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Spatial Distribution of Antibiotic Resistance in Soils Receiving Beef Feedlot Runoff

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SPATIAL DISTRIBUTION OF ANTIBIOTIC RESISTANCE IN SOILS RECEIVING BEEF FEEDLOT RUNOFF

by

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SPATIAL DISTRIBUTION OF ANTIBIOTIC RESISTANCE IN SOILS RECEIVING BEEF FEEDLOT RUNOFF

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A study was conducted to provide new insight on the potential contribution to antibiotic resistance from the land application of beef feedlot runoff to soil. This study reports the distribution and quantity of antibiotic resistant bacteria (ARBs), fecal indicator bacteria (FIB), and antibiotic resistance genes (ARGs) in soil from (i) a field receiving long-term application of beef feedlot runoff holding pond effluent and (ii) a cool-season pasture with no history of supplemental manure application.

Soil samples were collected June 2015 at the U.S. Meat Animal Research Center near Clay Center, Nebraska. A response surface sampling design (RSSD) model based on apparent soil electrical conductivity (EC_a) measured using electromagnetic induction (EMI) was used to identify six independent sampling locations in each field representing varying degrees of manure accumulation. At each location, intact soil cores were collected to a depth of 2.0 m, subsampled, and analyzed for ARBs (cefotaxime, erythromycin, and tetracycline resistance), FIB, and ARGs (*erm* and *tet*). Methods included culture-based, disc diffusion, Etest, and qPCR.

Results suggest the long-term application of beef feedlot runoff increased the soil microbial population, erythromycin resistant bacteria, *erm*(C), and *tet*(Q). The abundance of three cultured ARBs and *erm*(C) significantly decreased with depth in soil. Areas of high manure deposition had a positive correlation with erythromycin resistant bacteria.

The data produced will contribute to the body of knowledge impacting decisions and future research efforts of scientists, researchers, and policy-makers who are striving to effectively address the potential contribution to antibiotic resistance in humans from agricultural practices.

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CHAPTER 1. REVIEW OF LITERATURE

1.1 Introduction

This is a comprehensive literature review describing (i) beef cattle production and the associated antibiotic use and manure management in beef cattle feedlot operations as they specifically relate to the research described within this thesis, and (ii) an overview of antibiotic resistance including selection for, mechanisms of antibiotic resistance development, and current research relatable to the work described within this thesis.

1.2 Agricultural Systems and Practices

1.2.1 Beef Cattle Production

According to the USDA National Agricultural Statistics Service, there are currently an estimated 92.0 million cattle on feed in the United States with 30.3 million cattle raised annually for beef. The USDA Economic Research Service reported that the beef industry generates 95 billion dollars in the US and 6.5 billion dollars in revenue from exports in 2014 (USDA). The NRCS Waste Management Field Handbook reports that on average, a 450 kg steer will produce 27 kg of manure per day, with 10% comprised of fixed solids (NRCS). These fixed solids are composed of nitrogen (42%), potassium (32%), phosphorus (15%), and other minerals and salts (11%).

Feedlots, or land areas devoid of vegetation where livestock are confined and fed, are a common management practice for finishing beef cattle. While feedlots facilitate raising livestock on less land than is possible in a pastured setting, the concentration of manure from the confined animals at this high stocking density presents an environmental management challenge as the manure must be collected and contained until it can be land-applied at agronomic rates or otherwise treated or utilized. The collection and storage of manure from concentrated livestock production systems has given rise to concerns about safely handling and applying manure to agricultural fields (Chopra and Roberts, 2001). In particular, and relative to the research presented in this thesis, concerns have begun to focus on the role of land-applied manure from beef cattle feedlots as a means for introducing antibiotic resistance to the terrestrial environment.

1.2.2 Manure Management

The use of manure by as a soil amendment began over 8000 years ago as manure provides an abundant source of plant nutrients including nitrogen, phosphorus, potassium, micronutrients, organic matter, and microbes that positively impact soil quality (Balter, 2013). Recommended application practices and rates to meet the agronomic needs of agricultural crops are well established and accompanied by recognized guidelines or best management practices (BMPs) that provide a basis for developing economically and environmentally sound manure management.

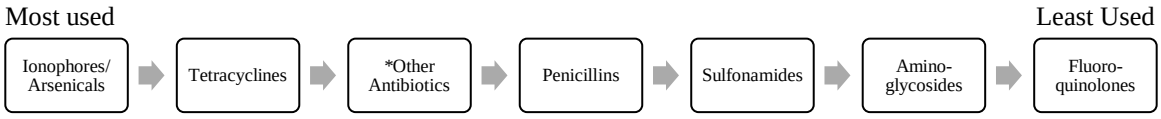
Regulatory oversight of manure management occurs at the federal, state, and sometimes local levels for livestock facilities meeting specific requirements based upon animal population and manure handling system type. Governing agencies include the

United States Environmental Protection Agency (USEPA) and state regulatory departments, which include Department of Natural Resources (DNR), Department of Environmental Quality (DEQ), and others. The provisions under the Clean Water Act (CWA), Effluent Limit Guidelines (ELG), and the National Pollution Discharge Elimination System (NPDES) limit the discharge of waste from permitted concentrated animal feeding operation[1] (CAFOs) based upon federal design standards implemented at the state level. For most liquid manure handling systems operating under a NPDES permit, a discharge of manure from the production area is only legal if it occurs as the results of precipitation exceeding the 25-year, 24-hour design storm event and if proper documentation reveals that the manure storage was properly managed to maintain the emergency storage volume (USEPA). Many operations require manure storage facilities capable of storing up to six months of manure and process wastewater as access to land for application of the waste is dependent upon weather, soil conditions, and crop production. Manure storage facilities designs vary depending on the species of livestock, housing system, and the intended use of the manure. Liquid and slurry manure are typically stored in engineered pits or tanks, lagoons, or earthen storage ponds. Solid waste is often stock-piled and applied to agricultural cropland during fallow periods. Liquid manure can be land applied by three primary methods: (i) surface application (broadcast), (ii) surface application followed by incorporation through tillage, and (iii) direct soil injection (Chee-Sanford et al., 2012). Solid manure is commonly surface applied with or without subsequent incorporation via tillage.

[1] A large beef cattle CAFO is defined as a facility housing at least 1,000 beef cattle or heifers or 1,000 veal calves (EPA, 2004)

1.2.3 Antibiotic Use in Beef Cattle Feedlot Systems

Antibiotics are routinely used in conventional beef cattle production systems to treat and prevent disease. There are three main antibiotic applications: (i) therapeutically to treat existing disease conditions, (ii) prophylactically at sub therapeutic doses to mitigate infection by bacterial pathogens to which animals may be more susceptible during periods of increased stress, and (iii) sub therapeutically to maintain growth at optimal levels (Chee-Sanford et al., 2009). There exist many discrepancies over the amount of antibiotics used in livestock production. The Animal Health Institute (AHI) estimated a total of 20.5 million pounds of antibiotics sold for all animal use in 1999 with 17.7 million pounds used for treatment and prevention of disease and only 2.8 million pounds for improving feed efficiency and maintain optimum growth (Chee-Sanford et al., 2009). The most commonly used antibiotic classes by weight in animal production according to a survey conducted by AHI include:



*Other antibiotics includes macrolides, lincosamides, polypeptides, streptogramins, and cephalosporins

A second source reports that 24.6 million pounds of antibiotics were used for non-therapeutic purposes alone in swine, poultry, and cattle industries (Mellon et al., 2001). Recent legislation has created new policies to govern the use of therapeutic applications and requiring veterinary oversight of antibiotics supplied in feed and water in an attempt to reduce the quantity of antibiotics administered to livestock (US Food and Drug Administration, 2013).

The World Health Organization's (WHO) list of antimicrobials of importance to human medicine contains 32 drug classes and nearly 260 individual pharmaceutical compounds. Each compound is classified as important, highly important, or critically important to human medicine. According to the Food Animal Residue Avoidance Database (FARAD), only 38 of the 260 compounds are recommended or registered for use in U.S. cattle, swine, and poultry (Papich, 2015). Of these 38 compounds (35 of which are approved for use in cattle), approximately 23 are considered critically important, 12 highly important, and 3 important to human medicine. The critically important drugs include those in the aminoglycoside class (gentamycin and streptomycin), macrolide class (erythromycin and tylosin), and the penicillin class (ampicillin and penicillin G). This classification system, developed by the WHO, has provided direction to researchers investigating AR in terms of human health.

1.2.4 Introduction of Antibiotics and Selection for Resistance into the Environment from Livestock Agriculture

The diagram below shows the many pathways antibiotics can enter into environmental systems:

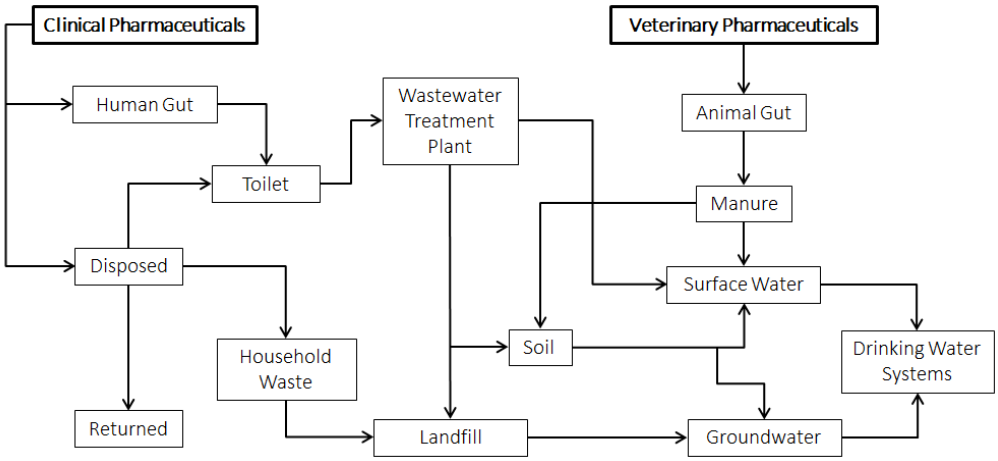


Figure 1.1 Available Pathways for Antibiotics Entering the Environment

Multiple studies have concluded that antibiotics are not completely absorbed in the gut of livestock and the parent compounds and their metabolites could act as selective pressure for microbes to harbor resistance (Halling-Sorensen et al., 1998; Chee-Sanford et al., 2009; Joy et al., 2014). It is estimated that 75% of antibiotics are excreted in waste as the parent compound or as active metabolites (Chee-Sanford et al., 2009). It has been suggested that about 25% of oral doses of tetracycline is excreted in feces and approximately 50-60% is excreted unchanged or as active metabolites in urine (Chee-Sanford et al., 2012). The same study reports oral administration of tylosin[2] in poultry operations resulted in a maximum of 67% of the antibiotic excreted, mainly in the feces. Recent research has now focused on the fate of antimicrobial compounds in common manure storage systems (i.e. anaerobic lagoons, slurry pits, stockpiled solids, composted solids, runoff holding ponds). Here, tylosin was discovered to degrade in a biphasic pattern with rapid initial loss followed by a slow degradation phase. Tylosin degraded

[2] An antibiotic commonly used in cattle, swine, and poultry husbandry to treat infections. Tylosin belongs to the macrolide drug class and has a bacteriostatic effect on susceptible organisms.

90% after 30-130 hours in aerobic slurries and after 12-26 hours in aerobic slurries, but residuals were still detected in low concentrations after eight months (Kolz et al., 2005) in both instances. It is also understood that residuals of tylosin in swine wastewater storage may exert selective pressure for resistance (Joy et al., 2014).

When manure is applied to land, remaining antibiotics and their active metabolites can be transferred with the manure. In the environment, antibiotics can be transported in a liquid phase or, more commonly, in solid phase adsorbed to colloids or soil particles (Campagnolo et al., 2002; Kolpin et al., 2002; Krapac et al., 2003; Yang and Carlson, 2003). However, half-life studies of antibiotics suggest that significant degradation of the parent compounds might occur before land application (Boxall et al., 2004). Quinolones and tetracyclines were reported to have the most persistent half-lives in manure of nearly 100 d (Kolz et al., 2005). One laboratory study reports the order of persistence of antibiotics in a soil-feces matrix as follows (Chee-Sanford et al., 2009):

Antibiotic degradation is widely studied and in the agriculture system, most antibiotics are assumed to enter the environment via water, so hydrolysis is an important degradation pathway (Huang et al., 2011). Beta-lactams, macrolides, and sulfonamides appear to be the most susceptible classes to hydrolysis. Another pathway is photolysis, or the decomposition or separation of molecules from light, but this process can be difficult to study due to complexities of the soil-atmosphere interface. Few studies have concluded

photolysis and the effects were negligible when compared with other abiotic processes (Beausse, 2004).

