare the transport of AMR (ARGs and ARB). Soil was collected from an abandoned farm field located next to the New River (N 37.335827, W 80.677768). The soil is mapped as Chavies variant sandy loam (13% clay, 67% sand), and was chosen for the sandy texture and relatively high permeability (Soil Survey Staff, 2019). The area

has not recently been in production and had no known previous exposure to antibiotics or manure. Soil was collected from the surface A horizon, sieved to 4mm to remove rocks and large roots, and homogenized. The sampled soil had a pH of 6.48, 2.1% organic matter, and the cation exchange capacity was 6.95 meq $100g^{-1}$.

Columns were made from PVC pipes that were 20 cm diameter and 25 cm deep. Rubber caps were clamped on the bottom of the tubes and included a drain hose to allow leachate to flow out. The drains were covered with a fine mesh filter to prevent soil from falling out. Approximately 1 cm of sand was added to the bottom of the columns to facilitate even drainage from the area of the soil column. Columns were filled with sieved soil to a depth of 20 cm, with an average bulk density of 1.25 g $cm^{-1}$. The edges of the columns were lined with bentonite clay to prevent water from preferentially flowing down the sides of the column.

*4.3.2 Preparation of bacteria and plasmids*

Ampicillin-resistant *Escherichia coli* were prepared by transforming a plasmid, pUCIDT (Integrated DNA Technologies, Coralville, IA), containing the ampicillin-resistant gene $bla_{TEM}$ via heat shock. *E. coli* were grown in LB broth containing 100 µg $L^{-1}$ ampicillin at 37°C overnight. Cells were then centrifuged at 5000 rpm for 5 minutes. The pellet was resuspended in 0.9% NaCl to a final concentration of approximately $1x10^9$ colony forming units (CFUs) $mL^{-1}$. CFU concentrations were verified by spread-plating the solution on Plate Count Agar (PCA) media containing 100 µg $L^{-1}$ ampicillin and incubating at 30°C for 48 hours, or until adequate growth was observed. Naked plasmids were prepared by growing ampicillin-resistant *E. coli* as described above and then extracting plasmids using QIAprep Spin miniprep kits (Qiagen, Hilden, Germany.). Concentrations of the plasmid were determined by measuring DNA concentration in

plasmid extracts using high-sensitivity dsDNA assays on a Qubit 3.0 fluorometer (Invitrogen, Waltham, MA) and correcting for plasmid mass. Plasmids were diluted to $1x10^9$ copies $mL^{-1}$ in 0.9% NaCl for loading into experimental columns.

*4.3.3 Column operation*

Soil columns were saturated from the bottom up with 0.005M $CaCl_2$ to prevent air pockets from forming. The low concentration of $CaCl_2$ was used to mimic the ionic strength of natural soil solutions (Radolinski et al., 2018), as pure water would impact the transport of bacteria and plasmids. Once columns were saturated, additional 0.005M $CaCl_2$ was added to the ponded water on top of the soil columns and a pressure head of 2 cm was maintained by use of Mariotte bottles (Figure 4.1). The first leachate sample was collected in a sterile glass flask from each column immediately before ARB or plasmid treatments were added. Bacterial treatments received 80 mL of the *E. coli* solution in NaCl described above to achieve a final concentration of $1x10^8$ CFU $g^{-1}$ soil. Plasmid treatments received 80 mL of the $bla_{TEM}$ plasmid solution in NaCl to give an equivalent final concentration of $1x10^8$ plasmid $g^{-1}$ soil. Control treatments received 80 mL 0.9% NaCl. Leachate samples were collected every 1/3 pore volume (1100 mL), with the last ~100 mL of each pore volume collected for each sample. The experiment was ended for each column when 3 pore volumes had flowed through. After the final sample was collected, Marriotte bottles were removed and no further $CaCl_2$ was added. Three pore volumes filtered through the soil columns in approximately 7-10 hr.

To facilitate final soil sampling by depth, columns were allowed to drain for approximately 3 days before soil was sampled. The soil in the columns was manually dug out, avoiding the bentonite clay liner, so that soil samples could be collected by depth in 5cm

increments. Instruments were cleaned and sterilized with 70% ethanol to avoid cross contamination. The edges of the column were not sampled to avoid any edge effect from the interface of soil and PVC pipe that would alter results. Leachate and soil samples were stored at 4 C until they were processed (<10 hours).

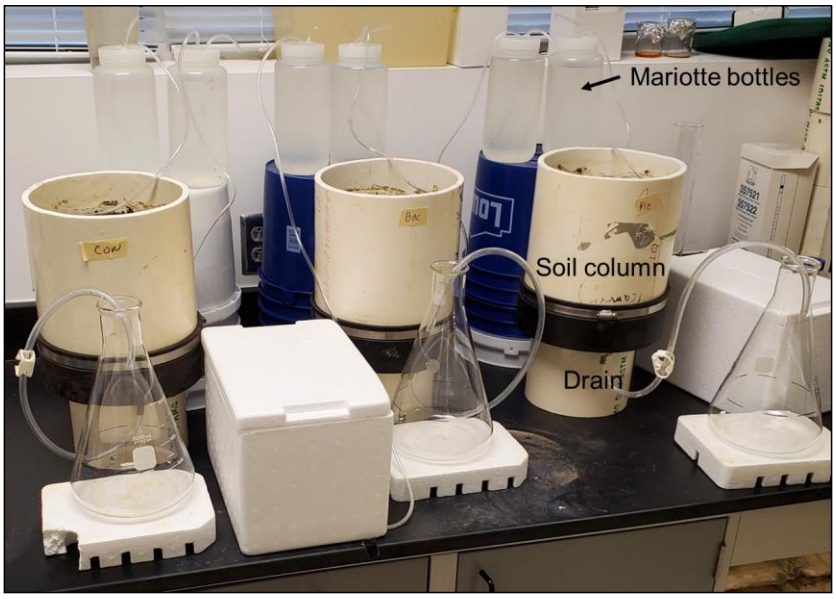


**Figure 4.1.** Column set up with Mariotte bottles to maintain a constant head on top of the soil columns.

*4.3.4 Enumeration of total and resistant bacteria*

Total and ampicillin-resistant culturable aerobic heterotrophic bacteria were quantified in soil and leachate samples by spread plating on the same day samples were collected. Samples were serially diluted 10-fold in 0.9% NaCl and spread on PCA plates for total bacteria and PCA

plates containing ampicillin at a concentration of 100 µL $L^{-1}$ for ampicillin-resistant bacteria. Plates were incubated at 30°C until adequate growth was observed (~48 hours). Samples were plated in duplicate, and the mean of the two plates, reported as log CFU g $soil^{-1}$ for soil or log CFU $mL^{-1}$ for leachate, was used for analysis.

*4.3.5 Quantification of resistance genes*

100 mL of each leachate sample was filtered on 0.45-micron nitrocellulose filters, and DNA was extracted from the filters using Qiagen PowerWater DNA extractions kits (Qiagen, Hilden, Germany). Qiagen PowerSoil DNA extraction kits (Qiagen, Hilden, Germany) were used to extract DNA from 0.25 g aliquots of soil samples. Quantitative PCR (qPCR) was used to quantify the 16S rRNA gene to estimate total bacterial abundance, and to quantify the $bla_{TEM}$ ampicillin-resistance gene. For 16s qPCR, the primers used were EUB518 (5'-ATT ACC GCG GCT GCT GG-3') and EUB338 (5'-ACT CCT ACG GGA GGC AGC AG-3'). Reaction mixtures and amplification conditions were followed as described in Fierer et al. (2005). For $bla_{TEM}$, reaction mixtures and amplification conditions followed Koukos et al. (2015), using 5'-CAG TGC TGC AAT GAT ACC GC-3 and 5'-AGA TCA GTT GGG TGC ACG AG-3 for forward and reverse primers, respectively. Each sample was amplified in triplicate, using the mean for statistical analysis. Standard curves were generated for each qPCR run, using known concentrations of $bla_{TEM}$, ranging from $10^2$-$10^8$ copies $\mu l^{-1}$. For quality controls, only data from plates with a standard curve with of $R^2 \geq 0.95$ and efficiency between 0.9 and 1.1 were used.

*4.3.6 Detection of plasmids in soil*

In order to determine whether extracellular plasmids sorb to the soil, and thus are unable to leach through soil, an additional experiment was conducted. Ten grams of soil were added to 6 250-ml glass beakers. Enough 0.005M $CaCl_2$ was added to each jar to completely saturate the soil. Half of the jars (n=3) also had sodium azide added to a final concentration of 128 µg $ml^{-1}$ to inhibit any microbial activity that could break down plasmids (Autry and Fitzgerald, 1993). Plasmids containing the $bla_{TEM}$ gene as described above were added to the soil to a final concentration of $1x10^8$ plasmid $g^{-1}$ soil to each jar. Subsamples of soil were collected at 1, 8, and 24 hours after plasmid additions and then frozen until further analysis. DNA was extracted from the soil samples, and the concentration of $bla_{TEM}$ was quantified using qPCR as described previously.

*4.3.7 Statistical analyses*

All statistical analyses were completed in R (version 3.6.1; R Core Team, 2018). The total load of bacteria or genes in leachate was calculated assuming the 100 mL sample had the same concentration for the entire 1/3 pore volume. For total load in soil, concentrations were multiplied by volume for each soil depth increment and summed. Loads were first totaled, then log-transformed and compared using ANOVAs or Kruskal Wallis nonparametric ANOVAs, if data were not normally distributed. Post hoc comparisons were made using pairwise t-tests, or Dunn tests if data was not normally distributed, with FDR corrected p-values to adjust for multiple comparisons.

## 4.4 Results

### *4.4.1 ARB and ARGs in leachate*

The Bacteria treatment had 100-fold more total loads of leached culturable bacteria and culturable ampicillin-resistant bacteria than either the Control or Plasmid treatments (Figure 4.2, Table 4.1). The average ratio of resistant to total bacteria was not different among treatments (Figure 4.2, $\chi^2 = 0.038$, $p = 0.981$). The Bacteria treatment also had 10-fold greater total abundance of leached 16S ($p = 0.010$) and 1000-fold greater total abundance of *bla*$_{TEM}$ ($p = 0.018$) gene copies compared to the Plasmid treatment (Fig. 2). However, there was not a significant difference between the Bacteria and Control or Control and Plasmid treatments for either 16S or *bla*$_{TEM}$ gene abundances (Table 4.1). The average ratio of *bla*$_{TEM}$ to 16S gene abundances was 1000-fold higher in the Bacteria treatment than in the Control or Plasmid treatments (Figure 4.2, Table 4.1).

**Table 4.1.** Comparisons of mean total loads and ratios of genes and bacteria among treatments in leachate samples with Kruskal-Wallis nonparametric ANOVAs and post-hoc dun tests with FDR adjusted p-values.

| **Parameter** | ANOVA | | Post-hoc Dunn test P-values | | |
|---|---|---|---|---|---|
| | $X^2$ | P-value | Bacteria: Control | Bacteria: Plasmid | Control: plasmid |
| Total Bacteria (log) | 7.423 | 0.024 | 0.036 | 0.043 | 0.844 |
| Amp-resistant Bacteria (log) | 7.423 | 0.024 | 0.036 | 0.043 | 0.844 |
| Resistant:Total Bacteria | 0.038 | 0.981 | - | - | - |
| 16S gene copies (log) | 8.769 | 0.012 | 0.116 | 0.010 | 0.239 |
| *Bla*$_{TEM}$ gene copies (log) | 8.000 | 0.018 | 0.075 | 0.018 | 0.433 |
| *Bla*$_{TEM}$ copy per 16S copy | 7.423 | 0.024 | 0.043 | 0.036 | 0.845 |

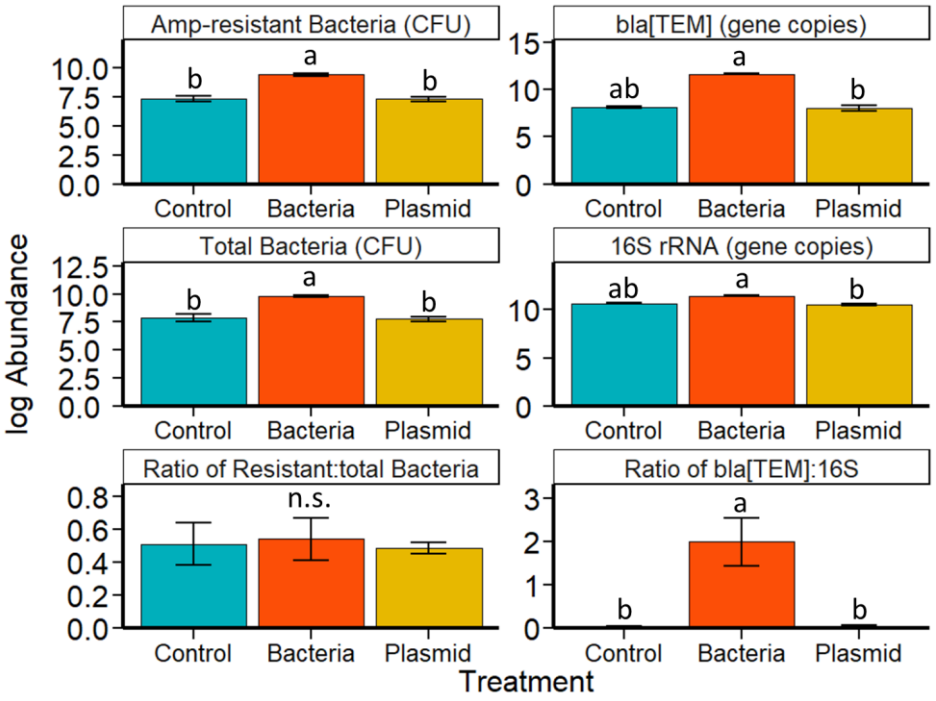


**Figure 4.2.** Mean (n=4) total load (sum of all 10 samples over 3 pore volumes) of bacteria (log CFU) and genes (log copies) in the leachate from saturated soil columns with either no additions (Control), ampicillin resistant *E. coli* (Bacteria), or a plasmid containing an ARG (Plasmid). Lowercase letters indicate significance among treatments. n.s. = not significant

Both total culturable bacteria and ampicillin-resistant culturable bacteria concentrations showed similar patterns of total culturable and culturable ampicillin-resistant bacteria in soil leachate. The total culturable bacteria concentration was initially $4.9x10^4 \pm 9.6x10^4$ CFU $ml^{-1}$ in the Bacteria treatment, increased 10-fold after only 1/3 pore volume, peaked at $1.2x10^6 \pm 4.8x10^5$ CFU $ml^{-1}$ at 1 pore volume, and then decreased to a concentration of $4.7x10^5 \pm 2.0x10^5$ CFU $ml^{-1}$ (Figure 4.3). The ampicillin-resistant culturable bacteria concentration was initially $5.7x10^3 \pm$

$1.1x10^4$ CFU $ml^{-1}$ in the Bacteria treatment, peaked at $4.6x10^5 \pm 1.4x10^5$ CFU $ml^{-1}$ at 1 pore volume, and then decreased to a concentration of $1.7x10^5 \pm 8.6x10^4$ CFU $ml^{-1}$. In contrast, the Plasmid and Control treatments had relatively low and stable concentrations of total and resistant bacteria in all leachate samples. In the Plasmid treatment, total bacteria ranged from $4.0x10^3 \pm 4.4x10^3$ CFU $ml^{-1}$ to $1.2x10^4 \pm 1.8x10^4$ CFU $ml^{-1}$, while culturable resistant bacteria ranged from $2.0x10^3 \pm 1.9x10^3$ CFU $ml^{-1}$ to $4.2x10^3 \pm 5.0x10^3$ CFU $ml^{-1}$. In the control treatment, total bacteria ranged from $4.8x10^3 \pm 5.2x10^3$ CFU $ml^{-1}$ to $7.8x10^4 \pm 1.5x10^5$ CFU $ml^{-1}$, while culturable resistant bacteria ranged from $2.8x10^3 \pm 3.4x10^3$ CFU $ml^{-1}$ to $5.5x10^3 \pm 8.2x10^3$ CFU $ml^{-1}$. This is not surprising, as we did not add live bacteria to the Control or Plasmid treatments, and the short time frame of the experiment would not allow bacteria to take up and express the $bla_{TEM}$ gene in the Plasmid treatment. Thus, there should not be a detectable increase in live resistant bacteria. The ratio of resistant to total bacteria among all three treatments was relatively similar at a ratio of 0.51 resistant bacteria:total bacteria. However, there was a large amount of variation, particularly at 2/3 and 2 2/3 pore volumes.

Next, we quantified concentrations of 16S rRNA and $bla_{TEM}$ gene copies in leachate as molecular indicators of total bacteria and the specific plasmid added in this experiment. The concentrations of 16S and $bla_{TEM}$ gene copies increased rapidly in the Bacteria treatment by ~100-fold of the initial concentration for 16S copies and ~100,000-fold of the initial concentration for $bla_{TEM}$ copies, and then declined after 1.67 pore volumes. In the plasmid treatment, the concentration of $bla_{TEM}$ increased 14-fold of the initial concentration after 0.67 pore volumes, then the concentrations slowly decreased in subsequent pore volumes of leachate. However, this difference is negligible, with concentrations still within the same order of

magnitude as the Control treatment and roughly 100-1,000x lower than values seen in the Bacteria treatment. Thus, even though plasmid containing $bla_{TEM}$ was added to the soil surface, it did not readily leach out of the soil column. The ratio of $bla_{TEM}$ to 16S genes increased from 0.0074 ± 0.0078 to 3.8 ± 2.6 in the Bacteria treatment throughout sampling. The ratio of $bla_{TEM}$ to 16S genes ranged from $4.1x10^{-4} \pm 1.8x10^{-4}$ to 0.19 ± 0.37 in the Plasmid treatment and ranged from $5.5x10^{-4} \pm 9.0x10^{-4}$ to 0.22 ± 0.44 in the Control treatment.

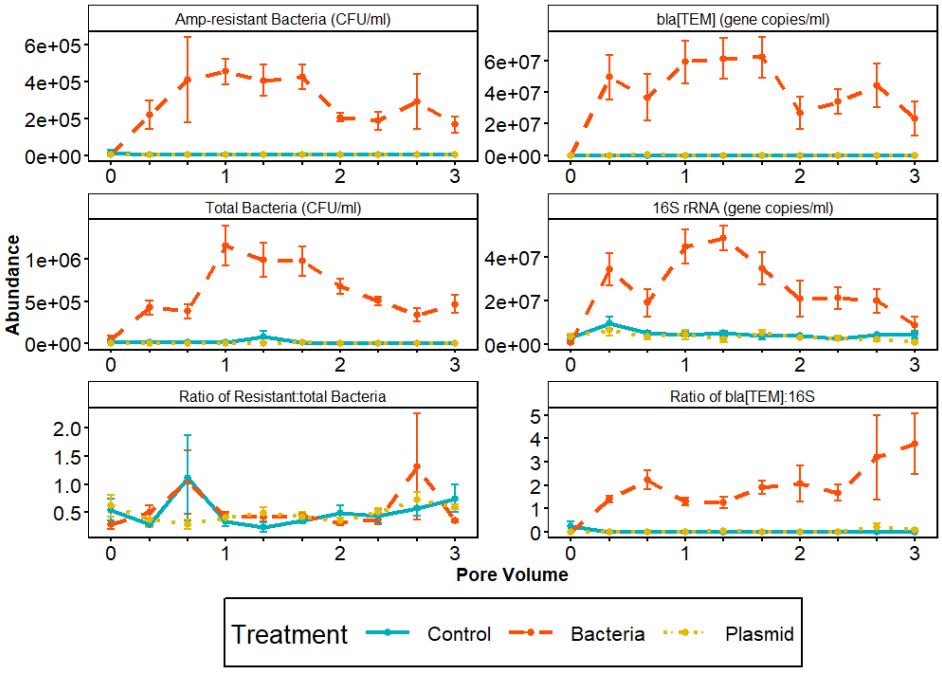


**Figure 4.3.** Mean (n=4) concentration of bacteria (CFU $ml^{-1}$) or genes (copies $ml^{-1}$) in leachate over 3 pore volumes in saturated soil columns with either no additions (Control), ampicillin resistant *E. coli* (Bacteria), or a plasmid encoding for ampicillin resistance (Plasmid).

*4.4.2 ARB and ARGs in soil*

Differences in the total loads of bacteria and genes throughout the soil columns were compared to determine how much of the added bacteria or gene was retained within the soil (Table 4.2). Culturable ampicillin-resistant bacteria were twice as abundant in the Bacteria treatment than the Plasmid (P=0.007) or Control treatments (P = 0.004, Fig 4.4). However, there were no differences among treatments for total bacteria (P = 0.382), or the ratio of resistant : total bacteria (P = 0.174, Table 4.2). In the Bacteria treatment, the total abundance of 16S gene copies was 30% higher, *bla*$_{TEM}$ gene copies were almost 1000-fold higher, and the ratios of *bla*$_{TEM}$:16S were almost 3-fold greater in the Bacteria treatment than the Control treatment (P < 0.05, Table 4.2), although there was not a significant difference between the Bacteria and Control or between the Plasmid and Control treatments.

**Table 4.2.** Comparisons of mean total loads and ratios of genes and bacteria among treatments in soil with ANOVAs and post-hoc pairwise t-tests with FDR adjusted p-values.

| **Parameter** | ANOVA | | Post-hoc t-test P-values | | |
|---|---|---|---|---|---|
| | F | P-value | Bacteria: Control | Bacteria: Plasmid | Control: plasmid |
| Total Bacteria (log) | 1.073 | 0.382 | - | - | - |
| Amp-resistant Bacteria (log) | 11.65 | 0.003 | 0.004 | 0.007 | 0.424 |
| Resistant:Total Bacteria* | 3.500 | 0.174 | - | - | - |
| 16S gene copies (log) | 4.48 | 0.045 | 0.046 | 0.185 | 0.232 |
| *Bla*$_{TEM}$ gene copies (log)* | 9.846 | 0.007 | 0.005 | 0.117 | 0.175 |
| *Bla*$_{TEM}$ copy per 16S copy* | 9.846 | 0.007 | 0.005 | 0.117 | 0.175 |

* Kruskal-Wallis nonparametric ANOVAs and post-hoc Dunn tests were used since data violated assumptions of normality.

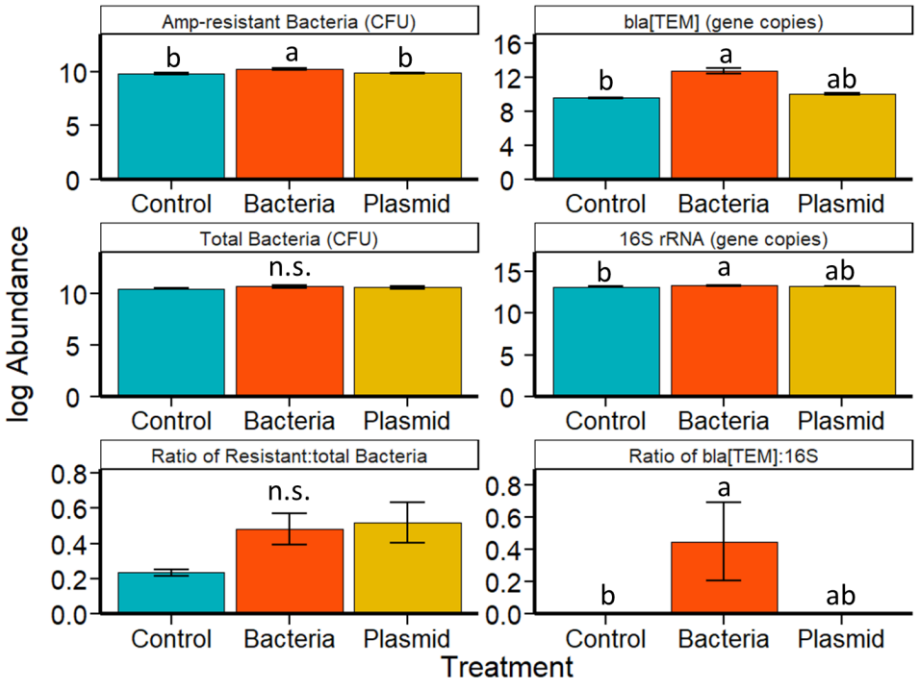


**Figure 4.4.** Mean (n=4) total load (for entire soil column) of bacteria (log CFU) and genes (log copies) in the soil from saturated soil columns with either no additions (Control), antibiotic resistant *E. coli* (Bacteria), or a plasmid containing an ARG (Plasmid). Lowercase letters indicate significance among treatments. N.s. = not significant.

We examined the ARGs and ARB in the soil to determine if and where the bacteria and plasmids were retained in the soil columns (Figure 4.5). Overall, based on the 2-way ANOVA, there were no differences in total culturable bacteria among treatments ($F_{2,36} = 1.78$, $P = 0.183$), or with depth ($F_{2,36} = 2.12$, $P = 0.115$). However, the abundance of bacteria in the Plasmid treatment was much lower at 5-10 cm than at 0-5 cm ($P = 0.014$, Fig. 4.5). There were significant differences with treatment ($F_{2,36} = 6.03$, $P = 0.006$) and depth ($F_{2,36} = 2.96$, $P = 0.045$) in

culturable resistant bacteria. Notably, the post hoc analysis showed the Bacteria treatment had more culturable resistant bacteria than the Control (P = 0.007) or Plasmid treatment (P = 0.027), and there were more culturable resistant bacteria in the top 5 cm of the column compared to the bottom 5 cm (P = 0.042). The ratio of resistant to total bacteria was highly variable and there were no differences with treatment ($F_{2,36}$ = 2.12, P = 0.138), or depth ($F_{2,36}$ = 0.87, P = 0.466).