1.3 Antibiotic Resistance

1.3.1 Overview

The term antibiotic resistance (AR) is used both in the clinical setting as well as the agricultural and environmental health community; however a standard definition of the term has not been established. Clinicians refer to AR as disease treatment failure, whereas scientists, public health officials, and policy makers use AR as a parameter to observe a system, without clear evidence of a direct linkage to disease treatment failure in humans (Calero-Cáceres and Muniesa, 2016). The study presented in this thesis uses AR as a parameter within an environmental system.

Two main constituents are typically measured to determine AR in agricultural settings: (i) antibiotic resistance genes (ARGs) and (ii) antibiotic resistant bacteria (ARBs).

ARGs refer to the genetic materials that encode for resistance to antibiotics. ARGs can be carried by a bacterial host and can be "traded" between bacteria, though the rates at which this occurs in field settings is unknown. Once a cell contains a resistance gene, it can transfer the gene using two primary mechanisms, (i) horizontal gene transfer or (ii) lateral gene transfer. The pathways of these mechanisms, however, are not universal for all ARGs (Ashbolt et al., 2013). There is strong evidence supporting the

idea that ARGs can persist in the environment even if the host is dead (Calero-Cáceres and Muniesa, 2016). ARBs are the living bacterium that contain an ARG, and in environmental literature, are typically described as displaying a reduced susceptibility to a specific antibiotic.

It has been reported there has been an increase in total antibiotic resistance in archived soils from 1940 to modern soils (Ehlert, 2010). However, the cause(s) for this increase are not yet well defined. Also, it is important to note ARBs and ARGs are frequently detected in environments with no history of human alterations to the soil (Frankel et al., 2006; Bhullar et al., 2012; Durso et al., 2012). Most clinical antibiotics are derived from soil-dwelling actinomycetes[3] (Kieser et al., 2000) and this may explain one driver for ARGs to persist in "natural" environments. Heavy metals and important survival co-functions of ARGs may also explain the ubiquity of some ARBs and ARGs. Because of this, the greater concern is not the presence of ARBs or ARGs, but instead which ARBs and ARGs are present and whether or not agricultural practices have altered the naturally occurring ARBs and ARGs (Pruden et al., 2006; Durso and Cook, 2014; Agga et al., 2015).

There exists a correlation between ARGs and bacterial density (Sui et al., 2015), suggesting the soil microbial community plays an important role in the presence and dissemination of ARBs and ARGs in the environment. Multiple studies has shown that the types of ARBs and ARGs in soil samples is a function of the microbial community structure (Durso et al., 2012; Forsberg et al., 2014; Peng et al., 2016), and research tends

[3] Actinomycetes are gram positive, generally anaerobic bacteria noted for a filamentous and branching growth pattern that results, in most forms, in an extensive colony, or mycelium. They belong to the order Actinomycetales and contain more than a dozen suborders.

to agree that any alteration to the microbial community in the environment will impact the ARBs and ARGs. Recent studies have also shown transfer rates are directly related to the microbial community (Subbiah et al., 2016), and some animal gut bacteria (Clostridia) persist in the environment long after excretion and can have a direct impact on the dissemination of ARGs to neighboring bacteria. In general, researchers are beginning to understand the complexities of AR from livestock manure management systems, but there still exists a significant knowledge gap.

1.3.2 Selection for Antibiotic Resistance in the Presence of Antibiotics

Entrance of antibiotics into the environment can occur via drug manufacturing processes, improper disposal of unused human and veterinary medications, land application of municipal waste treatment biosolids, and land application of livestock manure. Upon the introduction of antibiotics to the environment, bacteria will begin to interact with the drug compounds and their metabolites. The interaction that contributes to AR is largely complex and likely occurs in many different settings. Research suggests it could happen in the intestinal tract of animals, in excreted waste, in waste management systems, or in the environment long after it has left the animal (Sarmah et al., 2006). As reported in some studies, resistance may originate within commensal bacteria and then later transfer to other bacteria upon introduction to a new environment (SØrum and Sunde, 2001; Salyers et al., 2004). Analyses of the bacterial communities in the intestinal ecosystem of humans found large number of commensal bacteria (often more than 10^{14} colony forming units (CFU)) from several hundred species (Andremont et al., 2003). Fecal indicator bacteria such as enterobacteria and enterococci are considered relatively minor contributors to resistance due to lower intestinal quantities ranging from 10^{6} to 10^{8}

cells per gram of intestinal content (Chee-Sanford et al., 2009). The same study found the commensal genetic pool in the gut is so large and encompasses potential for multiple mechanisms conferring antibiotic resistance that antibiotic-resistant commensal bacterial may be selected each time an antibiotic is administered regardless of the health status of the animal (Chee-Sanford et al., 2009). Although still disputed, research has yielded strong evidence that the intestinal tract is an ideal ecosystem for the selection of antibiotic resistance. Though this phenomenon is not a simple relationship of cause and effect, research has demonstrated that the presence of antibiotics has the potential to alter the genetic resistome of bacterial communities in contact with antibiotics (Chee-Sanford et al., 2009).

The functional role of antibiotic resistance genes in antibiotic-producing bacteria is obvious (self-protection against the antibiotic synthesized), but the presence and function of these genes in bacteria from other ecological niches is not as clear from the literature. Numerous incidences of antibiotic resistance genes in presumably antibiotic-free environments suggest other factors drive the cells to maintain these functional genes (Allen et al., 2010). One plausible explanation for harboring these genes may be attributed to other co-metabolic housekeeping functions needed for the fitness of the organism (Chee-Sanford et al., 2009).

1.3.3 Mechanisms of Resistance

There are four known mechanisms of antibiotic resistance and, depending on the environmental conditions and cell structure, resistant genes will code for one or more mechanisms. These mechanisms are: (i) impermeable barriers, where some bacteria are

intrinsically resistant to certain antibiotics simply because they have an impermeable membrane or lack the target of the antibiotic; (ii) multidrug resistance efflux pumps, where pumps secrete antibiotics from the cell; some transporters, such as those of the resistance-nodulation-cell divisions family, can pump antibiotics directly outside the cell, whereas others, such as those of the major facilitator superfamily, secrete them into the periplasm; (iii) resistance mutations, where the mutations modify the target protein, for example by disabling the antibiotic-binding site but leave the cell functionality of the protein intact; and (iv) inactivation of the antibiotic, which can occur by covalent modification of the antibiotic, such as that catalyzed by acetyltransferases acting on aminoglycosides antibiotics (Poole, 2005; Allen et al., 2010).

1.3.4 Antibiotic Resistance in Similar Studies

Commonly studied antibiotic resistance gene classes, based on current literature and the relations of these genes to antibiotics commonly used in modern animal husbandry include *tet* (ten-eleven-translocation), *erm* (erythromycin ribosomal methylase), and *sul* (sulfonamide) genes. Today, scientists have identified approximately 558 *tet* genes, 129 *erm* genes and 180 *sul* genes (Liu and Pop, 2009; McArthur et al., 2013). **Table A.1** and **Table A.2** located in the appendix summarize *tet* and *erm* gene data compiled from the Comprehensive Antibiotic Resistance Database (CARD) and the Antibiotic Resistance Gene Database (ARDB). Genes are commonly subcategorized by mechanisms of resistance. The three known mechanisms are (i) efflux pump, (ii) ribosomal protection, and (iii) enzymatic. When studying ARGs transport, researchers commonly study gene groups by mechanisms as their fate and transport may be more closely related, though this is not always the case.

Since ARBs and ARGs are naturally occurring in the environment, it can be difficult to accurately define the impact of manure management practices on receiving soils. Because of this, a recent study was conducted to quantify the "background" abundance of 14 *tet* genes and 2 *sul* genes in the same geographical region presented in this thesis (Durso et al., 2016). The study found a large detection percentage of *tet*(A) and *tet*(D) in ungrazed prairies and very low detection percentages or no traces of *tet*(Q) or *tet*(X) in the same ungrazed prairies. These results are important to consider when choosing and analyzing gene abundance in soils within the sampling regions of eastern Nebraska. To our knowledge, there is no data available on background abundance of *erm* genes in relevant ungrazed prairies at this time.

Reports of the dissemination of ARGs in beef cattle systems are not as common as studies reporting on swine systems (Krapac et al., 2003; Koike et al., 2007a; Zhou et al., 2010; Sui et al., 2015). One study reported the frequency of ARBs seem to be especially high for swine as compared to cattle or sheep which correlates with the amount of antibiotics used in the production of these animals (Enne et al., 2008). In one case, swine lagoon and pit effluent was reported to contain tetracycline resistance efflux genes (*tet* B, C, E, H, Y, Z) and the ribosomal protection protein genes (*tet* W, O, Q, M, S, T, B(P), and *ort* A) (Aminov and Mackie, 2001). A three-year monitoring study reported the detection of *tet* (M, O, Q, W, C, H, and Z) consistently directly under two swine farms. Furthermore, *tet*(W) was detected in groundwater at approximately the same concentrations (99.8%) as the corresponding lagoon (Koike et al., 2007b).

Another study compared tetracycline and sulfonamide antibiotic residuals versus *tet* and *sul* ARGs in waste holding ponds of various animal operations (McKinney et al.,

2010). This research found that the relative abundances of *tet* genes decreased over time, but were still three to five orders of magnitude greater when compared to pristine river sediment. It was also reported that ARGs had the highest absolute abundance at the bottom of the lagoons, but when normalized to the 16S-rRNA genes, there was no significant difference between the relative abundances at different depths within the lagoon. Another recent publication on *tet* and *erm* genes reports losses in relative abundance of ARGs of approximately one to three orders of magnitude over a 40 day storage period in swine manure slurry under anaerobic conditions (Joy et al., 2014).

Past research has focused on the introduction of new bacteria (including pathogens) into the terrestrial environment following land application, and it has been shown that many microorganisms (possibly ARBs) can survive the transition from lagoon to soil surface (Boes et al., 2005). In one study, an increase in concentrations of ARBs was seen following manure application with a greater increase occurring in the move heavily manure soils (Andrews et al., 2004). Five months following application, the proportion of tetracycline resistant bacteria in all of the treated soils had returned to concentrations within the range of the non-manured control samples.

In summary, research has been focused on investigating the impacts of manure storage on AR distribution, but little research exists to describe AR dissemination in soils after long-term manure application, especially in beef cattle feedlot operations.

1.4 Objectives

Environmental routes of antibiotic resistance are largely unknown and the complexities of antibiotic resistance in agricultural systems are not well understood. While some research has been focused on identifying strategies to mitigate potential human health risks associated with antibiotic resistance originating from livestock production, a greater understanding of how livestock manure application to soil impacts the occurrence and dissemination of antibiotic resistance in soil is still needed. Therefore, the objective of the research presented in this thesis was as follows:

Quantify the concentrations of selected antibiotic resistant bacteria and antibiotic resistance genes as a function of manure accumulation and depth in soils (i) receiving long-term application of beef feedlot runoff holding pond effluent and (ii) utilized occasionally for beef cattle grazing with no history of supplemental manure application.

1.5 Thesis Presentation

This thesis is presented in manuscript form as a draft for publication. Chapter 1 is comprised of a comprehensive literature review of the current beef cattle manure management practices and the existing research on antibiotic resistance in the beef cattle agricultural system. Chapter 2 contains a summary of the thesis research project prepared for submission to the journal *Science of the Total Environment* under the manuscript titled, "Spatial Distribution of Antibiotic Resistance in Soils Receiving Beef Feedlot Runoff". Chapter 3 is an effective summary of the conclusions drawn from this study and suggestions for future research initiatives.

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CHAPTER 2. MANUSCRIPT DRAFT

Spatial Distribution of Antibiotic Resistance in Soils Receiving Beef Feedlot Runoff

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Abstract

Application of beef cattle manure to soil has been identified as a potential source of antibiotic resistant bacteria and antimicrobial resistance genes in the environment with subsequent risk for contamination of ground and surface waters. The objective of this study was to quantify and compare concentrations of antibiotic resistant bacteria and antibiotic resistance genes by soil depth and degree of manure accumulation within an agricultural field receiving long-term beef feedlot runoff holding pond effluent and a nearby field with no history or manure amendment. Soil dwelling and fecal indicator (*Enterococcus* and *Escherichia coli*) bacteria resistant to three antibiotics (cefotaxime, erythromycin, and tetracycline), and two classes of antibiotic resistant genes (*erm* and *tet*) were quantified by soil depth to 1.8 m and at six locations per field representing areas of varying manure accumulation determined by a response surface sampling design (RSSD) model based on apparent soil electrical conductivity measured using electromagnetic induction (EMI).

A significantly greater abundance of soil dwelling bacteria, erythromycin resistant bacteria, fecal indicator bacteria (FIB), *erm*(C), and *tet*(Q) were found on the field receiving the effluent. All ARBs decreased significantly ($p < 0.001$) by depth on both fields with an average 2-log reduction in CFU g^{-1} from the surface to a depth of 1.8 m. On the manured field, trace amounts of *erm*(C) were detected in 31% of samples throughout the soil profile with quantifiable averages of 10^4 copies g_{dw}^{-1}. Similarly, trace amounts of *tet*(Q) were detected in 58% of samples throughout the soil profile with quantifiable averages of 10^8 copies g_{dw}^{-1}.