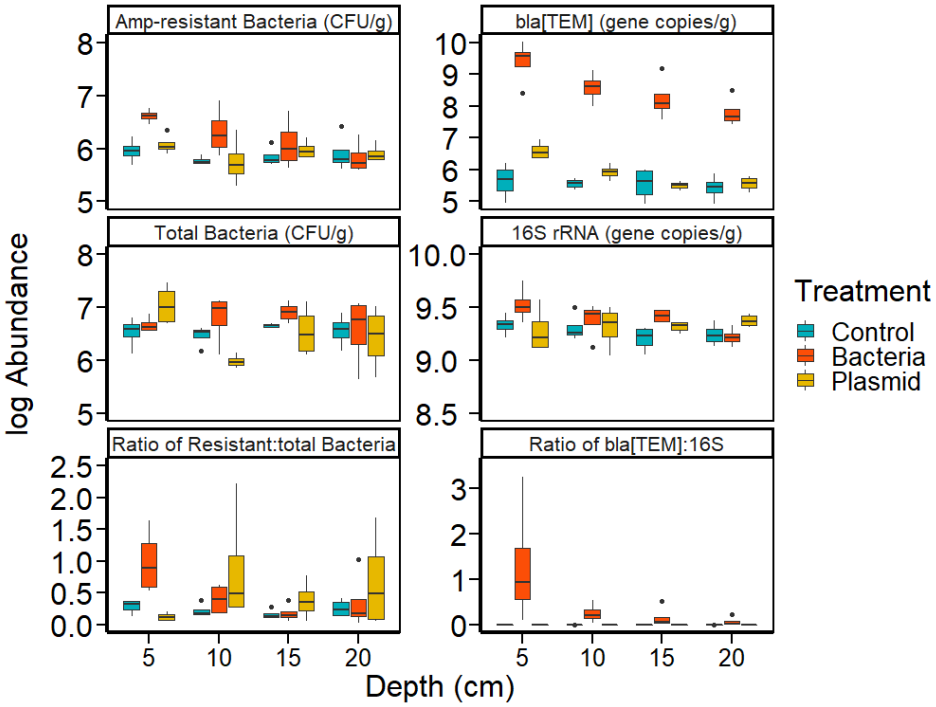


**Figure 4.5.** Mean (n=4) concentration of bacteria (log CFU g soil$^{-1}$) and genes (log copies g soil$^{-1}$) by depth in saturated soil columns with either no additions (Control), antibiotic resistant *E. coli* (Bacteria), or a plasmid containing an ARG (Plasmid).

The concentrations of 16S and *bla*$_{TEM}$ gene copies in the soil were compared to track molecular indicators of total bacteria and ARG plasmid copies retained in the column (Figure 4.5). The abundance of 16S gene copies did not vary by depth ($F_{2,36}$ = 1.18, P = 0.33) or by the interaction of treatment and depth ($F_{6,36}$ = 1.8, P = 0.13). However, the ANOVA resulted in a P-value of 0.069 for treatment ($F_{2,36}$ = 2.88), and no post-hoc comparisons were significantly different. In contrast, the abundance of *bla*$_{TEM}$ gene copies varied with treatment ($F_{2,36}$ = 214, P < $2x10^{16}$) and depth ($F_{3,36}$ = 10.4, P = $4.6x10^{-5}$). The concentration of *bla*$_{TEM}$ genes was higher in the Bacteria treatment compared to Control and Plasmid treatments (P < $2x10^{16}$) and was higher in the top 5cm compared to 5-10 cm (P = 0.034), 10-15 cm (P = 0.0007), and 15-20 cm (P = $4.4x10^{-5}$). The ratio of *bla*$_{TEM}$ to 16S varied with treatment ($F_{2,36}$ = 6.44, P = 0.004) and the interaction of treatment and depth ($F_{6,36}$ = 2.69, P = 0.029). However, variation by depth was only marginally significant ($F_{3,36}$ = 2.70, P = 0.060). Notably, Bacteria had a higher *bla*$_{tem}$:16S ratio than the Control treatment (P = 0.01) and the Plasmid treatment (P = 0.01). Additionally, within the Bacteria treatment, all depth increments were significantly different from one another (P<0.05), with decreasing values with depth (Appx C, Figure C.1).

Given that there were no detectable increases in plasmids in the soil or the leachate, the follow-up experiment was designed to investigate mechanisms for loss of detectable plasmids from the columns. Specifically, incubation experiments were designed to detect whether plasmids were adsorbing to soil particles, thus preventing recovery during DNA extraction, or breaking down via microbial degradation. While concentrations of detected plasmids varied over time ($F_2$ = 6.14, P = 0.011; Table 4.3) recovered concentrations were very close to the $10^8$ copies $g^{-1}$ soil that were initially added. Within the 24 hr incubation, there were no significant

differences in $bla_{TEM}$ gene copies between 1 and 8 hours or 1 and 24 hours, but the concentration of detected plasmid was 3.5 times lower at 24 hours compared to 8 hours (P = 0.009). Additionally, detected plasmid concentrations in soils without sodium azide, which would inhibit microbial degradation, were approximately 2.5-fold higher compared to soils with sodium azide (P = 0.004).

**Table 4.3**. Mean ± standard deviation of detected plasmids containing $bla_{TEM}$ (log copies $g^{-1}$) extracted from soil with or without sodium azide 1,8, and 24 hours after adding plasmids. Italic letters represent significant differences over time. Significant differences were also detected between the control and sodium-azide soils across all time points.

| Time (hrs) | Control | Na-azide |
|---|---|---|
| 1 | 7.75 ± 0.09 *ab* | 7.28 ± 0.12 *ab* |
| 8 | 7.95 ± 0.12 *a* | 7.50 ± 0.14 *a* |
| 24 | 7.35 ± 0.12 *b* | 7.07 ± 0.20 *b* |

**4.5 Discussion**

Our objectives were to determine whether biological elements of antibiotic resistance (viable bacteria and extracellular DNA containing ARGs) moved through or were retained in a natural soil column in a similar manner. One key finding of this work is that increased concentrations of viable ampicillin-resistant bacteria both remained in the soil matrix and readily transported and leached in pore water passing through the column. However, when extracellular plasmids containing the same ampicillin resistance gene were added to soil columns at the same concentrations, there were no increases in ARG copies in the leached porewater that moved through the soil or in the soil 3 days after adding them. Thus, we observed evidence of distinctly different fate and transport of different elements of AMR - naked DNA did not readily leach or persist for long in the soil, whereas ARB and the ARGs they carried did both. In other words, these results suggest that ARB showed significantly greater risk for potentially spreading AMR,

either by remaining in the soil or potentially leaching through soil into groundwater, where they could subsequently replicate or transfer resistance genes to other organisms (Poté et al., 2010).

There was consistent evidence of increased transport and persistence in the columns that had viable bacteria added. In the leachate, there was a much greater abundance of total bacteria, resistant bacteria, $bla_{TEM}$ gene copies, and 16S gene copies in the Bacteria treatment compared to the Control and Plasmid treatments. Bacterial transport through soil matrices via porewater flow is commonly observed. Fecal coliforms can leach into groundwater following manure applications (Unc and Goss, 2003), and previous studies have also found high abundances of ARB in runoff following applications of manure (Luby et al., 2016; Barrios et al., 2020; Hilaire et al., 2022). Furthermore, bacteria can leach through soil quickly through macropores (Chee-Sanford et al., 2009). Although our columns were sieved and packed to avoid macropores and preferential flow, it is possible, albeit unlikely, that preferential flow might have occurred. Additionally, after draining the soil columns, there was still a detectable presence of the resistant *E. coli* in the soil itself, as evidenced by higher abundances of resistant bacteria, total bacteria, 16S genes, and $bla_{TEM}$ genes compared to the control. The sandy nature of the soil may have allowed for bacteria to be carried easily by the water. The highest concentrations of bacteria and gene copies in our study were retained primarily in the top 5 cm of soil, which could be a result of bacterial cells being physically filtered by soil particles. Previous studies have also found higher abundances of ARGs in the top 5 cm of soil following manure applications, suggesting that bacteria can be retained over relatively short distances (Joy et al., 2013).

The most surprising conclusion form this study was the lack of transport observed in the columns where ARG-containing plasmids were added. Specifically, we did not observe higher

concentrations of $bla_{TEM}$ genes in the Plasmid treatment compared to the control, suggesting that extracellular DNA did not persist or transport in these systems nearly as well as viable cells. In contrast, multiple previous studies have shown that plasmids can move through soil (Ceccherini et al., 2007; Poté et al., 2010; Chen et al., 2015, 2017), with one study even observing that plasmids can move more readily through soil columns than bacteria (Rysz and Alvarez, 2006). However, we suspect that differences in the soil matrix used to fill the columns played a key role in the difference in results. In contrast to the sand used to fill columns by Rysz and Alvarez (2006) we used a natural soil containing organic matter, which can impact movement of ARGs within soil (Gothwal and Thatikonda, 2018).

Given that the leachate from the Plasmid treatment did not contain detectable increases in the added ARG, we expected to detect plasmid containing the ampicillin resistance gene that had been retained in the soil surface, as ARGs have been shown to bind to soil (Barrios et al., 2021). However, there was also no detectable increase in $bla_{TEM}$ concentrations retained in the Plasmid treatment soil columns. In previous work using unsaturated soil columns, plasmids carrying an ARG were able to move quickly through the column and were detected in leachate (Poté et al., 2010). However, the concentrations of ARGs detected in soil in those columns was reduced after 3 hours of exposure time, likely due to sorption to soil particles and degradation by nucleases present in soil. It should be noted that if plasmids are bound to soil particles or organic matter, but not degraded, they may still be taken up by some bacteria (Chee-Sanford et al., 2009). Our follow-up experiment showed that any sorption of plasmids to soil particles was did not cause significant differences in detectability. Previous work has shown that increasing ionic strength of solutions can cause plasmids to sorb to quartz sand and soil; however, at similar concentrations

to our experiment, little sorption occurred (Chen et al., 2015, 2017). Given that we did not see evidence of poor recovery, or degradation within the first 24 hr, in our follow-up experiment, the results suggest that there may have been a lag in biological activity after the addition of plasmid and the majority of loss via degradation most likely occurred in the soil between 24 and 72 hours.

### 4.6 Conclusions

This study was designed to further the knowledge of the spread of AMR in soil by independently comparing the transport of both ARB and ARG in identical controlled experimental columns using a natural soil. The results suggest AMR is more likely to spread as ARB rather than extracellular ARGs, which contradicts previous studies. Of course, it is important to note that only one soil type was used in this experiment, and it is probable that transport of different elements of AMR will vary among soil types. As we chose a soil that was highly permeable, our results could be considered a worst-case scenario for bacterial transport. Additional controlled experimental work using a range of natural soil types are needed to fully understand the risks of AMR transmission in soil. However, the stark difference in transport and persistence of the two elements observed in this work highlight that it can be important at times to differentiate whether ARGs are present in soil as extracellular DNA or are contained within live bacteria. For example, molecular approaches such as qPCR and metagenomics are commonly used to track the fate and transport of ARGs in environmental systems, but neither of these methods easily distinguish between ARB and extracellular ARGs. Likewise, land application of manure might have significantly different risks for transport and persistence in

some soils depending on whether ARGs are present extracellularly or within viable ARB. Overall, AMR is a challenging problem in that risk exists from its spread via multiple mechanisms such as antibiotic compounds, resistant organisms, or copies of resistance genes. Improved understanding of the behaviors of each of these independent mechanisms will improve risk management of AMR in the future.

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Williams-Nguyen, J., Sallach, J.B., Bartelt-Hunt, S., Boxall, A.B., Durso, L.M., McLain, J.E., Singer, R.S., Snow, D.D., Zilles, J.L., 2016. Antibiotics and Antibiotic Resistance in Agroecosystems: State of the Science. Journal of Environmental Quality 45, 394–406. doi:10.2134/jeq2015.07.0336

## Chapter 5. Overall Conclusions

The overall goal of this dissertation was to gain a better understanding of the factors that contribute to the spread of AMR in soil. Specifically, the objectives were to 1) examine the impact of multi-year repeated additions of manure from cattle administered antibiotics on soil microbial communities, 2) determine the interactive effects of soil moisture and type on soil microbial communities exposed to antibiotics and manure, and 3) differentiate between vertical transport of AMR in the form of viable ARB or ARGs in extracellular plasmids.

We found that microbial community structures were impacted by manure and manure from cattle administered antibiotics. These impacts were seen across multiple years of manure additions and across soil types and moisture contents. Additionally, ARG abundances, microbial activity, microbial biomass, and inorganic soil nitrogen pools were all impacted by manure and manure from cattle administered antibiotics across soil type and moisture content. Furthermore, the environmental conditions (soil type and moisture) had an interactive effect on microbial community responses to exposure to manure and manure from cattle administered antibiotics.

Interestingly, we also found that manure, even from antibiotic-free cattle, can increase ARG abundance in soil. However, this effect was not consistent across experiments. There are a number of possible explanations for this discrepancy, including the timeline of the studies, differences between soils and conducting a field study compared to a microcosm lab study. Although we were not able to pinpoint the reason for the differences in findings between the experiments, it highlights that manure and antibiotic impacts may vary widely across environments.

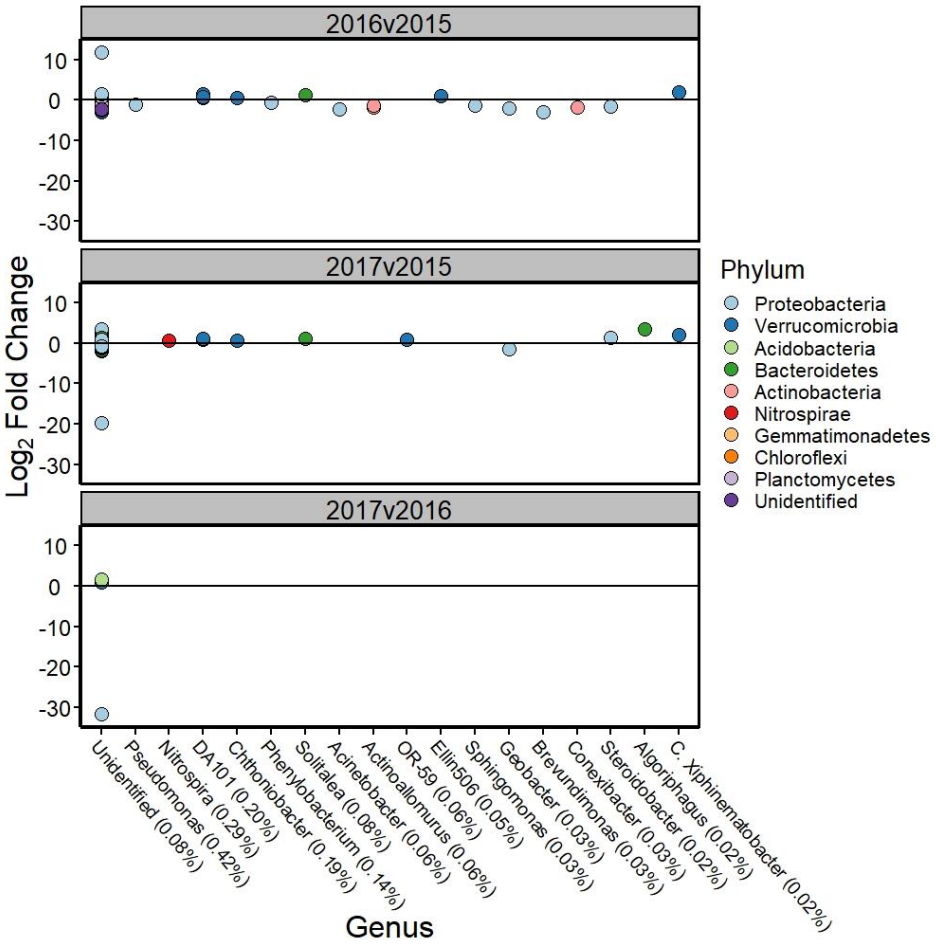
Furthermore, we used two antibiotics that had different modes of action (bacteriostatic and bactericidal) because there are a wide variety of antibiotic classes that target different bacteria. We found the impacts of manure from cattle administered antibiotics on soil microbial community structure, ARG abundances, microbial activity, microbial biomass, and inorganic soil nitrogen pools varied between antibiotics. The differences in microbial response between the two antibiotics were still detectable with two different soil types and across a wide range of soil moisture contents,

Finally, we found that viable ARB and plasmids containing ARGs do not move through soil in the same way. When added to a saturated soil, ARB quickly moved through the soil and were leached out of the soil column. However, some ARB were retained in near the soil surface. In contrast, plasmids did not move through the soil column, and were not detected in the soil surface, indicating that retained plasmids do not persist long in soil.

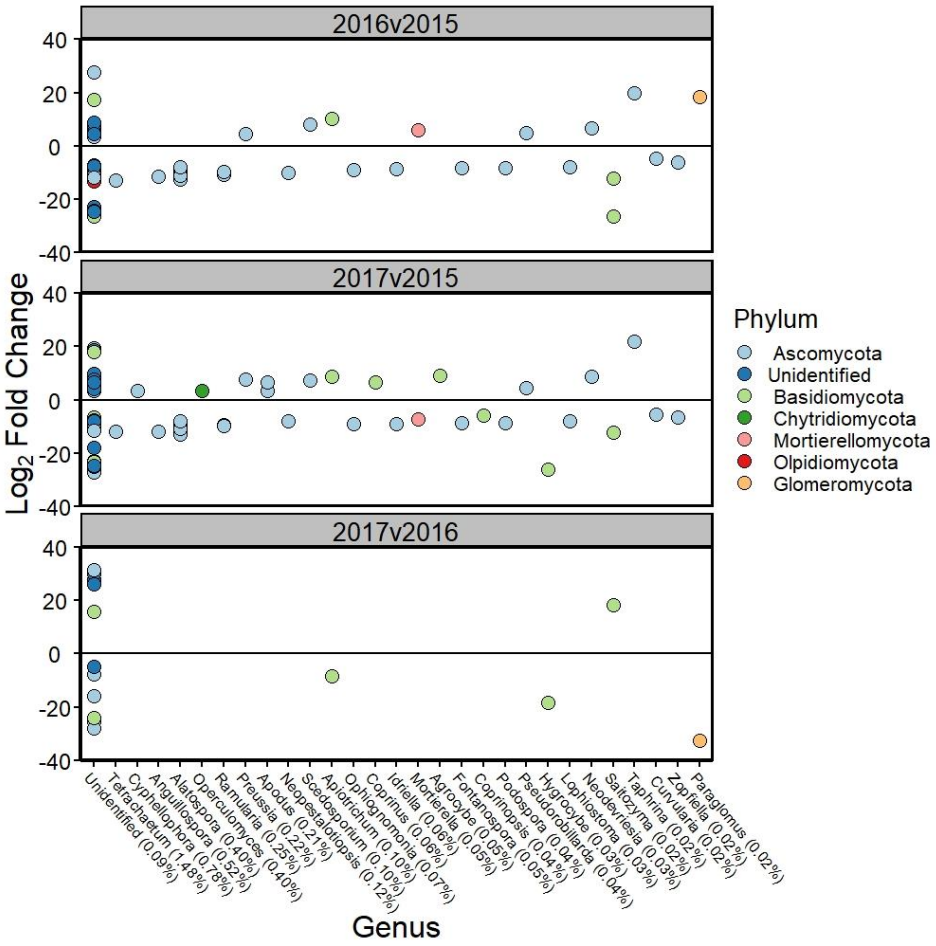
Overall, our findings indicate that management strategies to limit the spread of AMR in soil should consider the nuances of AMR in soils. It is critical to consider the implications of adding any manure to soil, as we saw even manure from antibiotic-free cattle caused an increase in ARG abundances. Furthermore, as there are large shifts in microbial responses in different environments, strategies should consider regional differences in soil environments. For example, we observed higher ARG abundances in higher moisture contents. Thus, spreading manure in drier fields when possible, may help limit persistence and spread of AMR. Additionally, our results suggest that AMR may spread more readily through natural soil matrices and persist in the environment longer than extracellular DNA. Thus, when evaluating the efficacy of manure management strategies – eg, manure composting, application, etc – the use of detection methods

that can specifically identify ARB will continue to be a valuable addition to the more commonly used molecular techniques that do not distinguish viable cells from extracellular DNA. Future research should continue evaluating the interactive relationship between soil environments and AMR in soil. Future AMR management practices that consider the diverse factors that affect the persistence and spread of AMR in the environment can help protect livestock productivity and maintain the efficacy of antibiotics to protect human and animal health.

**Appendix A: Supplementary Information for Chapter 2**




**Supplementary Figure A.1**. Fold changes between year pairs that significantly varied between year pairs for bacteria. Percent values in brackets show the relative abundance of each genus across all samples. Each point is one OTU, colored by phylum and grouped by genus.




**Supplementary Figure A.2.** Fold changes between year pairs that significantly varied between year pairs for fungi. Percent values in brackets show the relative abundance of each genus across all samples. Each point is one OTU, colored by phylum and grouped by genus.

**Supplementary Table A.1.** List of genes targeted in mfqPCR (Sandberg et al., 2018)

| Gene | Sequence (5'–3') | Amplicon length (primers excluded) | Reference |
|---|---|---|---|
| *16S rRNA* | CCTACGGGAGGCAGCAG<br>ATTACCGCGGCTGCTGG | 160 | Muyzer, G.; De Waal, E.C.; Uitterlinden, A.G. Profiling of Complex Microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S rRNA. Applied and Environmental Engineering 1993, 59, 695-700. |
| *aacA* | GTGTAACACGCAAGCACGAT<br>AGCCTCCGCGATTTCATAC | 158 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced |
| *aadA5* | ATCTTGCGATTTTGCTGACC<br>TGTACCAAATGCGAGCAAGA | 158 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. Microbiology 2009, 155:2306–2319. |
| *aadD* | ATGGGGATGATGTTAAGGCT<br>TCACTTCCACCTTCCACTCA | 113 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. Microbiology 2009, 155:2306–2319. |
| *acrD* | GGCAATCCTGTTGTGTCTGA<br>ACATGAGATTATCGAGGCCG | 145 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced |
| *ampC* | CCTCTTGCTCCACATTTGCT<br>ACAACGTTTGCTGTGTGACG | 149 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing |

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|---|---|---|---|
| | | | reduced susceptibility to selected antibiotics. Microbiology 2009, 155:2306–2319. |
| *arr2* | TTACAAGCAGGTGCAAGGAC<br>GCTCCATCAAGGCTGAAAAG | 100 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced |
| $bla_{ctx\text{-}m\text{-}32}$ | CGTCACGCTGTTGTTAGGAA<br>CGCTCATCAGCACGATAAAG | 116 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced |
| $bla_{KPC}$ | GATACCACGTTCCGTCTGG<br>GCAGGTTCCGGTTTTGTCTC | 213 | Hindiyeh M, Smollen G, Grossman Z, et al. Rapid Detection of blaKPC Carbapenemase Genes by Real-Time PCR. Journal of Clinical Microbiology 2008, 46:2879-2883. |
| $bla_{NPS}$ | GGACCATCGTCATCGAGTCT<br>ATTCGCAATCGAATACTGGG | 148 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced |
| $bla_{OXA}$ | TGATGATTGTCGAAGCCAAA<br>GCCTGTAGGCCACTCTACCC | 61 | Ross J, Topp E. Abundance of Antibiotic Resistance Genes in Bacteriophage following Soil Fertilization with Dairy Manure or Municipal Biosolids, and Evidence for Potential Transduction. Applied and Environmental Microbiology 2015, 81:7905-7913. |
| $bla_{SHV}$ | AACGGAACTGAATGAGGCGCT<br>TCCACCATCCACTGCAGCAGCT | 98 | Chia J-H, Chu C, Su L-H, et al. Development of a Multiplex PCR and SHV Melting-Curve Mutation Detection System for Detection of Some SHV and CTX-M β-Lactamases of Escherichia coli, Klebsiella pneumoniae, and Enterobacter cloacae in Taiwan. Journal of Clinical Microbiology 2005, 43:4486-4491. |
| $bla_{VIM}$ | CGCAGCTTTCTGGTTGGTAT<br>CGTGTCACCGAGTTTCTGAG | 140 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced |

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|---|---|---|---|
| *bla*$_{NDM-1}$ | ATTAGCCGCTGCATTGAT<br>CATGTCGAGATAGGAAGTG | 117 | Ahammad, Z.S.; Sreekrishnan, T.R.; Hands, C.L.; Knapp, C.W.; Graham, D.W. Increased Waterborne blaNDM-1 Resistance Gene Abundances Associated with Seasonal Human Pilgrimages to the Upper Ganges River. Environmental Science & Technology 2014, 48, 3014-3020. |
| *cadA* | GTGAGCAGGCCAGCACTGAA<br>TCTCGATGCGGTAGGTGGTC | 215 | Taghavi S, Lesaulnier C, Monchy S, et al. Lead(II) resistance in Cupriavidus metallidurans CH34: interplay between plasmid and chromosomally-located functions. Antonie van Leeuwenhoek 2009, 96:171–182. |
| *catB8* | GGGGAACTTCTTTCTGAGCA<br>CCCCGCTTCCTATAGAACAA | 135 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. Microbiology 2009, 155:2306–2319. |
| *chrA* | TCACGCCGGAATATAACTAC<br>CGTACCCTGATCAATCACTT | 229 | Patra RC, Malik S, Beer M, et al. Molecular characterization of chromium (VI) reducing potential in Gram positive bacteria isolated from contaminated sites. Soil Biology & Biochemistry 2010, 42:1857-1863. |
| *cmlB* | TAATTGGCGGTATTCCCTTG<br>AGCGTAAGCTGAATTGTGCC | 107 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced |
| *copA* | GTGTACGGTCCGCTGGTTAT<br>CTTGAACACTCCGGTCCAG | 297 | Shahla SN, Mohammad RK, Giti E, et al. Molecular analysis of copper resistance determinant (copA) in copper ixode nanoparticles resistant Pseudomonas fluorescens CuO-2 isolated from soil. International Journal of Biosciences 2014, 5:97-104. |
| *dfr13* | AATCGGTCCGCATTTATCTG<br>TTGGTAAGGGCTTGCCTATG | 134 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. Microbiology 2009, 155:2306–2319. |

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|---|---|---|---|
| *ereB* | TCTGCATTATGCCAACGGTA<br>TCTGCTCACTTTGTGGGTTTT | 117 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced |
| *ermB* | GATACCGTTTACGAAATTGG<br>GAATCGAGACTTGAGTGTGC | 324 | Chen, J.; Yu, Z.; Michel Jr., F.C.; Wittum, T.; Morrison, M. Development and Application of Real-Time PCR Assays for Quantification of erm Genes Conferring Resistance to Macrolides-Lincosamides-Streptogramin B in Livestock Manure and Manure Management System. Applied and Environmental Microbiology 2007, 73, 4407-4416. |
| *ermF* | CGACACAGCTTTGGTTGAAC<br>GGACCTACCTCATAGACAAG | 269 | Ma, Y.; Wilson, C.A.; Novak, J.T.; Riffat, R.; Aynur, S.; Murthy, S.; Pruden, A. Effect of Various Sludge Digestion Conditions on Sulfonamide, Macrolide, and Tetracycline Resistance Genes and Class I Integrons. Environmental Science & Technology 2011, 45, 7855-7861. |
| *floR* | TCGTCATCTACGGCCTTTTC<br>CTTGACTTGATCCAGAGGGC | 148 | Szczepanowski, R.; Linke, B.; Krahn, I. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria |
| *imp13* | AGGAGCGGCTTTACCTGATT<br>CGCTCCACAAACCAATTGAC | 158 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced |
| *intI1* | CCTCCCGCACGATGATC<br>TCCACGCATCGTCAGGC | 246 | Goldstein, C.; Lee, M.D.; Sanchez, S.; Hudson, C.; Phillips, B.; Register, B.; Grady, M.; Liebert, C.; Summers, A.O.; White, D.G.; Maurer, J.J. Incidence of Class 1 and 2 Integrases in Clinical and Commensal Bacteria from Livestock, Companion Animals, and Exotics. Antimicrobial Agents and Chemotherapy 2001, 45, 723-726. |
| *intI2* | GACGGCTACCCTCTGTTATCTC<br>TGCTTTTCCCACCCTTACC | 154 | Barraud O, Baclet MC, Denis F, et al. Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons. Journal of Antimicrobial Chemotherapy 2010, 65, 1642 – 1645. |