Depth had a significant effect on the detection of *erm*(C), though *tet*(Q) was persistent at all depths tested. Areas of high manure accumulation yielded a greater abundance of soil dwelling bacteria at the surface and erythromycin bacteria at a depth of 60 – 80 cm. No correlation was found between EMI and antibiotic resistance genes. This is the first study to report a strong correlation ($r = 0.777$) between EMI and erythromycin resistant bacteria.

KEYWORDS

Manure, antibiotic resistance genes, antibiotic resistant bacteria, beef cattle, feedlot, soil

2.1 Introduction

Livestock waste generated from animal feeding operations (AFOs) represents a potential pathway of antibiotics and antibiotic resistance genes (ARGs) entering the environment (Wang et al., 2012). According to the USDA Economic Research Service, nearly 30 million head of beef cattle were produced in the United States 2015; each 1000 pound beef animal can produce approximately 60 pounds of manure per day. A common method of managing manure-laden runoff from beef cattle feedlots is collection and storage in holding ponds until the effluent can be applied to land. Land application of beef feedlot runoff provides important nutrients to plants including nitrogen, potassium, phosphorus, and micronutrients (Eghball et al., 2004). However, the land application of this effluent may introduce antibiotic resistant bacteria (ARBs) and ARGs to the terrestrial environment.

ARBs and ARGs are ubiquitous in soils systems regardless of the input of pharmaceuticals; therefore, background quantification of ARGs and ARBs must accompany measured ARGs and ARBs abundances to better determine the effects of manure management practices (Bhullar et al., 2012; Durso et al., 2012). Durso et al., (2016) reported background ARG abundance data for the geographic region investigated during this study. This abundance data was used to provide insight to the expected naturally occurring ARGs.

Tetracyclines, macrolides, cephalosporins, and ionophores are common drug classes used in beef cattle production in the US to treat and prevent disease Excretion rates of 75% of pharmaceuticals have been reported (Halling-Sorensen et al., 1998; Chee-

Sanford et al., 2009), and approximately 80% of these excreted pharmaceuticals were used for maintaining growth performance (Zhou et al., 2013). These excreted pharmaceuticals and their metabolites can persist in the manure, runoff holding ponds, and in the soil after manure has been land applied and create selective pressure for bacteria to harbor ARGs (Chee-Sanford et al., 2009).

Selective pressure is needed for bacteria to harbor ARGs and the literature has suggested pharmaceuticals and their metabolites in trace amounts provide enough stress to promote the proliferation of ARGs (Joy et al., 2014). Smith et al., 2004 reported a correlation of ARGs in a cattle feedlot lagoon and *tet*(O), *tet*(W), and *tet*(Q) gene copy numbers and tetracycline concentrations. Additionally, Koike et al., (2007a) reported similar ARGs concentrations in a swine manure lagoon and in groundwater directly beneath the lagoon suggesting the potential of groundwater contamination from the stored manure. Few studies have described fate and transport of ARGs following land application of swine wastewater (Koike et al., 2007b; Joy et al., 2014).

Although contributions of ARBs and ARGs from swine manure to the environment have been more extensively studied (Zhang et al., 2013), the contribution of these compounds to the environment from land application of beef feedlot runoff is not well known. Therefore, the objectives of this study was to quantify the concentrations of three ARBs important to human and animal treatment (cefotaxime, erythromycin, and tetracycline), fecal indicator bacteria (FIB), and two classes of ARGs (*erm* and *tet*) as a function of soil depth and manure accumulation in soils receiving long-term application of beef cattle feedlot runoff holding pond effluent.

2.2 Materials and Methods

2.2.1 Study Site Description

Two fields at the U.S. Meat Animal Research Center (USMARC) near Clay Center, Nebraska were utilized in this study (**Figure 2.1)**. One field has received furrow irrigation of beef feedlot runoff holding pond effluent from a 5000-head beef feedlot through a gated pipe for at least 25 years. The second field is a naturalized cool-season pasture used rotationally for cattle grazing with no history of supplemental manure application. Feedlot cattle were fed to finish weight using a diet of either corn or wet distillers grain and treated with antibiotics to maintain herd health. During the time of sampling, the manured field was plated to alfalfa and irrigated from the feedlot runoff hold pond as needed to meet crops water requirements. The field utilized as pasture is comprised of cool-season forage mixture. The fields were approximately 0.5 km apart and the soils at both sites are classified as Hastings silt loam (fine, smectitic, mesic Udic Artistolls).

2.2.2 Field Sampling Locations

Six sampling locations (**Figure 2.2**) were identified in each field by evaluating salt accumulation patterns resulting from disproportionate manure laden runoff irrigation using electromagnetic induction (EMI) combined with a response surface sampling design (RSSD) (Eigenberg et al., 2008; Woodbury et al., 2009b)**.** Briefly, a Dualem-1S meter (Dualem Inc., Milton, ON, Canada) was used to collect soil apparent electrical

conductivity (EC_a) data from each field. The meter was positioned on a nonmetallic sled and pulled approximately 1.5 m s^{-1} in a serpentine pattern across the surface of each site on 6 m path intervals. Path spacing was maintained using a Trimble EZ-Guide global positioning system (GPS)/Guidance System (Trimble Navigation Limited, Sunnydale, CA). The Dualem-1S meter simultaneously recorded both perpendicular (PRP) and horizontal coplanar (HCP) orientations. Positional coordinates of the meter were determined using an AgGPS 332 receiver with real-time kinematic (RTK) correction.

Data were collected at a rate of five measurements per second and stored in a Juniper System Allegro (Juniper System, Inc., Logan, UT) data logger. A spatial response surface sampling design (RSSD) program contained in the USDA-ARS ESAP (EC_a Sampling, Assessment, and Prediction) software package was used on the PRP array to select sampling locations that optimized the estimation of the various soil measures/EC_a calibration equations. Specific coordinates and relevant information regarding the sampling locations are reported in **Table C.1**.

2.2.3 Soil Sampling and Preparations

Intact soil cores were collected in 5 cm diameter thin-walled plastic tubes from six locations per field to a depth of 2.4 m using a Giddings hydraulic soil probe (Giddings Machine Co., Windsor, CO). The cores were segmented into 20 cm segments using a reciprocating saw, capped on the ends, and immediately placed on ice. The blade of the saw was sterilized between cuts using 100% ethanol to ensure no cross-contamination.

Moisture content (**Table C.2**) was determined gravimetrically following the American Society of Agricultural of Biological Engineers (ASABE) protocol. Briefly, 10

g of homogenized soil was placed in a pre-weighed aluminum boat, dried in an oven at 105°C for 24 h, and re-weighed. All samples were processed in triplicate and the average was reported.

2.2.4 Phenotypic Analysis and Bacterial Isolation

Soils for ARB analysis from three segments (0-20, 60-80, and 160-180 cm) of each core were processed within 24 h of collection. Samples were diluted by adding 10 g of soil to 90 ml 1X Phosphate Buffered Saline (PBS) (Fisher Scientific, Pittsburgh, PA) in a WhirlPak Filter Bag (Nasco, Atkinson, Wisconsin). The mixture was thoroughly mixed by hand and serially diluted in PBS to prepare for plating. Three media, R2A (Becton Dickenson, Franklin Lakes, NJ), m-Enterococcus (ME) (Becton Dickenson, Franklin Lakes, NJ), and ChromAgar E. coli (CEC) (CHROMagar, Paris, France) were prepared each with the addition of either none or one of three antibiotics (cefotaxime, erythromycin, or tetracycline) at concentrations of 4, 10, and 16 μg mL^{-1}, respectively. Antibiotic concentrations were based on the Clinical Laboratory Standards Institute (CLSI) thresholds for resistance classifications of bacteria. The media and antibiotic combinations are defined as follows: R2A with no antibiotic (R2A), R2A with cefotaxime (R2A+c), R2A with erythromycin (R2A+e), R2A with tetracycline (R2A+t), ME with no antibiotic (ME), ME with cefotaxime (ME+c), ME with erythromycin (ME+e), ME with tetracycline (ME+t), CEC with no antibiotic (CEC), CEC with cefotaxime (CEC+c), CEC with erythromycin (CEC+e), and CEC with tetracycline (CEC+t). 50 μL of homogenized soil solution was spiral plated in duplicate onto each media-antibiotic combination using an Eddy Jet Spiral Plater (Neutec Group Inc,.

Farmingdale, NY)., R2A, m-Enterococcus, and ChromAgar E. coli media were incubated at 25, 42, and 37°C, respectively, and for 72, 48, and 24 h, respectively.

2.2.5 Bacterial Isolate Analysis

Select isolates were collected, re-suspended, and stored in glycerol at -80°C until further analyzed to determine the diversity and magnitude of the resistome. Up to four isolates were picked from the soil dwelling bacteria (R2A) cultured from each field and fecal indicator bacteria (ME, CEC, CEC+e, CEC+t) cultured from the runoff-amended field. Soil dwelling bacteria isolates were suspended in Trypic Soy Broth (TSB) (Becton Dickenson, Franklin Lakes, NJ), and FIB were suspended in R2A broth (HiMedia Laboratories, Mumbai, India) with either 10 µg mL^{-1} erythromycin or 16 µg mL^{-1} tetracycline depending on the source of the isolate.

Disc diffusion analysis was performed according to Clinical Laboratory Standards Institute (CLSI) standard methods on the fecal indicator bacterial cultures for 12 antibiotics (**Table C.3)** described as highly or critically important to human medicine by the World Health Organization (WHO). Isolates were taken from the freezer stock and grown in TSB with none, 10 µg mL^{-1} erythromycin, or 16 µg mL^{-1} tetracycline depending on the source of the isolate. *Enterococcus* isolates were incubated at 42°C for 48 h and adjusted to an optical density (OD) of 0.900 ± 0.25 using a BioMate3 Spectrophotometer (Thermo Scientific, Waltham, MA). *E. coli* isolates were incubated at 37°C for 18-24 h and adjusted to an OD of 0.300 ± 0.25 using a BioMate3 Spectrophotometer. All diluted cultures were swabbed onto Mueller-Hinton II Agar (Becton Dickinson, Franklin Lakes, NJ) and incubated at 37°C (*E. coli*) or 42°C (*Enterococcus*) for 18-24 h. Zones of

inhibition were measured using Flash & Go (IUL Instruments, Barcelona, Spain) and characterized as resistant, intermediate, or susceptible based on standards given by Clinical Laboratory Standards Institute (CLSI).

The minimum inhibitory concentrations (MIC) were determined for the soil dwelling bacteria (R2A) using Etest strips (Biomerieux, Marcy-I'Etoile, France) for gentamicin (256 µg mL^{-1}), sulfamethoxazole with trimethoprim (32 µg mL^{-1}), ceftriaxone (32 µg mL^{-1}), nalidixic acid (256 µg mL^{-1}), erythromycin (256 µg mL^{-1}), and tetracycline (256 µg mL^{-1}). Isolates were thawed and cultured on R2A agar at 25°C for 72 h then re-suspended in R2A broth. Isolates were normalized to an OD of 0.900 ± 0.25 using a BioMate3 Spectrophotometer (Thermo Scientific, Waltham, MA) and adjusted cultures were swabbed onto R2A agar. E-strips were added to the inoculated plates and incubated at 25°C for 18-24 h.

2.2.6 DNA Extraction and Real-Time Polymerase Chain Reaction Analysis

Soils (approximately 5 g) for ARG analysis from six segments (0-20, 20-40, 40-60, 60-80, 100-120, and 160-180 cm) of each core were lyophilized for 48 hours and homogenized using a roller mill homogenizer with sterile metal bars and amber vials for 18 hours. Genomic DNA from approximately 250 mg of dry soil was extracted using the MoBio PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) with the modification of replacing the garnet beads with approximately 0.5 g of 0.1 mm sterile glass beads to more effectively lyse to cells from the high clay content soil matrix. The modification was determined by experimentally optimizing DNA yields from soil matrices with relatively high clay content and occasional low biomass in deeper soils.

DNA was released by bead beating at 4.5 m s^{-1} for 40 s twice using an Omni Bead Ruptor 24 (OMNI International, Kennesaw, Georgia). DNA extracts were confirmed and quantified with exACTGene 24 kb Max DNA Ladder (Fisher Scientific, Pittsburgh, PA) and gel electrophoresis imaging software (Kodak, Rochester, Ney York).