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|---|---|---|---|
| *intI3* | GGATGTCTGTGCCTGCTTG<br>GCCACCACTTGTTTGAGGA | 100 | Barraud O, Baclet MC, Denis F, et al. Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons. Journal of Antimicrobial Chemotherapy 2010, 65, 1642 – 1645. |
| *mefE* | CCTGCAAATGGCGATTATTT<br>AATAGCAAGCACTGCACCAG | 159 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced |
| *merA* | GTGCCGTCCAAGATCATG<br>GGTGGAAGTCCAGTAGGGTGA | 321 | Pitkanen LK, Tamminen M, Hynninen A, et al. Fish Farming Affects the Abundance and Diversity of the Mercury Resistance merA in Marine Sediments. Microbes and Environments 2001, 26:205-211. |
| *mexB* | GTGTTCGGCTCGCAGTACTC<br>AACCGTCGGGATTGACCTTG | 204 | McNamara P, LaPara T, Novak P. The Impacts of Triclosan on Anaerobic Community Structures, Function, and Antimicrobial Resistance. Environmental Science & Technology 2014, 48:7393-7400. |
| *nikA* | AATATCAGGCAGACGGTTCG<br>AGGGTGAAGGTCCAGGTTTT | 45 | Fantino, J-R, Py B, Fontecase M, et al. A genetic analysis of the response of Escherichia coli to cobalt stress. Environmental Microbiology 2010, 12:2846–2857. |
| *qacF* | TGGCTGTTTCAATCTTTGGC<br>GCCCATACAGCGTAAGCAAT | 132 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. Microbiology 2009, 155:2306–2319. |
| *qnrA* | AGGATTTCTCACGCCAGGATT<br>CCGCTTTCAATGAAACTGCA | 83 | Cummings DE, Archer KA, Arriola DJ, et al. Broad Dissemination of Plasmid-Mediated Quinolone Resistance Genes in Sediments of Two Urban Coastal Wetlands. Environmental Science & Technology 2011, 45:447-454. |
| *qnrB* | AAATATGGCTCTGGCACTCG<br>CTTTCAGCATCGCACGACTA | 151 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing |

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|---|---|---|---|
| | | | reduced susceptibility to selected antibiotics. Microbiology 2009, 155:2306–2319. |
| *rcnA* | GGGCACTCAAAAACGATGAT<br>GCGAAATAGTTGCTGCCAGT | 54 | Fantino, J-R, Py B, Fontecase M, et al. A genetic analysis of the response of Escherichia coli to cobalt stress. Environmental Microbiology 2010, 12:2846–2857. |
| *strB* | GCCTGTTTTTCCTGCTCATT<br>CGCAGTTCATCAGCAATGTC | 110 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. Microbiology 2009, 155:2306–2319. |
| *sul1* | CCGTTGGCCTTCCTGTAAAG<br>TTGCCGATCGCGTGAAGT | 29 | Heuer, H.; Smalla, K. Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. Environmental Microbiology 2007, 9, 657-666. |
| *sul2* | GACAGTTATCAACCCGCGAC<br>GTCTTGCACCGAATGCATAA | 107 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. Microbiology 2009, 155:2306–2319. |
| *sul3* | TCCGTTCAGCGAATTGGTGCAG<br>TTCGTTCACGCCTTACACCAGC | 83 | Pei, R.; Kim, S.-C.; Carlson, K.H.; Pruden, A. Effect of River Landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). Water Research 2006, 40, 2427-2435. |
| *tet*(A) | GCTACATCCTGCTTGCCTTC<br>CATAGATCGCCGTGAAGAGG | 170 | Ng, L.-K.; Martin, I.; Alfa, M.; Mulvey, M. Multiplex PCR for the detection of tetracycline resistant genes. Molecular and Cellular Probes 2001, 15, 209-215. |
| *tet*(L) | TCGTTAGCGTGCTGTCATTC<br>GTATCCCACCAATGTAGCCG | 227 | Ng, L.-K.; Martin, I.; Alfa, M.; Mulvey, M. Multiplex PCR for the detection of tetracycline resistant genes. Molecular and Cellular Probes 2001, 15, 209-215. |
| *tet*(M) | GTGGACAAAGGTACAACGAG<br>CGGTAAAGTTCGTCACACAC | 366 | Ng, L.-K.; Martin, I.; Alfa, M.; Mulvey, M. Multiplex PCR for the detection of tetracycline resistant genes. Molecular and Cellular Probes 2001, 15, 209-215. |

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|---|---|---|---|
| *tet*(S) | CAAGGATTGTACGGTTGGAAA<br>TTTCGAAGCTAAGATATGGCTC | 129 | Szczepanowski R, Linke B, Krahn I, et al. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced |
| *tet*(W) | GAGAGCCTGCTATATGCCAGC<br>GGGCGTATCCACAATGTTAAC | 126 | Aminov, R. I.; Garrigues-Jeanjean, N.; Mackie, R. I. Molecular ecology of tetracycline resistance: Development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. App. Environ. Microbiol. 2001, 67, 22-32. |
| *tet*(X) | AGCCTTACCAATGGGTGTAAA<br>TTCTTACCTTGGACATCCCG | 237 | Ghosh, S.; Sadowsky, M.J.; Roberts M.C.; Gralnick, J.A.; LaPara, T.M. Sphingobacterium sp. strain PM2-P1-29 harbours a functional tet(X) gene encoding for the degradation of tetracycline. Journal of Applied Microbiology 2009, 106, 1336-1342. |
| *vanA* | GTAGGCTGCGATATTCAAAGC<br>CGATTCAATTGCGTAGTCCAA | 189 | Bell JM, Paton JC, Turnidge J. Emergence of Vancomycin-Resistant Enterococci in Australia: Phenotypic and Genotypic Characteristics of Isolates. Journal of Clinical Microbiology 1998, 36:2187-2190. |
| *vanB* | TTGCATGGACAAATCACTGG | 319 | Graham M, Ballard A, Grabasch EA, et al. High Rates of Fecal Carriage of Nonenterococcal vanB in both Children and Adults. Antimicrobial Agents and Chemotherapy 2008, 52:1195-1197. |

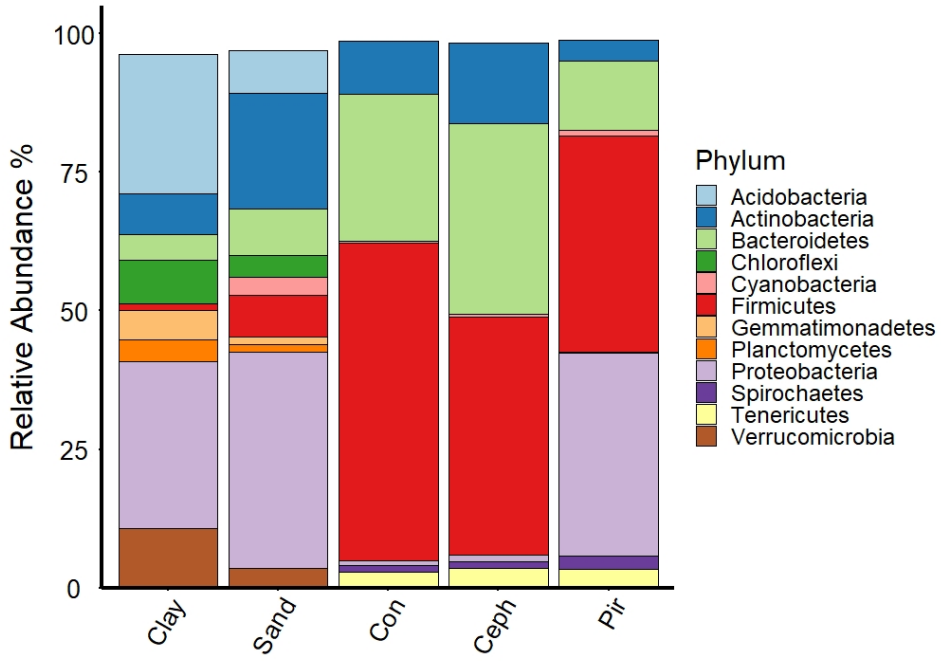
**Supplementary Table A.2**. Mean abundances of ARGs (log10 copies g-1 soil) present in at least 20% of all samples by Treatment, and the FDR adjusted p-values from Mann-Whitney U pairwise comparisons.

| | $log_{10}$ Mean Abundance | | | | FDR-adjusted p-value | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| ARG | NM | CON | CEPH | PIR | CON & NM | CEPH & NM | PIR & NM | CEPH & CON | PIR & CON | PIR & CEPH |
| *aadA5* | 0.00 | 2.17 | 2.90 | 3.33 | **0.0013** | **0.0001** | **< 0.0001** | 0.9653 | 0.2035 | 0.7485 |
| *aadD* | 0.24 | 1.32 | 2.92 | 3.19 | 0.0715 | **0.0004** | **0.0002** | 0.2139 | 0.0639 | 0.7485 |
| *bla$_{SHV}$* | 8.09 | 7.88 | 7.03 | 7.33 | 0.0881 | 0.2378 | **0.0151** | 1.0000 | 0.6816 | 0.7485 |
| *cadA* | 1.00 | 1.89 | 1.49 | 1.64 | 0.3192 | 0.5385 | 0.4494 | 0.9653 | 0.6975 | 1.0000 |
| *ermF* | 0.00 | 3.50 | 3.87 | 4.53 | **0.0001** | **< 0.0001** | **< 0.0001** | 0.9653 | 0.2035 | 0.7485 |
| *floR* | 0.65 | 5.46 | 5.59 | 5.81 | **< 0.0001** | **< 0.0001** | **< 0.0001** | 0.9653 | 0.2836 | 0.7485 |
| *intI1* | 5.15 | 6.49 | 6.60 | 6.67 | **0.0001** | **0.0001** | **< 0.0001** | 0.9653 | 0.2836 | 0.7485 |
| *intI2* | 0.27 | 3.46 | 3.57 | 3.75 | **0.0002** | **0.0002** | **0.0001** | 0.9653 | 0.5544 | 0.7485 |
| *merA* | 0.79 | 0.59 | 2.63 | 2.57 | 0.7755 | **0.0369** | **0.0417** | 0.2139 | 0.1261 | 0.9733 |
| *mexB* | 4.91 | 4.25 | 4.47 | 3.64 | 0.9486 | 0.8599 | 0.2010 | 0.9653 | 0.2836 | 0.7485 |
| *strB* | 0.66 | 5.12 | 5.30 | 5.49 | **< 0.0001** | **< 0.0001** | **< 0.0001** | 0.9653 | 0.7879 | 0.7485 |
| *sul1* | 0.41 | 5.00 | 5.16 | 5.67 | **< 0.0001** | **< 0.0001** | **< 0.0001** | 0.9653 | 0.1855 | 0.7485 |
| *sul2* | 0.20 | 4.53 | 4.78 | 4.73 | **< 0.0001** | **< 0.0001** | **< 0.0001** | 0.9653 | 0.2836 | 0.7485 |
| *tet*(A) | 1.07 | 3.05 | 2.82 | 1.87 | **0.0310** | 0.0506 | 0.3219 | 0.9653 | 0.2836 | 0.7485 |
| *tet*(L) | 0.21 | 0.41 | 1.77 | 2.65 | 0.6467 | **0.0207** | **0.0003** | 0.2139 | **0.0120** | 0.7485 |
| *tet*(M) | 0.20 | 3.09 | 3.18 | 3.66 | **0.0002** | **0.0002** | **0.0001** | 0.9653 | 0.3908 | 0.7485 |
| *tet*(W) | 1.14 | 4.76 | 5.13 | 5.08 | **< 0.0001** | **< 0.0001** | **< 0.0001** | 0.7685 | 0.2836 | 0.8894 |
| *tet*(X) | 0.00 | 4.43 | 4.70 | 4.81 | **< 0.0001** | **< 0.0001** | **< 0.0001** | 0.9653 | 0.4500 | 0.7485 |