Three *erm* genes (*erm*(A), *erm*(B), *erm*(C)) and three *tet* genes (*tet*(A), *tet*(X), and *tet*(Q)) were analyzed. Only *erm*(A), *erm*(C), *tet*(X), and *tet*(Q) generated consistent results. ARGs were quantified using quantitative polymerase chain reaction (qPCR) and standards prepared as described in previous studies (Zhou et al., 2013; Joy et al., 2014). Briefly, the PCR products of ARGs were purified using a QIAquick PCR Purification Kit, cloned, and transformed using the TOPOP® TA Cloning Kit for Sequencing with One Shot TOP10 (Invitrogen, Carlsbad, CA). Plasmids were extracted from the transformed *E. coli* cells using Qiagen's Plasmid Midi Kit (Qiagen Sciences, Germantown, MD). The plasmid extracts containing target ARG amplicons were quantified using the NanoDrop spectrometer (Thermo Scientific, Waltham, MA) and calculated using a published equation (Li et al., 2012). Standards were serially diluted using Sigma water (Sigma-Aldrich, St. Louis, Missouri).

All qPCR reactions used Sigma Aldrich KiCqStart SYBR Green qPCR ReadyMix (Sigma-Aldrich, St. Louis, Missouri) and optimized primer concentrations between 200 – 600 nM and quantified using an Eppendorf MasterCycler RealPlex^4 (Eppendorf, Hamburg, Germany). All ARGs were normalized to the abundance of the 16S rRNA gene from each sample. Relevant qPCR conditions, primer selections, linear ranges, and reaction efficiencies are provided in supporting information (**Table C.7**).

2.2.7 Statistical Analysis

A repeated measures analysis of variance (ANOVA) with cores as the experimental unit and depths as the repeated measures factor was used to assess the effects of depth, field, and depth x field interactions on ARBs. Correlation was used to assess the association of manure accumulation with soil dwelling bacteria and ARBs. Fisher's exact test was used to test for effects of site and depth on the presence/absence of ARGs. All statistical computations were performed with SAS (SAS, Cary, NC.)

2.3 Results and Discussion

2.3.1 Antibiotic Resistant Bacteria Analysis

Soil dwelling ARBs were recovered from 94% of the runoff-amended field samples and 100% of the pasture samples (**Table C.4**, **Table C.5**). Previous work (Popowska et al., 2012) also reported greater detection of ARBs in non-manured than manure-amended soils, and found that non-manured soils yielded lower MICs, contained fewer ARGs, and did not display multidrug resistance (MDR).

Across all depths, there were significantly more (p = 0.026) soil dwelling bacteria in the manured-field compared to the pasture with means of 6.49 log CFU g^{-1} and 6.12 log CFU g^{-1} respectively. This is consistent with previous research (Andrews et al., 2004) reporting swine manure application to soils increased the soil dwelling bacteria populations.

Of the three ARB populations cultured (cefotaxime, erythromycin, and tetracycline resistant), only erythromycin resistant bacteria showed significant differences ($p = 0.023$) between fields when pooling all depths at a sampling location. Although there is a history of chlortetracycline (CTC) routinely used as a feed additive at the study site and CTC is commonly detected in manure and manure storage systems (Campagnolo et al., 2002), there was no evidence of an alteration to tetracycline resistant bacteria on the manured-field. However, Inglis et al. (2005) report a correlation between tetracycline concentrations and an increase of tetracycline resistance. Furthermore, CTC has been reported as the most persistent antibiotic in a soil-feces matrix (Chee-Sanford et al., 2009) with a half-life of nearly 100 days (Kolz et al., 2005), suggesting that in this study, tetracycline compounds did not persist after application of effluent from the runoff holding pond to create selective pressure for the proliferation of tetracycline resistance. One possible explanation suggested by Kim et al. (2011) is extractable concentrations of tetracycline and their metabolites decline with time in organic matrices.

When pooling all depths by each sampling location (**Table C.6**), the abundance of erythromycin resistant bacteria from the manured-field and the pasture were 4.93 log CFU g^{-1} and 4.30 log CFU g^{-1} ($p = 0.023$) respectively. Population of tetracycline resistant bacteria from the manured field and pasture were 3.92 log CFU g^{-1} and 3.89 log CFU g^{-1} ($p = 0.828$) respectively, and the population of cefotaxime resistant bacteria from the manured field and pasture were 5.78 log CFU g^{-1} and 5.71 log CFU g^{-1} ($p = 0.678$) respectively. These results support recent research (Subbiah et al., 2016) suggesting that not all antibiotic practices afford the same risk for proliferation of resistant bacteria in the environment.

Depth had a significant effect ($p < 0.0001$) on bacterial populations across both fields (**Figure 2.4**, **Table C.6**). There was an average 2-log CFU g^{-1} reduction among all measured bacteria across both fields observed from the soil surface to a depth of 1.8 m. The ANOVA among the mean concentrations of erythromycin resistant bacteria by depth in the manured field and the pasture revealed a significant difference at the middle (60 – 80 cm) and bottom (160 – 180 cm) depths, but not difference at the surface (0 – 20 cm). This suggests the vertical transport of erythromycin resistant bacteria could pose an increased risk in shallow groundwater from soils receiving beef feedlot runoff. A similar ANOVA revealed the bottom depth (160 – 180 cm) was not significant ($p = 0.004$) in the differences in non-resistant soil dwelling bacteria between fields. It is noteworthy that the major differences of bacterial populations were not significant at the surface, but instead at the middle (60 – 80 cm) depth (erythromycin resistant bacteria) and bottom (160 – 180 cm) (erythromycin resistant bacteria and non-resistant soil dwelling bacteria). One possible explanation for this phenomenon is as the deep root systems biodegrade in the soil profile on the manured field, new avenues are created for the transport of ARBs and ARGs deep into the soil profile. The relatively shorter root system of the naturalized cool-season pasture would not the same vertical transport.

Concentrations of soil dwelling bacteria at the soil surface and erythromycin resistant bacteria at 60 – 80 cm depth correlated positively with manure accumulation (r = 0.598 and 0.777 respectively) . Other studies have reported correlations between EMI data and nitrate, total nitrogen, and volatile fatty acids using the geospatial methods described in this study (Woodbury et al., 2009a; Tripathi and Mishra, 2014), but this is the first study reporting correlation between ARBs and EMI measurements.

Tylosin is routinely used in US beef cattle and has reported half-lives of an average of 4 – 8 days in manure and 10 – 40 days in surface-water simulation systems (Kolz et al., 2005). Loke et al. (2000) also reports tylosin and its degradation products are relatively stable in the manure matrix. The literature also finds tylosin compounds in addition to the parent antimicrobial may exert selective pressure for erythromycin ribosomal methylase resistance (Joy et al., 2014). Assuming the holding pond effluent contains concentrations of organic matter, tylosin-laced organic matter applied to the soils analyzed in this study may contribute to the phenomenon observed. There was no correlation found between manure accumulation and cefotaxime resistant bacteria ($p > 0.62$) or tetracycline resistant bacteria ($p > 0.42$).

2.3.2 Fecal Indicator Bacteria and Isolated Culture Analysis

Fecal indicator bacteria (FIB) (*Enterococcus* and *E. coli*) were cultured from approximately 50%, 33%, and 0% of the top (0 – 20 cm), middle (60 – 80 cm), and bottom (160 – 180 cm) depths respectively on the runoff-amended field, while the cool-season pasture yielded approximately 6% culturable FIB from all samples (**Figure 2.4**). This suggests FIB did not leach through the soil profile and, subsequently, did not appear to pose a risk for contamination of groundwater. This contradicts two past studies (Krapac et al., 2003; Cook et al., 2014) reporting detecting manure-borne FIB in groundwater as a result of animal production.

Disc diffusion analysis on 11 FIB isolates (four *Enterococcus* and seven *E. coli*) revealed several MDR isolates among both species (**Table 2.3**). Previous research (Popowska et al., 2012) found that MDR isolates were more prevalent in agricultural

environments, with the highest frequency in vegetable garden soil, and no detections in forest soils. On average, the 11 isolates displayed resistance to at least three out of 12 antibiotics classified as critically or highly important to human medicine according to the WHO priority list (**Table C.3**). The most widespread resistance illustrated by an *Enterococcus* isolate was an isolate demonstrating resistance to eight of 12 the antibiotics tested, while *E. coli* demonstrated resistance to just two of the 12 antibiotics. These results suggest that *Enterococcus* may possess a greater resistome to the antibiotic agents tested. Ten of 11 (91%) isolates displayed resistance to erythromycin and four of 11 (36%) isolates displayed resistance to tetracycline, which is consistent with our evaluation of soil dwelling ARBs, but considerably higher than previous work. One study (Inglis et al., 2005) of *Campylobacter* isolates from beef feedlots found 10% and 11% of isolates displayed resistance to erythromycin and tetracycline, respectively. While these incidences of resistant bacteria are considerably less than those found in this study, it is noteworthy that Inglis et al. (2005) cultured bacteria from feedlot surfaces while the cultures in this study were from soil receiving beef cattle runoff holding pond effluent. This may suggest that resistant bacteria flourish in runoff holding ponds or in the soil environment following manure application, or that ARGs present in the feedlot surface are acquired by soil dwelling bacteria following manure application.

The minimum inhibitory concentration (MIC) results from 35 non-resistant soil dwelling bacterial isolates determined by the Etest yielded additional insight on the diversity of the resistome and differences between fields (**Table 2.4**). The runoff-amended field had a higher median MIC for co-trimoxazole, erythromycin, tetracycline, and nalidixic acid. A similar study (Popowska et al., 2012) of soil dwelling isolates

cultured from soils receiving livestock manure application reported MIC ranges of 8 - 256 µg mL^{-1} for tetracycline and 0.094 - 256 µg mL^{-1} for erythromycin. The MIC of tetracycline reported in this study are 2-log µg mL^{-1} lower than Popowska et al. (2012), while the MIC of erythromycin in this study is within their reported range.

2.3.3 Antibiotic Resistance Genes Analysis

Quantifiable *erm*(C) genes (**Table C.8**) were detected in seven samples from the runoff-amended field (n = 36) averaging 6.11 x 10^4 copies g_{dw}^{-1} (5.17 x 10^{-5} copies g^{-1} relative to the 16S rRNA gene). Four of the seven samples were from the soil surface and three were at various depths down to 100 – 120 cm, all with similar magnitudes of absolute abundance. Trace amounts of *erm*(C) were found below the detection limit (100 copies μL^{-1}) in four samples (n = 36) from the runoff-amended field at various depths, though primarily found within the same soil core. Previous work primarily investigated *erm*(C) in swine facilities and Chen et al. (2007) reported a significantly greater abundance of *erm*(C) in swine manure compared to cattle manure. The same study reported the greatest detection of *erm*(C) in a swine lagoon and no detection in fresh cattle manure samples.

Quantifiable *tet*(Q) genes (**Table C.8**) were detected in three samples from the surface of the runoff-amended field (n = 36) averaging 2.48 x 10^8 copies g_{dw}^{-1} (3.88 x 10^{-2} copies g^{-1} relative to the 16S rRNA gene). Trace amounts of *tet*(Q) were found below the detection limit (10 copies μL^{-1}) in 18 samples of various depths from the manured-field (n = 36) and in two samples from the pasture (n = 18). There was a 7-log reduction of absolute abundance from the surface to a depth of 20 cm. This agrees with previous

research (Joy et al., 2013) reporting 5-log reductions of *tet*(Q) from the surface to a depth of 10 cm. Similar to *erm*(C), the same sampling location was observed to have the highest abundance of *tet*(Q) at the surface and 100% *tet*(Q) detections at the tested depths within the soil profile.

tet(X) and *erm*(A) were not detected in any of the soil samples from either field. Durso et al. (2016) reported similar findings of no detection of *tet*(Q) and very few detections of *tet*(X) on 20 different natural prairies, supporting our *tet*(X) results and providing evidence that the *tet*(Q) detected on the runoff-amended field is the result of long-term application of beef feedlot runoff holding pond effluent.

Because the majority of samples yielded *erm*(C) and *tet*(Q) quantities below the detection limit, statistical analysis was performed on the presence/absence datasets to determine the effects of depth (**Table 2.5**) and manure accumulation patterns (**Table 2.6**) on the dissemination of ARGs. There was a significant ($p < 0.05$) increase of *erm*(C) and *tet*(Q) on the surface (0 – 20 cm) of the manured-field compared to the pasture. Fisher's exact test comparing the surface with all other depths within a core suggested a significant difference in detection of *erm*(C) ($p = 0.053$) but not *tet*(Q) ($p = 0.820$). This suggests *erm*(C) may not transport vertically through the soil profile as willingly as *tet*(Q). Koike et al., (2007b) also found similar results of *tet*(Q) absolute abundances of approximately 10^7 copies g_{dw}^{-1} in agricultural soil samples and has detected *tet*(Q) frequently in groundwater adjacent to swine production facilities indicating depth does not have a strong effect on the vertical transport of *tet*(Q).

Manure accumulation did not have a significant effect on the dissemination of *erm*(C). This is inconsistent with our analysis of erythromycin resistant bacteria previously described. According to the Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al., 2013) and the Antibiotic Resistance Gene Database (ARDB) (Liu and Pop, 2009), there exist nearly 129 known genes conferring enzymatic resistance to *erm* compounds. This suggests other *erm* ARGs other than *erm*(C) could be responsible for the erythromycin resistance phenotypic expression we observed previously. Statistical analysis did indicate correlation ($p = 0.080$) between manure accumulation and *tet*(Q) detection suggesting areas of high manure accumulation could pose a greater risk to the proliferation of *tet*(Q), though a larger sample size ($n \geq 12$) could prove useful for future investigations.