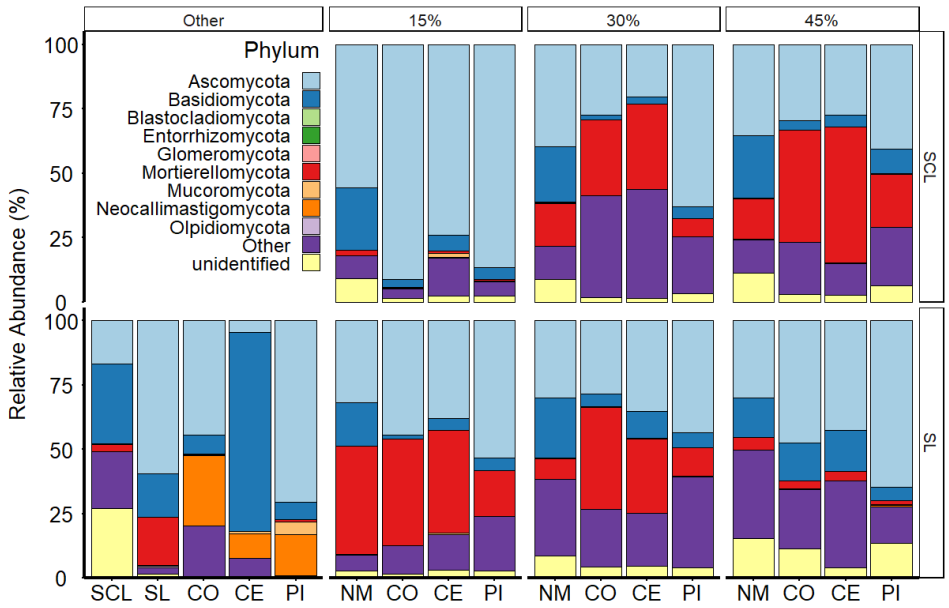
**Supplementary Table A.3.** Mean normalized abundances of ARGs (copy/16S copy) present in at least 20% of all samples by Treatment, and the FDR adjusted p-values from Mann-Whitney U pairwise comparisons.

| | Mean Abundance (copy/16S copy) | | | | FDR-adjusted p-value | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| ARG | NM | CON | CEPH | PIR | CON & NM | CEPH & NM | PIR & NM | CEPH & CON | PIR & CON | PIR & CEPH |
| *aadA5* | 0.0E+00 | 3.8E-06 | 3.7E-06 | 9.3E-06 | **0.0013** | **0.0001** | **< 0.0001** | 0.9369 | 0.2477 | 0.7151 |
| *aadD* | 1.9E-07 | 1.1E-06 | 1.4E-05 | 8.6E-06 | 0.0642 | **0.0004** | **0.0002** | 0.2139 | 0.0779 | 0.8849 |
| *bla*$_{SHV}$ | 3.1E-02 | 2.0E-02 | 2.0E-02 | 1.7E-02 | 0.1520 | 0.1790 | 0.0610 | 0.9369 | 0.6648 | 0.9749 |
| *cadA* | 2.6E-06 | 1.9E-06 | 1.9E-06 | 1.6E-06 | 0.3390 | 0.6829 | 0.4265 | 0.9369 | 0.6845 | 1.0000 |
| *ermF* | 0.0E+00 | 4.0E-05 | 3.1E-04 | 3.2E-04 | **0.0001** | **< 0.0001** | **< 0.0001** | 0.9369 | 0.2370 | 0.7151 |
| *floR* | 6.7E-07 | 1.4E-04 | 5.7E-04 | 4.9E-04 | **< 0.0001** | **< 0.0001** | **< 0.0001** | 0.9369 | 0.2626 | 0.7151 |
| *intI1* | 1.7E-04 | 8.3E-04 | 1.5E-03 | 1.4E-03 | **0.0008** | **0.0004** | **0.0001** | 0.9369 | 0.2370 | 0.7151 |
| *intI2* | 4.4E-07 | 1.3E-05 | 3.2E-05 | 6.3E-05 | **0.0002** | **0.0001** | **0.0001** | 0.9369 | 0.4200 | 0.7151 |
| *merA* | 2.1E-06 | 3.8E-06 | 3.2E-05 | 2.7E-05 | 0.7755 | **0.0369** | **0.0369** | 0.2139 | 0.1137 | 1.0000 |
| *mexB* | 1.9E-03 | 1.1E-03 | 1.4E-03 | 6.3E-04 | 1.0000 | 0.6883 | 0.2665 | 0.9369 | 0.3957 | 0.7151 |
| *strB* | 5.3E-07 | 1.8E-04 | 4.1E-04 | 3.0E-04 | **< 0.0001** | **< 0.0001** | **< 0.0001** | 0.9369 | 0.5811 | 0.7151 |
| *sul1* | 1.4E-07 | 1.1E-04 | 2.3E-04 | 2.9E-04 | **< 0.0001** | **< 0.0001** | **< 0.0001** | 0.9369 | 0.1590 | 0.7151 |
| *sul2* | 7.5E-08 | 3.0E-05 | 1.6E-04 | 1.0E-04 | **< 0.0001** | **< 0.0001** | **< 0.0001** | 0.9369 | 0.2626 | 0.7151 |
| *tet*(A) | 6.5E-06 | 2.2E-05 | 6.7E-06 | 8.2E-06 | **0.0408** | 0.0705 | 0.3416 | 0.9369 | 0.2626 | 0.7151 |
| *tet*(L) | 2.7E-08 | 1.3E-07 | 4.3E-06 | 2.3E-06 | 0.6467 | **0.0183** | **0.0003** | 0.2139 | **0.0177** | 0.7151 |
| *tet*(M) | 5.7E-08 | 2.3E-05 | 2.0E-05 | 4.5E-05 | **0.0003** | **0.0003** | **0.0001** | 0.9369 | 0.3818 | 0.7151 |
| *tet*(W) | 1.4E-05 | 2.7E-05 | 4.5E-05 | 4.4E-05 | **< 0.0001** | **< 0.0001** | **< 0.0001** | 0.9369 | 0.2795 | 1.0000 |
| *tet*(X) | 0.0E+00 | 8.9E-05 | 2.1E-04 | 2.7E-04 | **< 0.0001** | **< 0.0001** | **< 0.0001** | 0.9369 | 0.4200 | 0.7151 |

**Appendix B: Supplementary Information for Chapter 3**




**Supplementary Figure B.1.** Mean relative abundance (%) of the 12 most abundant phyla in bacterial communities in sandy clay loam (SCL) or sandy loam (SL) soil and in the manure from antibiotic-free cattle (Con), cattle administered cephapirin (Ceph), or cattle administered pirlimycin (Pir) before the start of the experiment.

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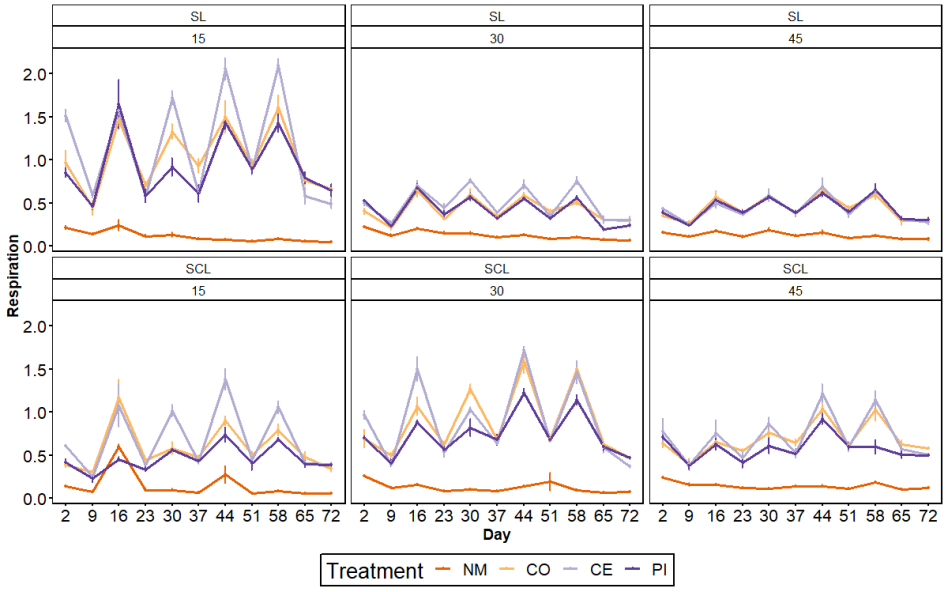
Supplementary Figure B.2. Mean relative abundance (%) of the 9 most abundant phyla in soil fungal communities in a sandy clay loam (SCL) or sandy loam (SL) soil, in the manure from antibiotic-free cattle (CO), cattle administered cephapirin (CE), or cattle administered pirlimycin (PI) before the start of the experiment; and after 10 weeks in a SCL or SL soil. Soils were kept at 15, 30, or 45% moisture content, and treatments of no manure, antibiotic-free manure, or manure from cattle administered either cephapirin or pirlimycin was added every other week.




**Supplementary Figure B.3.** Average ± SE (n=5) respiration over 10 weeks in a sandy clay loam (SCL) or sandy loam (SL) soil. Soils were kept at 15, 30, or 45% moisture content by mass, and treatments of no manure (NM), antibiotic-free manure (CO), or manure from cattle administered either cephapirin (CE) or pirlimycin (PI) was added every other week.

**Supplementary Table B.1.** Soil testing data from the two soil types.

| Soil | pH | BpH | P ppm | K ppm | Ca ppm | Mg ppm | Zn ppm | Mn ppm | Cu ppm | Fe ppm | B ppm | % OM | CEC meq/100g | % Base Sat | % Ca Sat | % Mg Sat | % K Sat |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SL | 6.26 | 6.23 | 3 | 57 | 497 | 184 | 2.1 | 13.1 | 0.8 | 15.5 | 0.3 | 3.8 | 5.2 | 80.4 | 48.2 | 29.4 | 2.8 |
| SCL | 5.8 | 6.25 | 42 | 94 | 319 | 48 | 1.1 | 15.3 | 0.9 | 13.1 | 0.1 | 0.8 | 3.1 | 71.4 | 51.1 | 12.6 | 7.7 |

**Supplementary Table B.2.** Aligned rank ANOVA with FDR-adjusted p-values and average gene abundances for each moisture content. Lowercase letters indicate significant differences for post-hoc analyses.

| | Aligned rank ANOVA | | Average ± se log copies/g soil | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Gene** | **F-value** | **Adj. P-value** | **15%** | | **30%** | | **45%** | |
| *aacA* | 23.70 | **4.66E-07** | 0.82 ± 0.81 | c | 1.72 ± 0.93 | a | 1.59 ± 0.94 | b |
| *aadA5* | 39.63 | **3.23E-09** | 1.89 ± 0.7 | c | 2.58 ± 0.37 | b | 2.85 ± 0.23 | a |
| *aadD* | 27.57 | **8.38E-08** | 1.95 ± 0.65 | b | 2.9 ± 0.23 | a | 2.86 ± 0.23 | a |
| *acrD* | 23.08 | **5.54E-07** | 2.38 ± 0.76 | b | 3.14 ± 0.08 | a | 3.06 ± 0.16 | a |
| *ampC* | 27.98 | **8.29E-08** | 2.16 ± 0.85 | b | 3.11 ± 0.09 | a | 3.03 ± 0.2 | a |
| *arr2* | 10.34 | **5.74E-04** | 0.39 ± 0.54 | b | 0.23 ± 0.37 | c | 0.86 ± 0.57 | a |
| $bla_{NDM1}$ | 3.47 | 9.60E-02 | 0.56 ± 0.57 | | 0.79 ± 0.67 | | 1.25 ± 0.69 | |
| $bla_{CTX\text{-}M32}$ | 1.63 | **4.82E-02** | 0.91 ± 0.75 | a | 0.78 ± 0.73 | a | 1.55 ± 0.82 | a |
| $bla_{imp13}$ | 9.07 | 2.26E-01 | 0.66 ± 0.67 | | 0.96 ± 0.75 | | 1.71 ± 0.89 | |
| $bla_{KPC}$ | 2.61 | **1.02E-03** | 0.88 ± 0.72 | a | 1.34 ± 0.8 | a | 1.65 ± 0.76 | a |
| $bla_{NPS}$ | 33.40 | **1.92E-08** | 0.52 ± 0.67 | b | 0.48 ± 0.64 | c | 1.4 ± 0.9 | a |
| $bla_{OXA}$ | 9.45 | **8.59E-04** | 1.13 ± 0.78 | b | 1.17 ± 0.75 | b | 2.24 ± 0.71 | a |
| $bla_{SHV}$ | 10.06 | **6.20E-04** | 3.09 ± 0.12 | b | 3.18 ± 0.05 | a | 3.19 ± 0.07 | a |
| $bla_{VIM}$ | 9.17 | **9.93E-04** | 0.08 ± 0.22 | b | 0.16 ± 0.32 | a | 0.47 ± 0.48 | a |
| *cadA* | 8.61 | **1.36E-03** | 2.62 ± 0.49 | b | 2.87 ± 0.09 | a | 2.95 ± 0.09 | a |
| *catB8* | 8.18 | **1.79E-03** | 0.35 ± 0.54 | b | 0.68 ± 0.64 | b | 1.55 ± 0.73 | a |
| *chrA* | 22.86 | **5.54E-07** | 1.97 ± 0.79 | b | 2.67 ± 0.35 | a | 2.76 ± 0.35 | a |
| *cmlB* | 6.58 | **5.12E-03** | 1.39 ± 0.67 | ab | 1.03 ± 0.67 | b | 1.91 ± 0.6 | a |
| *copA* | 6.00 | **7.63E-03** | 1.16 ± 0.78 | b | 1.88 ± 0.71 | ab | 2.1 ± 0.72 | a |
| *dfr13* | 3.78 | **3.89E-02** | 0.55 ± 0.63 | b | 1.07 ± 0.76 | a | 0.87 ± 0.73 | ab |
| *ereB* | 29.08 | **6.26E-08** | 0.34 ± 0.54 | c | 0.5 ± 0.66 | b | 1.39 ± 0.9 | a |
| *ermB* | 5.42 | **1.10E-02** | 1.8 ± 0.88 | b | 2.32 ± 0.8 | ab | 2.74 ± 0.62 | a |
| *ermF* | 0.95 | 3.95E-01 | 1.67 ± 0.87 | | 1.97 ± 0.83 | | 2.17 ± 0.83 | |
| *floR* | 5.39 | **1.10E-02** | 2.03 ± 0.69 | b | 2.61 ± 0.5 | a | 2.63 ± 0.62 | a |
| *intI1* | 1.23 | 3.13E-01 | 3.15 ± 0.14 | | 3.06 ± 0.4 | | 3.1 ± 0.4 | |
| *IntI2* | 10.26 | **5.74E-04** | 1.22 ± 0.84 | c | 2.09 ± 0.82 | b | 2.45 ± 0.76 | a |
| *IntI3* | 7.27 | **3.29E-03** | 1.39 ± 0.8 | b | 2.05 ± 0.64 | ab | 2.33 ± 0.63 | a |
| *mefE* | 3.31 | 5.43E-02 | 2.04 ± 0.81 | | 2.49 ± 0.61 | | 2.63 ± 0.64 | |
| *merA* | 5.85 | **8.35E-03** | 2.66 ± 0.62 | b | 2.96 ± 0.37 | b | 3.15 ± 0.11 | a |
| *mexB* | 9.84 | **6.84E-04** | 0.96 ± 0.79 | b | 1.41 ± 0.84 | b | 2.51 ± 0.67 | a |
| *nikA* | 10.44 | **5.74E-04** | 2.34 ± 0.77 | b | 3.12 ± 0.09 | a | 3.07 ± 0.11 | a |
| *qacF* | 7.63 | **2.59E-03** | 1.17 ± 0.77 | b | 0.62 ± 0.65 | b | 1.63 ± 0.83 | a |
| *qnrA* | 1.57 | 2.33E-01 | 0.35 ± 0.46 | | 0.76 ± 0.59 | | 0.85 ± 0.68 | |
| *qnrB* | 3.75 | **3.89E-02** | 0.79 ± 0.73 | a | 1.05 ± 0.71 | ab | 1.68 ± 0.78 | b |
| *rcnA* | 30.17 | **5.12E-08** | 2.14 ± 0.84 | b | 3.05 ± 0.12 | a | 3 ± 0.14 | a |