2.4 Conclusions

A summary (**Table 2.7**) is provided showing the key factors that influence the dissemination of ARBs and ARGs investigated in this study. The long-term application of beef feedlot runoff holding pond effluent increased culturable soil dwelling bacteria, erythromycin resistant bacteria, *erm*(C), and *tet*(Q) in the soils analyzed. ARBs and ARG abundances were quantified by soil depth (0 to 1.8 m) and by degree of manure accumulation using a response surface sampling design model based on apparent soil electrical conductivity measured using EMI. Depth was determined to have a significant effect on the measured differences between ARB populations with an average 2-log CFU g_{dw}^{-1} reduction from the soil surface to a depth of 1.8 m. Areas of high manure deposition

strongly correlate (r = 0.777) with erythromycin resistant bacteria warranting further research to investigate the abundance of pharmaceutical compounds relative to manure accumulation as a potential source of selective pressure for maintaining resistance.

This study represents the first investigation of the influences of soil depth and degree of manure accumulation on the dissemination of ARBs and ARGs in soils receiving long-term beef cattle feedlot manure runoff effluent, though the exact mechanisms and source(s) of selective pressure leading to these results are unknown. Analysis of analysis of archived soil samples from this study to quantify concentrations of antibiotic compounds and their metabolites may yield results that improve understanding of the potential selective pressure contributing to the study results.

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2.7 Appendix

List of Figures

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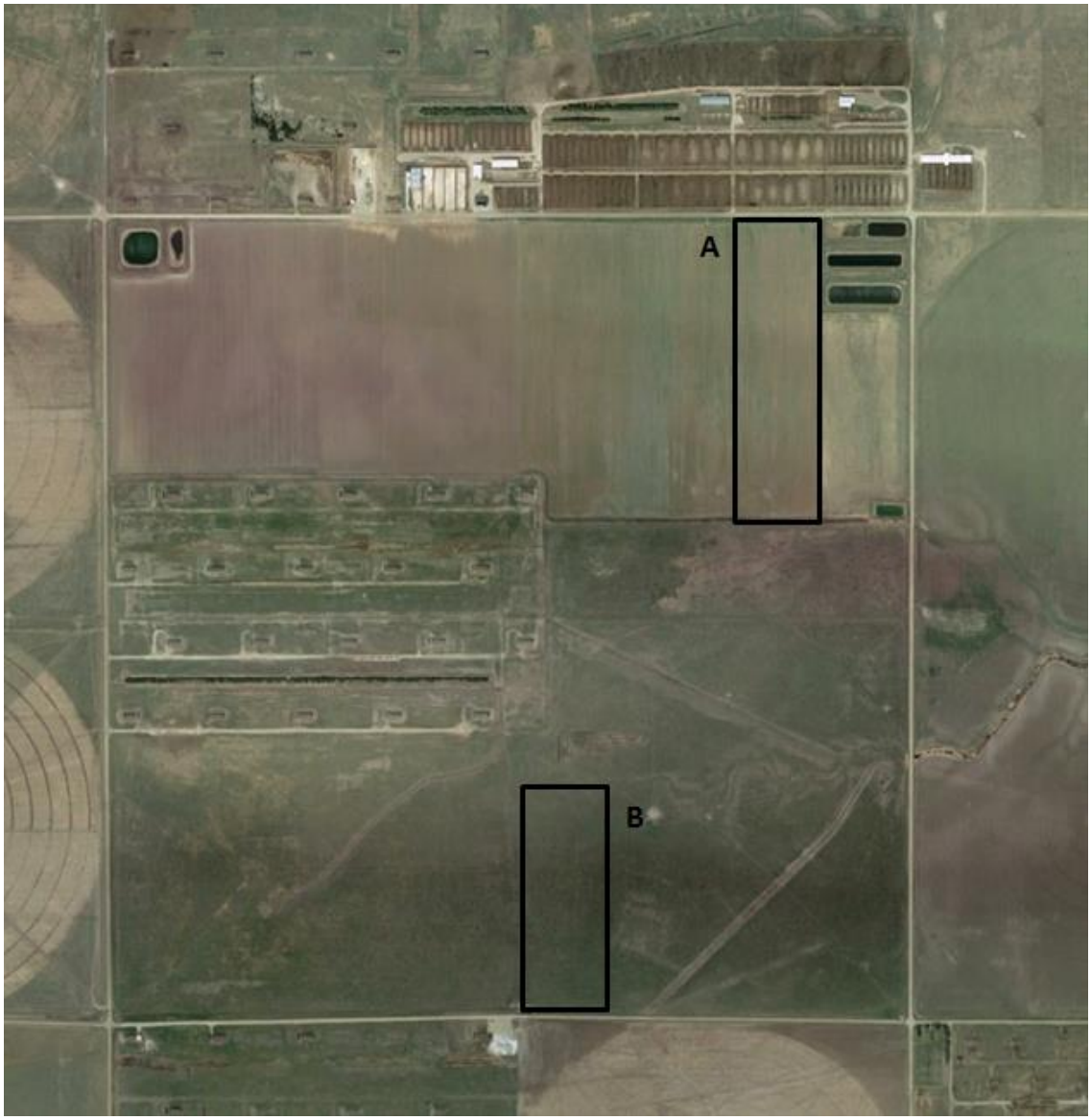

Figure 2.1 Aerial Image of the Sampling Field Boundaries at the U.S. Meat Animal Research Center near Clay Center, Nebraska

(A) Runoff-amended field
(B) Cool-season pasture

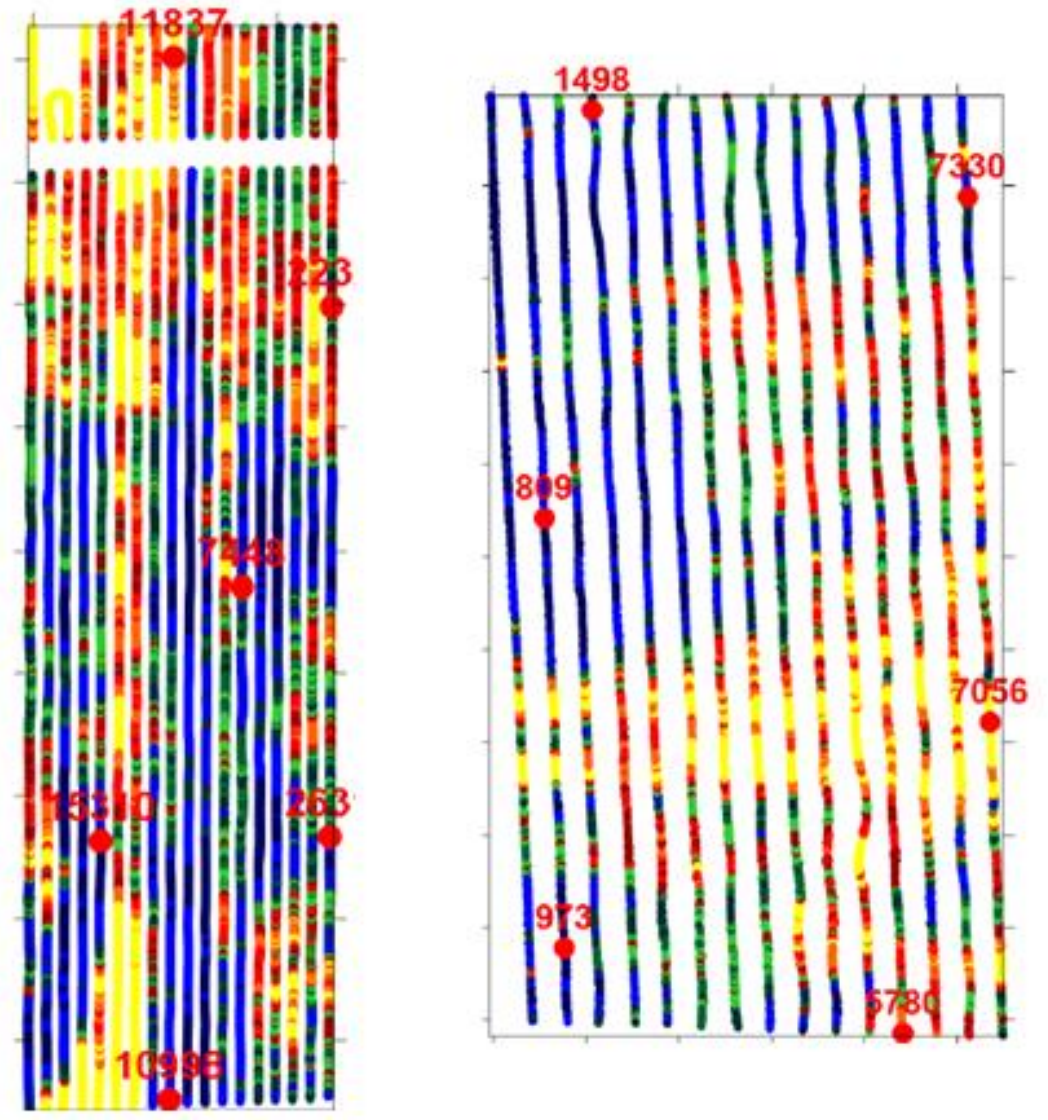

Figure 2.2 Manure Accumulation Patterns from the Runoff-Amended Field (left) and Cool-Season Pasture (right)

Manure accumulation patterns are represented by salt accumulation patterns shown above. High manure areas are shown in red, while low manure areas are shown in blue. EC_a ranges are (28.5, 44.7) for the runoff-amended field and (12.9, 29.3) for the pasture. The sampling locations determined by the response surface sampling design (RSSD) are shown in red.

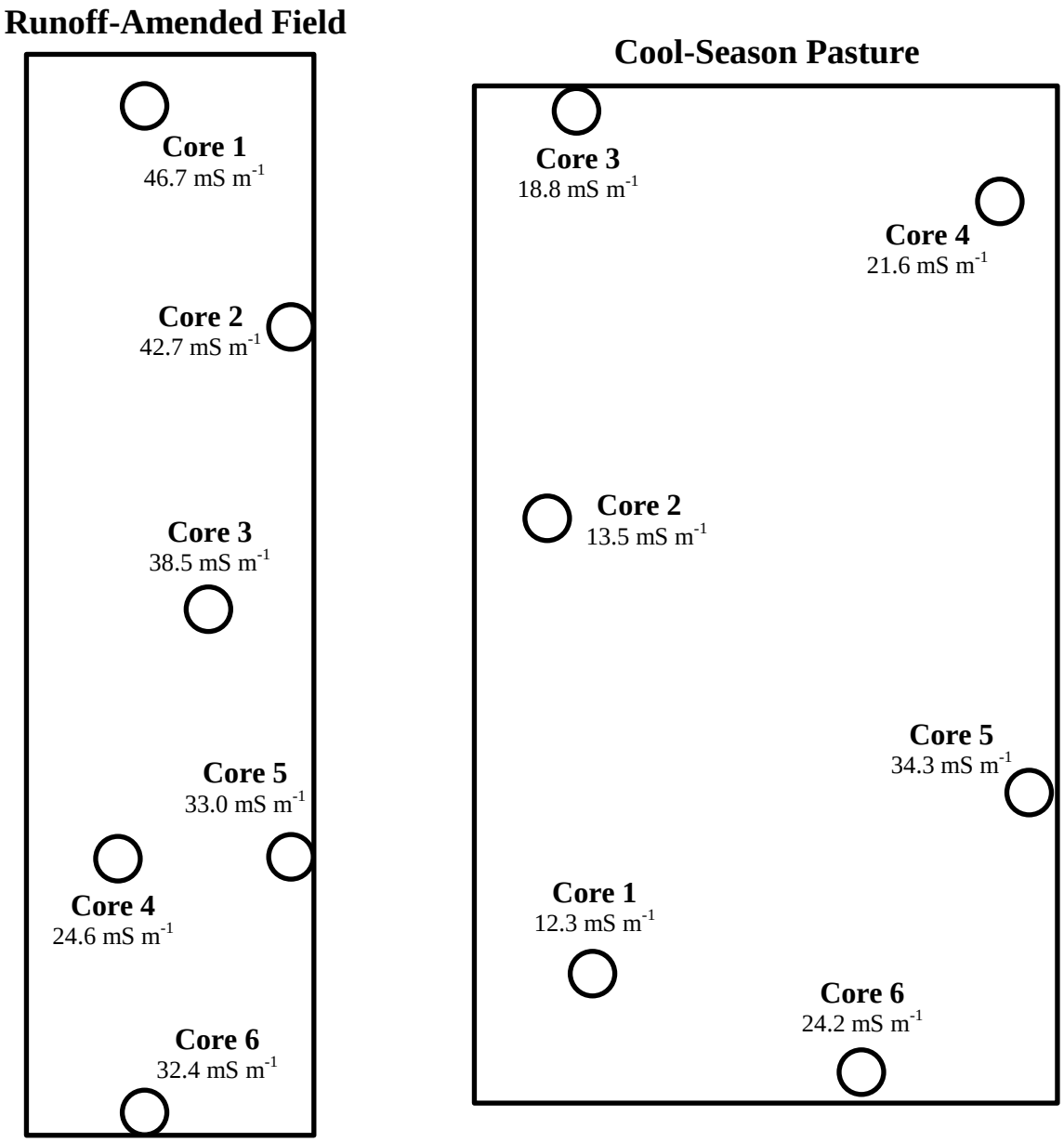

Figure 2.3 Schematic of Sampling Locations and Electrical Conductivity Measurements

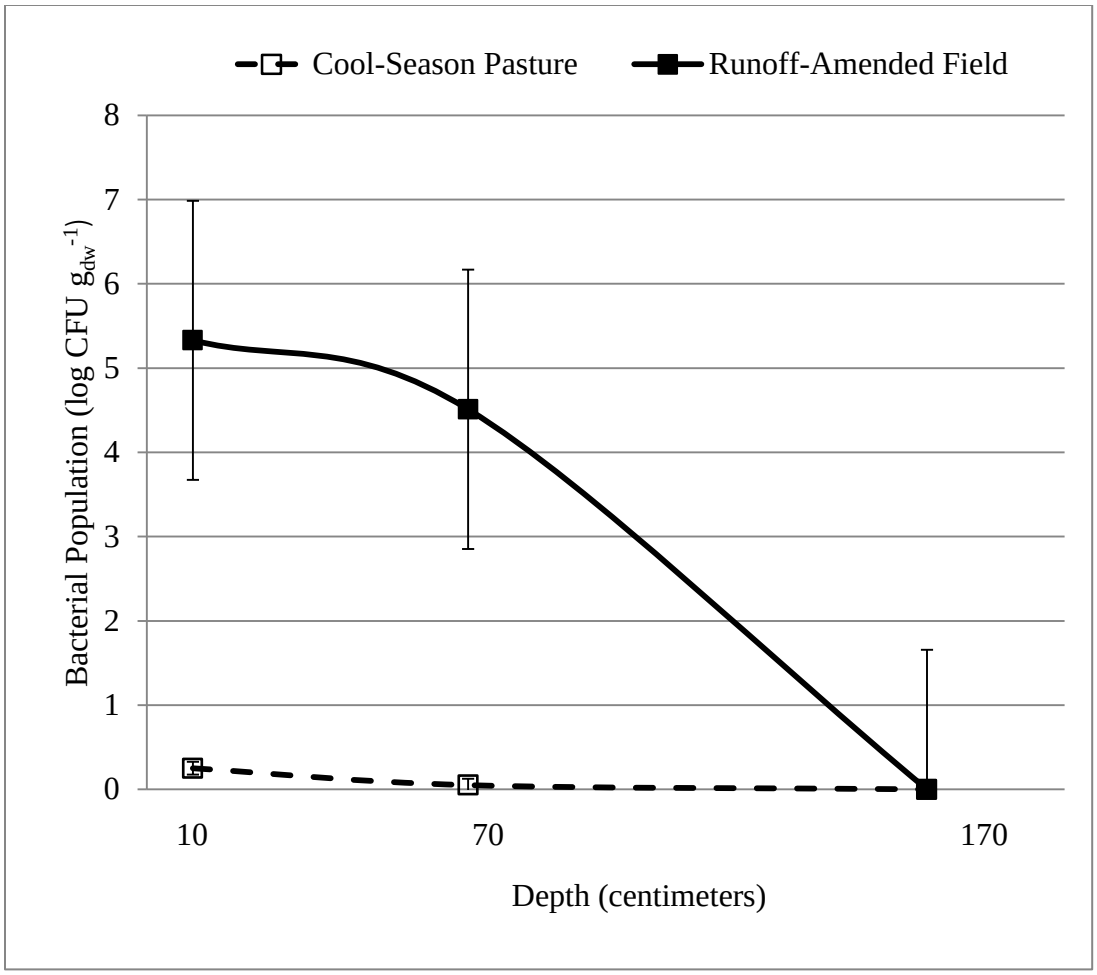

Figure 2.4 Mean Population of Fecal Indicator Bacteria (FIB) by Soil Depth

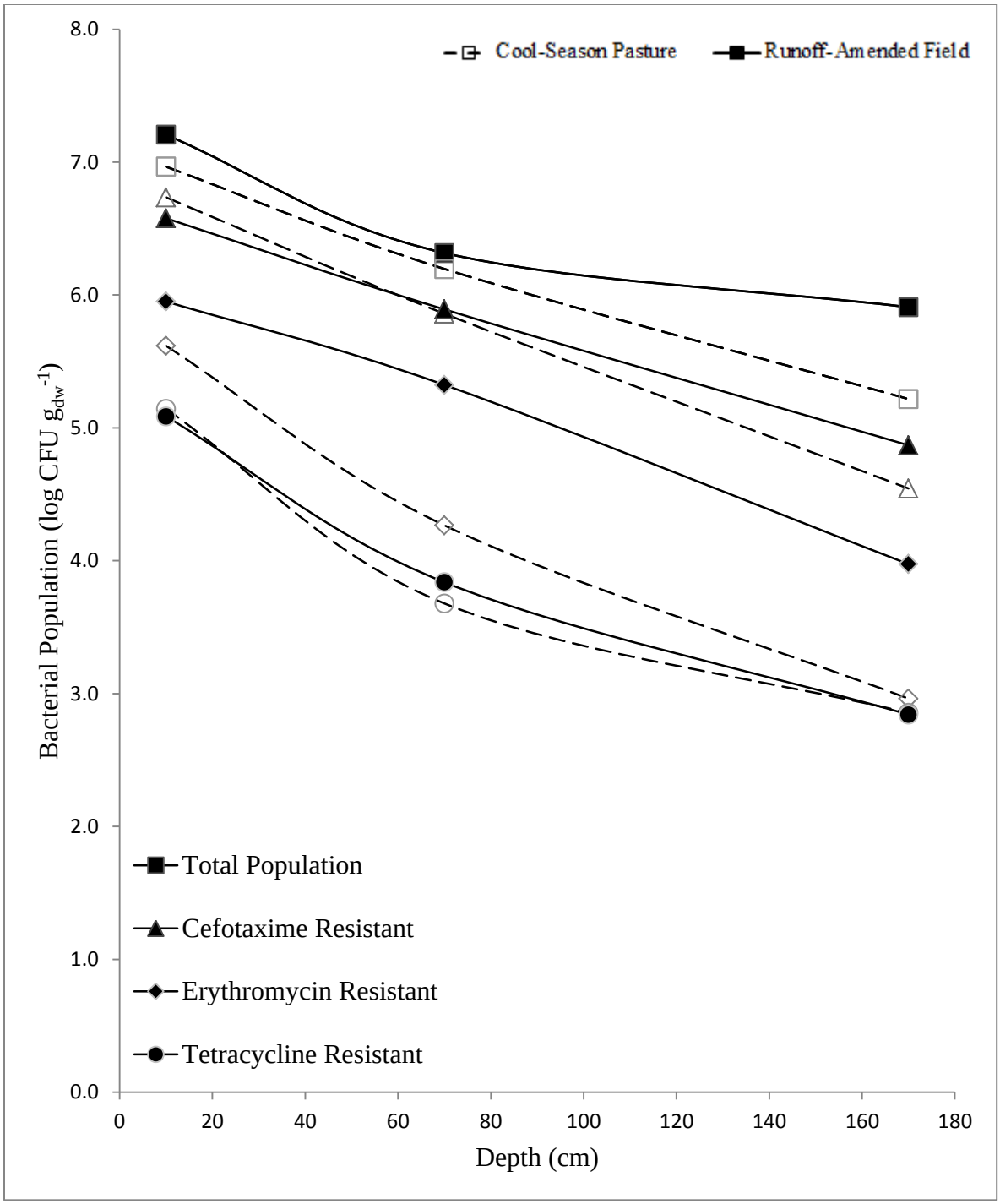

Figure 2.5 Mean Concentrations of ARBs by Soil Depth Among All Cores

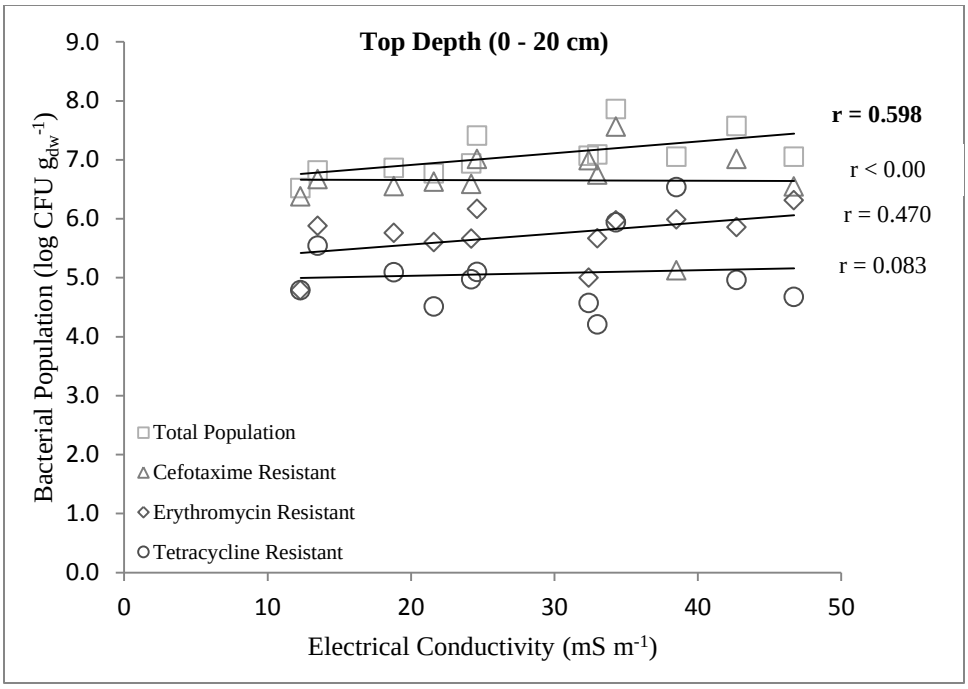

Figure 2.6 Correlations between Manure Accumulation and ARBs (Top Depth, 0 – 20 cm)

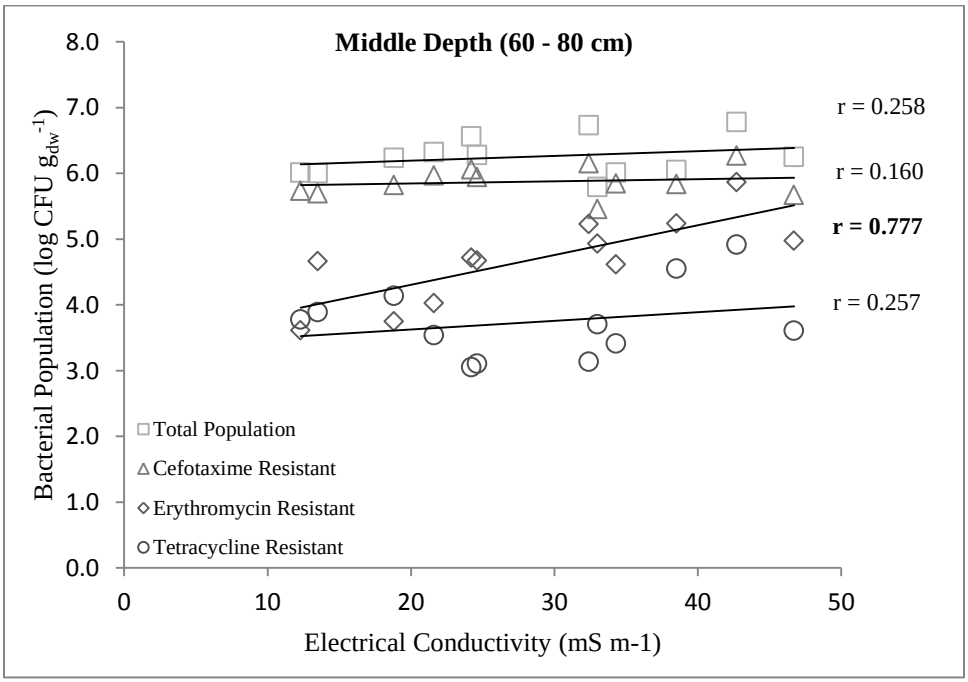

Figure 2.7 Correlations Between Manure Accumulation and ARBs (Middle Depth, 60 – 80 cm)