| | Aligned rank ANOVA | | Average ± se log copies/g soil | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Gene** | **F-value** | **Adj. P-value** | **15%** | | **30%** | | **45%** | |
| *strB* | 2.12 | 1.47E-01 | 2.8 ± 0.53 | | 2.65 ± 0.55 | | 2.79 ± 0.49 | |
| *sul1* | 6.55 | **5.12E-03** | 2.96 ± 0.39 | b | 2.93 ± 0.42 | b | 3.09 ± 0.18 | a |
| *sul2* | 5.73 | **8.92E-03** | 2.58 ± 0.61 | a | 2.17 ± 0.84 | b | 2.49 ± 0.77 | a |
| *sul3* | 12.59 | **1.45E-04** | 2.27 ± 0.44 | c | 2.57 ± 0.11 | b | 2.71 ± 0.1 | a |
| *tetA* | 2.83 | 8.09E-02 | 3.14 ± 0.08 | | 3.11 ± 0.1 | | 3.17 ± 0.1 | |
| *tetL* | 4.88 | **1.58E-02** | 2.09 ± 0.83 | b | 2.94 ± 0.41 | a | 2.87 ± 0.53 | a |
| *tetM* | 7.14 | **3.48E-03** | 2.46 ± 0.78 | b | 2.94 ± 0.42 | ab | 3.07 ± 0.39 | a |
| *tetS* | 18.09 | **6.58E-06** | 1.8 ± 0.86 | b | 2.23 ± 0.78 | b | 3.03 ± 0.38 | a |
| *tetW* | 5.00 | **1.47E-02** | 2.59 ± 0.72 | b | 2.77 ± 0.64 | ab | 2.92 ± 0.54 | a |
| *tetX* | 0.95 | 3.95E-01 | 1.37 ± 0.86 | | 1.93 ± 0.89 | | 1.96 ± 0.91 | |
| *vanA* | 13.03 | **1.18E-04** | 0.08 ± 0.23 | b | 0.33 ± 0.52 | a | 0.39 ± 0.52 | a |
| *vanB* | 15.36 | **2.97E-05** | 1.26 ± 0.76 | c | 1.92 ± 0.67 | b | 2.44 ± 0.34 | a |

**Supplementary Table B.3.** Aligned rank ANOVA with FDR-adjusted p-values and average gene abundances for each treatment. Lowercase letters indicate significant differences for post-hoc analyses.

| Aligned rank ANOVA | | | Average ± se log copies/g soil | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Gene | F.value | Adjusted P-value | NM | | CO | | CE | | PI | |
| *aacA* | 14.58 | **5E-06** | 2.02 ± 0.89 | a | 1.06 ± 0.89 | b | 1.05 ± 0.88 | b | 1.42 ± 0.94 | a |
| *aadA5* | 11.40 | **5E-05** | 2.75 ± 0.24 | a | 2.11 ± 0.61 | b | 2.34 ± 0.64 | b | 2.6 ± 0.44 | a |
| *aadD* | 15.42 | **5E-06** | 2.82 ± 0.28 | a | 2.25 ± 0.65 | b | 2.38 ± 0.57 | b | 2.89 ± 0.18 | a |
| *acrD* | 17.47 | **2E-06** | 3.11 ± 0.12 | a | 2.68 ± 0.6 | b | 2.62 ± 0.7 | b | 3.06 ± 0.13 | a |
| *ampC* | 14.97 | **5E-06** | 3.09 ± 0.12 | a | 2.52 ± 0.71 | b | 2.6 ± 0.69 | b | 2.91 ± 0.43 | a |
| *arr2* | 3.15 | 0.0557 | 0.68 ± 0.66 | | 0.35 ± 0.48 | | 0.34 ± 0.39 | | 0.63 ± 0.54 | |
| $bla_{NDM1}$ | 1.12 | **0.0203** | 1.06 ± 0.69 | a | 0.26 ± 0.44 | a | 1.32 ± 0.71 | a | 0.85 ± 0.65 | a |
| $bla_{CTX\text{-}M\text{-}32}$ | 3.61 | 0.3641 | 1.52 ± 0.86 | | 0.9 ± 0.72 | | 0.9 ± 0.77 | | 1.04 ± 0.79 | |
| $bla_{imp13}$ | 1.70 | **0.0345** | 1.31 ± 0.86 | a | 0.59 ± 0.67 | a | 1.06 ± 0.82 | a | 1.52 ± 0.85 | a |
| $bla_{KPC}$ | 4.27 | 0.2337 | 0.97 ± 0.79 | | 1.39 ± 0.75 | | 1.04 ± 0.78 | | 1.76 ± 0.75 | |
| $bla_{NPS}$ | 19.38 | **1E-06** | 1.25 ± 0.89 | a | 0.35 ± 0.59 | b | 0.64 ± 0.71 | b | 1.01 ± 0.85 | a |
| $bla_{OXA}$ | 1.46 | 0.2923 | 1.66 ± 0.85 | | 1.31 ± 0.8 | | 1.27 ± 0.78 | | 1.85 ± 0.77 | |
| $bla_{SHV}$ | 5.70 | **0.0053** | 3.19 ± 0.05 | a | 3.17 ± 0.07 | a | 3.08 ± 0.12 | b | 3.19 ± 0.08 | a |
| $bla_{VIM}$ | 5.46 | **0.0064** | 0.31 ± 0.4 | a | 0.13 ± 0.32 | ab | 0 ± 0 | b | 0.52 ± 0.5 | a |
| *cadA* | 6.66 | **0.0027** | 2.92 ± 0.11 | b | 2.71 ± 0.4 | b | 2.72 ± 0.4 | b | 2.91 ± 0.09 | a |
| *catB8* | 1.23 | 0.3312 | 1.09 ± 0.79 | | 0.64 ± 0.63 | | 0.72 ± 0.69 | | 1.05 ± 0.71 | |
| *chrA* | 7.42 | **0.0014** | 2.49 ± 0.58 | bc | 2.25 ± 0.72 | c | 2.49 ± 0.54 | b | 2.67 ± 0.41 | a |
| *cmlB* | 1.26 | 0.328 | 1.38 ± 0.7 | | 1.44 ± 0.69 | | 1.3 ± 0.71 | | 1.65 ± 0.63 | |
| *copA* | 3.85 | **0.0309** | 1.87 ± 0.72 | ab | 1.52 ± 0.81 | b | 1.19 ± 0.8 | b | 2.3 ± 0.62 | a |
| *dfr13* | 1.65 | 0.2421 | 1.08 ± 0.79 | | 0.81 ± 0.69 | | 0.73 ± 0.7 | | 0.73 ± 0.71 | |

| Aligned rank ANOVA | | | Average ± se log copies/g soil | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Gene | F.value | Adjusted P-value | NM | | CO | | CE | | PI | |
| *ereB* | 14.79 | **5E-06** | 1.01 ± 0.83 | a | 0.33 ± 0.56 | b | 0.68 ± 0.76 | b | 1 ± 0.84 | a |
| *ermB* | 2.31 | 0.1261 | 2.72 ± 0.61 | | 1.82 ± 0.89 | | 2.31 ± 0.85 | | 2.35 ± 0.76 | |
| *ermF* | 1.99 | 0.1772 | 2.31 ± 0.78 | | 1.32 ± 0.88 | | 2.23 ± 0.83 | | 1.92 ± 0.82 | |
| *floR* | 3.64 | **0.0345** | 2.78 ± 0.45 | a | 2.03 ± 0.73 | ab | 2.23 ± 0.73 | b | 2.69 ± 0.46 | a |
| *intI1* | 9.14 | **0.0003** | 3.26 ± 0.1 | a | 2.94 ± 0.46 | b | 3 ± 0.45 | b | 3.22 ± 0.11 | a |
| *IntI2* | 2.80 | 0.0781 | 2.53 ± 0.72 | | 1.42 ± 0.87 | | 1.79 ± 0.87 | | 2.01 ± 0.87 | |
| *IntI3* | 2.57 | 0.096 | 2.23 ± 0.66 | | 2.05 ± 0.66 | | 1.33 ± 0.8 | | 2.13 ± 0.69 | |
| *mefE* | 1.31 | 0.3236 | 2.49 ± 0.71 | | 2.15 ± 0.73 | | 2.32 ± 0.75 | | 2.6 ± 0.62 | |
| *merA* | 3.64 | **0.0345** | 2.9 ± 0.45 | b | 2.92 ± 0.43 | ab | 2.78 ± 0.59 | b | 3.12 ± 0.11 | a |
| *mexB* | 1.38 | 0.3126 | 1.83 ± 0.83 | | 1.46 ± 0.87 | | 1.17 ± 0.87 | | 2.09 ± 0.78 | |
| *nikA* | 7.69 | **0.0012** | 3.1 ± 0.11 | a | 2.67 ± 0.63 | b | 2.73 ± 0.59 | b | 2.92 ± 0.43 | a |
| *qacF* | 1.28 | 0.3276 | 1.38 ± 0.88 | | 0.85 ± 0.7 | | 0.99 ± 0.75 | | 1.35 ± 0.82 | |
| *qnrA* | 2.81 | 0.0781 | 0.72 ± 0.68 | | 0.5 ± 0.57 | | 0.49 ± 0.55 | | 0.93 ± 0.58 | |
| *qnrB* | 4.71 | **0.0131** | 1.62 ± 0.81 | a | 1.27 ± 0.78 | a | 0.48 ± 0.56 | b | 1.38 ± 0.76 | a |
| *rcnA* | 12.90 | **2E-05** | 3.03 ± 0.13 | a | 2.54 ± 0.7 | b | 2.54 ± 0.68 | b | 2.85 ± 0.42 | a |
| *strB* | 0.02 | 0.9957 | 2.71 ± 0.63 | | 2.76 ± 0.46 | | 2.74 ± 0.52 | | 2.78 ± 0.5 | |
| *sul1* | 6.03 | **0.0045** | 3.11 ± 0.19 | a | 2.92 ± 0.45 | b | 3.05 ± 0.18 | a | 2.91 ± 0.45 | b |
| *sul2* | 5.07 | **0.0093** | 2.46 ± 0.82 | ab | 2.76 ± 0.45 | a | 2.2 ± 0.83 | b | 2.22 ± 0.84 | b |
| *sul3* | 5.73 | **0.0053** | 2.59 ± 0.16 | b | 2.44 ± 0.36 | b | 2.4 ± 0.38 | b | 2.66 ± 0.11 | a |
| *tetA* | 0.34 | 0.8116 | 3.14 ± 0.12 | | 3.16 ± 0.08 | | 3.13 ± 0.09 | | 3.14 ± 0.07 | |
| *tetL* | 1.81 | 0.2117 | 2.42 ± 0.81 | | 2.72 ± 0.59 | | 2.59 ± 0.7 | | 2.83 ± 0.45 | |
| *tetM* | 6.20 | **0.004** | 3 ± 0.46 | a | 2.76 ± 0.61 | a | 2.52 ± 0.8 | b | 3.04 ± 0.19 | a |
| *tetS* | 5.70 | **0.0053** | 2.55 ± 0.71 | a | 2.4 ± 0.77 | a | 1.76 ± 0.94 | b | 2.74 ± 0.45 | a |
| *tetW* | 2.61 | 0.0939 | 2.95 ± 0.49 | | 2.8 ± 0.6 | | 2.65 ± 0.72 | | 2.65 ± 0.72 | |

| Aligned rank ANOVA | | | Average ± se log copies/g soil | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Gene | F.value | Adjusted P-value | NM | | CO | | CE | | PI | |
| *tetX* | 1.34 | 0.3193 | 1.68 ± 0.95 | | 1.36 ± 0.91 | | 2.17 ± 0.82 | | 1.83 ± 0.88 | |
| *vanA* | 11.12 | **6E-05** | 0.21 ± 0.35 | b | 0.14 ± 0.35 | c | 0.15 ± 0.37 | b | 0.57 ± 0.64 | a |
| *vanB* | 3.76 | **0.0327** | 1.83 ± 0.63 | ab | 1.76 ± 0.71 | b | 1.75 ± 0.74 | b | 2.17 ± 0.6 | a |