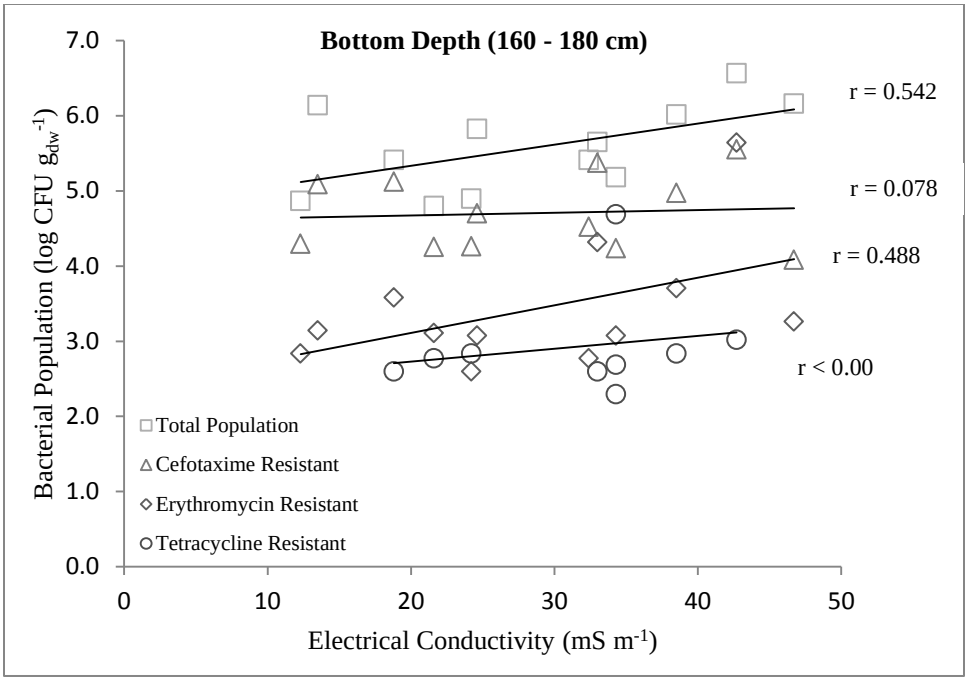

Figure 2.8 Correlations Between Manure Accumulation and ARBs (Bottom Depth, 160 – 180 cm)

Table 2.1 Mean Populations (log CFU g_{dw}^{-1}) of ARBs by Field and Depth

Depth[1]	Statistic	Total Population	Cefotaxime Resistant	Erythromycin Resistant	Tetracycline Resistant
Pooled Depths and Sites (n = 18, 16)	Mean	**6.49^{a} (6.12)^{b}**	**5.78 (5.71)**	**4.93^{a} (4.30)^{b}**	**3.87 (3.92)**
	Std. Error	*0.098*	*0.111*	*0.165*	*0.193, 0.171*
Top (n = 6,6)	Mean	**7.21^{x} (6.96)^{x}**	**6.57^{x} (6.73)^{x}**	**5.83^{x} (5.61)^{x}**	**5.01^{x} (5.14)^{x}**
	Std. Error	*0.157*	*0.191*	*0.237*	*0.271*
Middle (n = 6,6)	Mean	**6.32^{y} (6.19)^{y}**	**5.89^{y} (5.86)^{y}**	**5.15^{ya} (4.23)^{yb}**	**3.84^{y} (3.64)^{y}**
	Std. Error	*0.157*	*0.191*	*0.237*	*0.271*
Bottom (n = 4,6)	Mean	**5.94^{za} (5.22)^{zb}**	**4.87^{z} (4.54)^{z}**	**3.80^{za} (3.06)^{zb}**	**2.75^{z} (2.99)^{z}**
	Std. Error	*0.157*	*0.191*	*0.238*	*0.382, 0.277*

[1] Top = 0 - 20 cm, Middle = 60 - 80 cm, Bottom = 160 - 180 cm.
Runoff-amended field means (left) followed by the cool-season pasture means in parenthesis (right).
Superscripts represent significance ($\alpha < 0.05$) between depths (x,y,z), and between fields (a,b).

Table 2.2 Simple Pearson Correlations of Manure Accumulation and ARB Populations

Depth	Total Population	Cefotaxime Resistant	Erythromycin Resistant	Tetracycline Resistant
Top (0 - 20 cm)	**0.598**	< 0.00	0.470	0.083
	(0.04)	(0.97)	(0.12)	(0.80)
Middle (60 - 80 cm)	0.258	0.160	**0.777**	0.257
	(0.42)	(0.62)	**(0.00)**	(0.42)
Bottom (160 - 180 cm)	0.542	0.078	0.488	< 0.00
	(0.07)	(0.81)	(0.11)	(0.89)

Correlation coefficients are in **bold** if they are significant ($\alpha < 0.05$). The *p-value* is shown in parenthesis.

Table 2.3 Proportion of Fecal Indicator Bacteria Isolates Cultured from the Runoff-Amended Field Displaying Resistance to 12 WHO Priority List Antibiotic Compounds

Antibiotic Compound	WHO Priority	*Enterococcus* (n=4)	*Escherichia coli* (n=7)
Ceftriaxone	Critical	100	-
Chloramephenicol	n/a	-	-
Cefoxitin	High	50	-
Erythromycin	Critical	75	100
Tetracycline	High	50	29
Co-trimoxazole	High	-	-
Nalidixic Acid	Critical	100	-
Ciprofloxacin	Critical	25	-
Gentamicin	Critical	-	-
Streptomycin	Critical	100	43
Meropenem	Critical	75	-
Ampicillin	Critical	-	-

Table 2.4 Median and Mean[1] Minimum Inhibitory Concentrations (MIC) for Soil Dwelling Bacteria in µg mL^{-1}

Antibiotic (Drug Class)	Co-trimoxazole (Antifolate)		Ceftriaxone (Cephalosporin)		Erythromycin (Macrolide)		Tetracycline (Tetracycline)		Nalidixic Acid (Quinolone)		Gentamicin (Aminoglycoside)	
Detection Limits (min, max)	**(0.002,32)**		**(0.002,32)**		**(0.016,256)**		**(0.016,256)**		**(0.016,256)**		**(0.016,256)**	
Runoff-Amended Field (n=17)	0.09	(5.43)	6.00	(11.69)	0.25	(32.03)	0.06	(0.72)	24.00	(22.50)	4.00	(12.51)
Cool- Season Pasture (n=18)	0.08	(2.32)	7.50	(15.01)	0.08	(16.97)	0.03	(2.82)	12.00	(19.32)	4.00	(7.20)

Median (Mean)

[1] Values outside of the detection limits were set equal to the minimum or maximum detection limits for the calculation of the mean.

Table 2.5 Effect of Depth in ARG Detection for the Runoff-Amended Field

Depth (cm)	*erm*(C)	*tet*(Q)
	Number of Detections (n=6)	Number of Detections (n=6)
0 - 20	5[a]	5[a]
20 - 40	0	4
40 - 60	1	3
60 - 80	2	3
100 - 120	2	3
160 - 180	1	3

Superscripts represent significance ($\alpha < 0.05$) compared to cool-season pasture (0 detections)

Table 2.6 Quantities of Samples Yielding Detection of *erm*(C) and *tet*(Q) as a Function of Degree of Manure Accumulation

EC (mS m^{-1})	*erm*(C)	*tet*(Q)
	Number of Detections (n=6)	Number of Detections (n=6)
24.6	1	3
32.4	1	1
33.0	2	3
38.5	1	3
42.7	5	6
46.7	1	5
Spearman's Rank Correlation	**r = 0.270**	**r = 0.759**
	***p* = 0.604**	***p* = 0.080**

Table 2.7 Summary of Key Factors Influencing ARBs and ARGs in Soil Based Upon Study Results

Factor	ARBs			ARGs	
	Cefotaxime Resistant	**Erythromycin Resistant**	**Tetracycline Resistant**	***erm*(C)**	***tet*(Q)**
Long-term Application of Beef Feedlot Runoff Holding Pond Effluent	No effect	Significant Increase[1]	No effect	Significant Increase[2]	Significant Increase[2]
Vertical Transport in Soil (Surface to 1.8 m)	Significant Decrease	Significant Decrease	Significant Decrease	Moderate Decrease	No effect
Manure Accumulation Patterns (Low to High)	No effect	Significant Strong Positive Correlation[1]	No effect	No effect	No effect

[1] Significant increase at pooled depths, 60 – 80 cm, and 160 – 180 cm

[2] Significant increase at the surface depth

[3] Significant correlation at depth 60 - 80 cm

CHAPTER 3. GENERAL CONCLUSIONS

3.1 Conclusions

The application of beef feedlot runoff holding pond effluent to agricultural soils offers beneficial nutrients to the receiving soils, but could also lead to increased concentrations of veterinary pharmaceuticals, their degradation products, antibiotic resistant bacteria (ARBs), and antibiotic resistance genes (ARGs). Veterinary pharmaceuticals and their degradation products have been shown to create a selective pressure for microbial communities to develop and harbor ARGs and lead to the proliferation of antibiotic resistance (AR) in agriculture systems. Research has suggested that the agricultural environment is a potential pathway for AR to impact humans creating a potential increased risk to human health.

Two fields were studied to determine the effects of land application of manure-laden runoff from beef feedlots on ARBs and ARGs in soil, and to identify key factors that influence the dissemination of ARBs and ARGs in soils. One field received long-term application of beef feedlot runoff holding pond effluent, while the second field was a cool-season pasture with no history of supplemental manure amendment. The quantification of three ARBs and two ARGs were reported as a function of soil depth and manure accumulation patterns from a response surface sampling design model based on apparent conductivity measured using electromagnetic induction (EMI).

From this research, the following conclusions were made:

1. The long-term application of beef feedlot runoff holding pond effluent increased culturable soil dwelling bacteria, erythromycin resistant bacteria, *erm*(C), and *tet*(Q) compared to a cool-season pasture.
    a. Differences in soil dwelling bacteria between fields were most significant at the bottom depth (160 – 180 cm).
    1. Differences in erythromycin resistant bacteria between fields were most significant at the depths of 60 – 80 cm and 160 – 180 cm.
    b. The differences in ARG detections between fields were only significant at the surface.
2. Soil depth significantly impacted concentrations of ARBs and some ARGs.
    a. Depth significantly reduced ARB populations with an average 2-log reduction from surface samples to samples at a depth of 1.8 m.
    b. *erm*(C) did not appear to be moving through the soil profile, whereas *tet*(Q) was abundant throughout all soil depths.
3. Erythromycin resistant bacteria abundance in soil appeared to be significantly impacted by manure accumulation.

a. Areas of high manure accumulation appear to pose a greater risk for proliferation of erythromycin resistance than areas with lower accumulation of manure.

b. Manure accumulation had a significant weak correlation with non-resistant soil dwelling bacteria.

c. Manure accumulation did not have an effect on the detection of *erm*(C) or *tet*(Q).

3.2 Recommendations

From this research, the following recommendations are offered for future research:

1. Sample more runoff-amended fields; and more cores ($n \geq 12$).
2. Quantify antibiotics and their metabolites in addition to ARBs and ARGs.
3. Use the summary of key factors table to guide future research initiatives.
4. Optimize DNA extraction methods for the specific characteristics of the soil.
5. Compare this data with other antibiotic resistance pathways to determine where the greatest increased risk exists.