**Supplementary Table B.4.** Aligned rank ANOVA with FDR-adjusted p-values and average gene abundances for each soil type.

| | Aligned rank ANOVA | | Average ± se log copies/g soil | |
|---|---|---|---|---|
| **Gene** | **F.value** | **Adjusted P-value** | **SL** | **SCL** |
| *aacA* | 197 | **1.04E-14** | 2.55 ± 0.75 | 0.25 ± 0.48 |
| *aadA5* | 13.9 | **0.001106** | 2.33 ± 0.73 | 2.56 ± 0.14 |
| *aadD* | 1.16 | 0.329007 | 2.67 ± 0.66 | 2.5 ± 0.19 |
| *acrD* | 59.1 | **5.54E-09** | 2.58 ± 0.64 | 3.14 ± 0.11 |
| *ampC* | 55.8 | **8.34E-09** | 2.41 ± 0.73 | 3.13 ± 0.1 |
| *arr2* | 4.96 | **0.039974** | 0.42 ± 0.51 | 0.58 ± 0.53 |
| $bla_{NDM1}$ | 6.76 | 0.099107 | 0.61 ± 0.61 | 1.12 ± 0.68 |
| $bla_{CTX\text{-}M\text{-}32}$ | 13.5 | **0.018117** | 1.47 ± 0.82 | 0.71 ± 0.68 |
| $bla_{imp13}$ | 0.39 | **0.001209** | 1.56 ± 0.9 | 0.68 ± 0.62 |
| $bla_{KPC}$ | 3.15 | 0.569262 | 1.24 ± 0.8 | 1.35 ± 0.76 |
| $bla_{NPS}$ | 97.4 | **5.64E-12** | 1.47 ± 0.89 | 0.17 ± 0.4 |
| $bla_{OXA}$ | 0.22 | 0.653288 | 1.65 ± 0.89 | 1.4 ± 0.69 |
| $bla_{SHV}$ | 4.77 | **0.043042** | 3.14 ± 0.11 | 3.17 ± 0.06 |
| $bla_{VIM}$ | 7.21 | **0.015513** | 0.22 ± 0.36 | 0.25 ± 0.38 |
| *cadA* | 20.7 | **9.74E-05** | 2.76 ± 0.41 | 2.86 ± 0.07 |
| *catB8* | 8.59 | **0.008368** | 1.26 ± 0.77 | 0.49 ± 0.55 |
| *chrA* | 45.9 | **7.71E-08** | 2.12 ± 0.69 | 2.82 ± 0.32 |
| *cmlB* | 14.9 | **0.000763** | 1.04 ± 0.71 | 1.84 ± 0.55 |
| *copA* | 5.54 | **0.031389** | 1.44 ± 0.82 | 1.98 ± 0.68 |
| *dfr13* | 36.4 | **8.03E-07** | 1.47 ± 0.8 | 0.21 ± 0.35 |
| *ereB* | 113 | **7.21E-13** | 1.36 ± 0.87 | 0.17 ± 0.4 |
| *ermB* | 0 | 0.977171 | 2.34 ± 0.81 | 2.25 ± 0.78 |
| *ermF* | 9.64 | **0.005348** | 2.46 ± 0.73 | 1.44 ± 0.85 |
| *floR* | 4.99 | **0.039974** | 2.62 ± 0.7 | 2.24 ± 0.52 |
| *intI1* | 42.3 | **1.81E-07** | 3.26 ± 0.34 | 2.95 ± 0.31 |
| *IntI2* | 13.3 | **0.001258** | 2.5 ± 0.8 | 1.38 ± 0.78 |
| *IntI3* | 0.37 | 0.569262 | 2.01 ± 0.76 | 1.86 ± 0.68 |
| *mefE* | 6.88 | **0.017678** | 2.62 ± 0.71 | 2.17 ± 0.67 |
| *merA* | 3.3 | 0.093458 | 2.89 ± 0.53 | 2.97 ± 0.31 |
| *mexB* | 0.64 | 0.466056 | 1.76 ± 0.85 | 1.52 ± 0.85 |
| *nikA* | 29.8 | **5.17E-06** | 2.57 ± 0.65 | 3.13 ± 0.12 |
| *qacF* | 20.2 | **0.00011** | 1.71 ± 0.83 | 0.58 ± 0.58 |
| *qnrA* | 2.34 | 0.15553 | 0.57 ± 0.6 | 0.74 ± 0.59 |
| *qnrB* | 11.1 | **0.003006** | 0.75 ± 0.67 | 1.6 ± 0.77 |

| | Aligned rank ANOVA | | Average ± se log copies/g soil | |
|---|---|---|---|---|
| **Gene** | **F.value** | **Adjusted P-value** | **SL** | **SCL** |
| *rcnA* | 58.8 | **5.54E-09** | 2.41 ± 0.71 | 3.06 ± 0.15 |
| *strB* | 38.2 | **5.13E-07** | 3.18 ± 0.33 | 2.32 ± 0.55 |
| *sul1* | 56.4 | **8.19E-09** | 3.33 ± 0.05 | 2.67 ± 0.4 |
| *sul2* | 47.7 | **5.33E-08** | 3.04 ± 0.45 | 1.8 ± 0.81 |
| *sul3* | 17.7 | **0.00026** | 2.42 ± 0.39 | 2.62 ± 0.07 |
| *tetA* | 96.1 | **5.64E-12** | 3.25 ± 0.07 | 3.04 ± 0.07 |
| *tetL* | 0.82 | 0.414417 | 2.69 ± 0.73 | 2.59 ± 0.55 |
| *tetM* | 11.8 | **0.002306** | 2.71 ± 0.67 | 2.94 ± 0.44 |
| *tetS* | 9.91 | **0.004922** | 2.62 ± 0.7 | 2.11 ± 0.78 |
| *tetW* | 6.01 | **0.025533** | 2.99 ± 0.55 | 2.53 ± 0.69 |
| *tetX* | 31.9 | **2.93E-06** | 2.68 ± 0.66 | 0.86 ± 0.77 |
| *vanA* | 28.3 | **8.02E-06** | 0.37 ± 0.54 | 0.17 ± 0.34 |
| *vanB* | 26.8 | **1.24E-05** | 1.38 ± 0.71 | 2.37 ± 0.49 |

**Supplementary Table B.5.** 3-way PERMANOVA for 16S microbial community structures

| | **Df** | **Sums Of Sqs** | **Mean Sqs** | **F Model** | **$R^2$** | **Pr(>F)** |
|---|---|---|---|---|---|---|
| Treatment | 3 | 0.7064 | 0.23545 | 7.58 | 0.07033 | 0.001 |
| Moisture | 2 | 1.0493 | 0.52464 | 16.891 | 0.10447 | 0.001 |
| Type | 1 | 2.3621 | 2.36208 | 76.049 | 0.23518 | 0.001 |
| Treatment:Moisture | 6 | 0.5994 | 0.09989 | 3.216 | 0.05968 | 0.001 |
| Treatment:Type | 3 | 0.5373 | 0.17911 | 5.767 | 0.0535 | 0.001 |
| Moisture:Type | 2 | 1.3055 | 0.65274 | 21.015 | 0.12998 | 0.001 |
| Treatment:Moisture:Type | 6 | 0.6264 | 0.1044 | 3.361 | 0.06237 | 0.001 |
| Residuals | 92 | 2.8575 | 0.03106 | 0.28451 | | |
| Total | 115 | 10.0438 | 1 | | | |

**Supplementary Table B.6.** 3-way PERMANOVA for ITS microbial community structures

| | Df | Sums Of Sqs | Mean Sqs | F Model | $R^2$ | Pr(>F) |
|---|---|---|---|---|---|---|
| Treatment | 3 | 0.7064 | 0.23545 | 7.58 | 0.07033 | 0.001 |
| Moisture | 2 | 1.0493 | 0.52464 | 16.891 | 0.10447 | 0.001 |
| Type | 1 | 2.3621 | 2.36208 | 76.049 | 0.23518 | 0.001 |
| Treatment:Moisture | 6 | 0.5994 | 0.09989 | 3.216 | 0.05968 | 0.001 |
| Treatment:Type | 3 | 0.5373 | 0.17911 | 5.767 | 0.0535 | 0.001 |
| Moisture:Type | 2 | 1.3055 | 0.65274 | 21.015 | 0.12998 | 0.001 |
| Treatment:Moisture:Type | 6 | 0.6264 | 0.1044 | 3.361 | 0.06237 | 0.001 |
| Residuals | 92 | 2.8575 | 0.03106 | 0.28451 | | |
| Total | 115 | 10.0438 | 1 | | | |

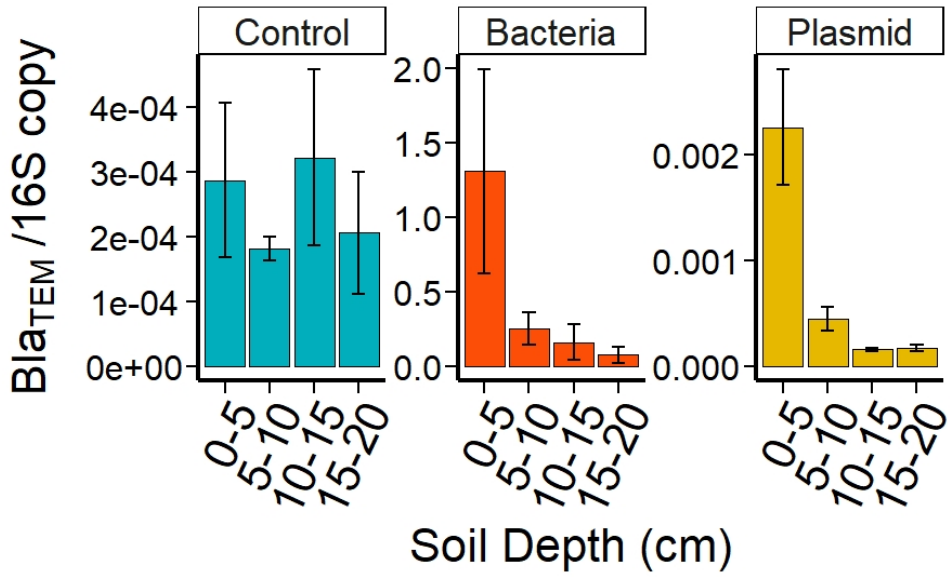
**Supplementary Table B.7.** 3-way ANOVA for respiration

| | Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|
| Treatment | 3 | 20.331 | 6.777 | 684.488 | < 2e-16 |
| Moisture | 2 | 2.345 | 1.172 | 118.418 | < 2e-16 |
| Type | 1 | 0.151 | 0.151 | 15.223 | 0.000177 |
| Treatment:Moisture | 6 | 0.986 | 0.164 | 16.604 | 4.78E-13 |
| Treatment:Type | 3 | 0.126 | 0.042 | 4.255 | 0.00723 |
| Moisture:Type | 2 | 6.728 | 3.364 | 339.757 | < 2e-16 |
| Treatment:Moisture:Type | 6 | 2.534 | 0.422 | 42.651 | < 2e-16 |
| Residuals | 96 | 0.95 | 0.01 | | |

**Supplementary Table B.8.** 3-way ANOVA for Substrate Induced Respiration (SIR)

| | Df | Sum Sq | Mean Sq | F value | Pr(>F) |
|---|---|---|---|---|---|
| Treatment | 3 | 33.09 | 11.03 | 98.849 | < 2e-16 |
| Moisture | 2 | 5.35 | 2.68 | 23.975 | 3.59E-09 |
| Type | 1 | 33.84 | 33.84 | 303.276 | < 2e-16 |
| Treatment:Moisture | 6 | 3.64 | 0.61 | 5.431 | 7.17E-05 |
| Treatment:Type | 3 | 5.86 | 1.95 | 17.505 | 3.82E-09 |
| Moisture:Type | 2 | 27.39 | 13.7 | 122.743 | < 2e-16 |
| Treatment:Moisture:Type | 6 | 8.54 | 1.42 | 12.76 | 1.52E-10 |
| Residuals | 96 | 10.71 | 0.11 | | |

**Appendix C: Supplementary Information for Chapter 4**




**Supplementary figure C.1.** Mean (n=4) ratio of *bla*$_{TEM}$ to 16S gene abundance in soil with depth.