Appendices

Appendix A. *tet* and *erm* Gene Information

Table A.1 Compiled Summary of *tet* Gene Data

Gene[1]	Definition[1]	Number of Genes[2]	Synonyms(s)[1]	Mechanism
tet(A)	*tet(*A) is a tetracycline efflux pump found in many species of Gram-negative bacteria.	61	-	Efflux Pump
tet(B)	*tet*(B) is a tetracycline efflux protein expressed in many Gram-negative bacteria. It confers resistance to tetracycline, doxycycline, and minocycline, but not tigecycline.	53	-	Efflux Pump
tet(C)	*tet*(C) is a tetracycline efflux pump found in many species of Gram-negative bacteria. It is typically found in plasmid DNA.	35	-	Efflux Pump
tet(D)	*tet(*D) is a tetracycline efflux pump found exclusively in Gram-negative bacteria.	19	-	Efflux Pump
tet(E)	*tet(*E) is a tetracycline efflux pump found in many Gram-negative bacteria, especially those in water environments. The gene is found on large plasmids.	5	-	Efflux Pump
tet(G)	*tet(*G) is a tetracycline efflux protein found in Gram-negative bacteria. It is found in both chromosomal and plasmid DNA, and is linked to floR, sul1, and cmlA9 (florfenicol/chloramphenicol, sulfamethoxazole, and chloramphenicol resistance genes, respectively).	9	-	Efflux Pump
tet(H)	*tet(*H) is a tetracycline efflux protein expressed in Gram-negative bacteria (Actinobacillus, Acinetobacter, Gallibacterium, Histophilus, Mannheimia, Moraxella, Pasteurella, and Psychrobacter). Its gene is linked to the resistance genes sul2, and strAB, which confer resistance to sulfamethoxazole and streptomycin, respectively.	13	-	Efflux Pump
tet(J)	*tet(*J) is a tetracycline efflux protein expressed in Gram-negative bacteria (Escherichia, Morganella, and Proteus).	3	-	Efflux Pump
tet(K)	*tet(*K) is a tetracycline efflux protein found in both Gram-negative (Haemophilus and Gallibacterium) and Gram-positive (many species, including mycobacteria) bacteria.	9	-	Efflux Pump
tet(L)	*tet(*L) is a tetracycline efflux protein found in many species of Gram-negative and Gram-positive bacteria.	33	-	Efflux Pump
tet(M)	*tet(*M) is a ribosomal protection protein that confers tetracycline resistance. It is found on transposable DNA elements and its horizontal transfer between bacterial species has been documented.	95	-	Ribosomal Protection
tet(O)	*tet(*O) is a ribosomal protection protein. It is associated with conjugative plasmids.	41	-	Ribosomal Protection
*tet*A(P)	*tet*A(P) is a inner membrane tetracycline efflux protein found on the same operon as the ribosomal protection protein *tet*B(P). It is found in Clostridium, a Gram-positive bacterium.	18*	*tet*P	Efflux Pump

*tet*B(P)	*tet*B(P) is a tetracycline ribosomal protection protein found on the same operon as *tet*A(P), a tetracycline efflux protein.	18*	*tet*P	Ribosomal Protection
tet(Q)	*tet(*Q) is a ribosomal protection protein. Its gene is associated with a conjugative transposon and has been found in both Gram-positive and Gram-negative bacteria.	22	-	Ribosomal Protection
tet(S)	*tet*(S) is a ribosomal protection protein found in Gram-positive and Gram-negative strains. It is similar to *tet*(M) and *tet*(O).	12	-	Ribosomal Protection
tet(T)	*tet*(T) is a ribosomal protection protein of streptococci. It is similar to *tet*(Q).	2	-	Ribosomal Protection
tet(V)	*tet(*V) is a tetracycline efflux protein that has been found in Mycobacterium smegmatis and M. fortuitum.	2	-	Efflux Pump
tet(W)	*tet(*W) is a ribosomal protection protein. It is associated with both conjugative and non-conjugative DNA and has been found strains of C. difficile.	42	-	Ribosomal Protection
tet(X)	*tet(*X) is a flavin-dependent monooxygenase conferring resistance to tetracycline antibiotics. Hydroxylates at position 11a of the *tet*raketide.	5	-	Ezymatic
tet(Y)	*tet(*Y) is a tetracycline efflux pump found in Gram-negative bacteria (Aeromonas and Escherichia). It is associated with plasmid DNA.	8	-	Efflux Pump
tet(Z)	*tet(*Z) is a tetracycline efflux protein found in Gram-positive bacteria (Corynebacterium and Lactobacillus). It is associated with plasmid DNA.	1	-	Efflux Pump
tet(30)	*tet(*30) is a tetracycline efflux pump found in agrobacterium, a Gram-negative bacterium.	3	-	Efflux Pump
tet(31)	*tet(*31) is a tetracycline efflux pump found in Aeromonas salmonicida, a Gram-negative bacteria. It has also been shown to be expressed in Gallibacterium anatis.	1	-	Efflux Pump
tet(32)	*tet(*32) is a tetracycline resistance gene similar to *tet*(O), and binds to the ribosome to confer tetracycline resistance as a ribosomal protection protein.	13	-	Efflux Pump
tet(33)	*tet(*33) is a tetracycline efflux pump found in Gram-positive bacteria, including Arthrobacter and Corynebacterium.	3	-	Efflux Pump
tet(34)	*tet(*34) causes the activation of Mg^{2+}-dependent purine nucleotide synthesis, which protects the protein synthesis pathway. It is found in Gram-negative Vibrio	17		Enzymatic
tet(35)	*tet(*35) is a tetracycline efflux pump found in the Gram-negative Vibrio and Stenotrophomonas. It is unrelated to other *tet* resistance genes.	0*	effJ	Efflux Pump
tet(36)	*tet*(36) is a tetracycline resistance gene found in Bacteroides similar to *tet*(Q), and binds to the ribosome to confer antibiotic resistance as a ribosomal protection protein.	1	-	Ribosomal Protection
tet(37)	*tet(*37) is a chromosome-encoded oxidoreductase isolated from an uncultured bacterium that confers resistance to tetracycline	2		Enzymatic
tet(38)	*tet(*38) is a tetracycline efflux pump found in the Gram-positive Staphylococcus aureus. It is regulated by mgrA, which also regulates NorB.	22	-	Efflux Pump

tet(39)	*tet(*39) is a tetracycline efflux pump found in Gram-negative bacteria, including Brevundimonas, Stenotrophomonas, Enterobacter, Alcaligenes, Acinetobacter, and Providencia.	1	-	Efflux Pump
tet(40)	*tet(*40) is a tetracycline efflux pump found in the Gram-positive Clostridium. It is similar to *tet*A(P).	5	-	Efflux Pump
tet(41)	*tet(*41) is a tetracycline efflux pump found in Serratia, a Gram-negative bacterium. It is related to Acinetobacter *tet*(39).	1	*tet*A(41)	Efflux Pump
tet(42)	*tet(*42) is a tetracycline efflux pump found in both Gram-negative (Pseudomonas) and Gram-positive (Microbacterium, Bacillus, Staphylococcus, Paenibacillus) bacteria.	0[3]	*tet*A(42)	Efflux Pump
tet(43)	*tet*(43) is a tetracycline resistance gene with unknown origins, isolated from metagenomic DNA.	0[3]	-	N/A
tet(44)	*tet(*44) is a tetracycline resistance gene found in Campylobacter fetus, and binds to the ribosome to confer antibiotic resistance as a ribosomal protection protein.	0[3]	-	Ribosomal Protection
tet(45)	*tet(*45) is a tetracycline efflux pump found in Bhargavaea cecembensis strain previously isolated from a poultry-litter-impacted soil.	0[3]	-	Efflux Pump

[1]CARD - The Comprehensive Antibiotic Resistance Database (https://card.mcmaster.ca/home)
[2]ARDB - Antibiotic Resistance Genes Database (https://ardb.cbcb.umd.edu/)
[3]In question

Table A.2 Compiled Summary of *erm* Gene Data

Gene	Definition[1]	Number of Genes[2]	Synonyms(s)[1]	Mechanism
erm(A)	*erm*(A) confers the MLS_b[3] phenotype. Similar to *erm*(C), Expression of *erm*(A) is inducible by erythromycin. The leader peptide causes attenuation of the mRNA and stabilizes the structure preventing further translation. When erythromycin is present, it binds the leader peptide causing a change in conformation allowing for the expression of *erm*(A).	25	*erm*TR	Enzymatic
erm(B)	*erm*(B) confers the MLS_b phenotype. Similar to *erm*(C), expression of *erm*(B) is inducible by erythromycin. The leader peptide causes attenuation of the mRNA and stabilizes the structure preventing further translation. When erythromycin is present, it binds the leader peptide causing a change in conformation allowing for the expression of *erm*(B).	20	*erm*BC, *erm*, *erm*Z, *erm*BP, *erm*AM, *erm*BZ1, *erm*P, *erm*BZ2, *erm*IP, *erm*AMR, *erm*2	Enzymatic
erm(C)	*erm*(C) is a methyltransferase that catalyzes the methylation of A2058 of the 23S ribosomal RNA in two steps. Expression of *erm*(C) is inducible by erythromycin. The leader peptide causes attenuation of the mRNA and stabilizes the structure preventing further translation. When erythromycin is present, it binds the leader peptide causing a change in conformation allowing for the expression of *erm*(C).	25	*erm*(C)', *erm*IM, *erm*M	Enzymatic
erm(D)	*erm*(D) confers MLS_b phenotype.	4	*erm*K, *erm*J	Enzymatic
erm(F)	*erm*(F) confers the MLS_b phenotype.	10	*erm*FU, *erm*FS	Enzymatic
erm(G)	*erm*(G) is a rRNA adenine N-6-methyltransferase that protects the ribosome from inactivation due to antibiotic binding.	5	-	Enzymatic
erm(H)	*erm*(H) is a plasmid-mediated methyltransferase found in Streptomyces th*erm*otolerans	1	carB	Enzymatic
erm(N)	*erm*(N) is a methyltransferase found in the tylosin producer Streptomyces fradiae. Like other e*rm* enzymes, it catalyzes the methylation of A2058 of the 23S ribosomal RNA. Specifically, this enzyme transfers only one methyl group. The gene is found in the tylosin biosynthetic cluster and is responsible for self-resistance to tylosin.	1	tlrD	Enzymatic
erm(O)	*erm*(O) is a methyltransferase found in the spiramycin producer Streptomyces ambofaciens. Like other e*rm* enzymes, it catalyzes the methylation of A2058 of the 23S ribosomal RNA. Specifically, this enzyme transfers only one methyl group. The gene is responsible for self-resistance to spiramycin.	4	srmA, Irm	Enzymatic
erm(Q)	*erm*(Q) confers MLS_b phenotype.	2	-	Enzymatic
erm(R)	*erm*(R) is a methyltransferase found in the erythromycin producer Aeromicrobium erythreum. Like other *erm* enzymes, it catalyzes the methylation of A2058 of the 23S ribosomal RNA. The gene is found within the erythromycin biosynthetic cluster and is responsible for self-resistance.	3	-	Enzymatic

erm(S)	*erm*(S) is a methyltransferase found in the tylosin producer Streptomyces fradiae. Like other *erm* enzymes, it catalyzes the methylation of A2058 of the 23S ribosomal RNA. Specifically, this enzyme transfers two methyl groups. The gene is found within the tylosin biosynthetic cluster and is responsible for self-resistance	1	*erm*SF, tlrA	Enzymatic
erm(T)	*erm*(T) confers MLS_b phenotype.	5	*erm* GT	Enzymatic
erm(U)	*erm*(U) is a methyltransferase found in the lincomycin producer Streptomyces lincolnensis. Like other *erm* enzymes, it catalyzes the methylation of A2058 of the 23S ribosomal RNA. Specifically, this enzyme transfers only one methyl group. The gene is found in the lincomycin biosynthetic cluster and is responsible for self-resistance.	3	IrmB	Enzymatic
erm(V)	*erm*(V) is a plasmid-mediated methyltransferase found in Streptomyces viridochromogenes	2	*erm*SV	Enzymatic
erm(W)	*erm*(W) is a methyltransferase found in the mycinamicin producer Micromonospora griseorubida. Like other *erm* enzymes, it catalyzes the methylation of A2058 of the 23S ribosomal RNA. The gene is found within the mycinamicin biosynthetic cluster and is responsible for self-resistance.	1	myrB	Enzymatic
erm(X)	*erm*(X) is a rRNA methyltransferase that protects the ribosome from inactivation due to antibiotic binding.	10	*erm*(C)D, *erm*(C)X	Enzymatic
erm(Y)	*erm*(Y) is a plasmid-mediated methyltransferase found in Staphylococcus aureus	2	*erm*GM	Enzymatic
erm(30)	*erm*(30) confers a MLS_b resistant phenotype. Along with *erm*(31), these genes are responsible for self-resistance in the pikromycin/narbomycin/methymycin/neomethymycin producer, Streptomyces venezuelae.	N/A	-	Enzymatic
erm(31)	*erm*(31) confers a MLS_b resistant phenotype. Along with *erm*(30), these genes are responsible for self-resistance in the pikromycin/narbomycin/methymycin/neomethymycin producer, Streptomyces venezuelae.	N/A	-	Enzymatic
erm(33)	*erm*(33) confers MLS_b phenotype.	N/A	-	Enzymatic
erm(34)	*erm*(34) confers MLS_b phenotype.	N/A	-	Enzymatic
erm(35)	*erm*(35) confers MLS_b phenotype.	N/A	-	Enzymatic
erm(36)	*erm*(36) confers MLS_b phenotype.	N/A	-	Enzymatic
erm(37)	*erm*(37) is found in Mycobacterium species and confers the MLS_b phenotype. In addition to methylation of A2058 this erm methylates adjacent adenosines (A2057 and A2059) as well.	N/A	-	Enzymatic
erm(38)	*erm*(38) confers MLS_b phenotype.	N/A	-	Enzymatic
erm(39)	*erm*(39) confers MLS_b phenotype.	N/A	-	Enzymatic
erm(41)	*erm*(41) confers MLS_b phenotype.	N/A	-	Enzymatic
erm(42)	*erm*(42) confers MLS_b phenotype in Pasteurella multocida	N/A	-	Enzymatic

[1] CARD - The Comprehensive Antibiotic Resistance Database (https://card.mcmaster.ca/home)
[2] ARDB - Antibiotic Resistance Genes Database (https://ardb.cbcb.umd.edu/)
[3] MLS_b = cross-resistance to macrolides, lincosamides, and streptogramins B

Appendix B. Project Images

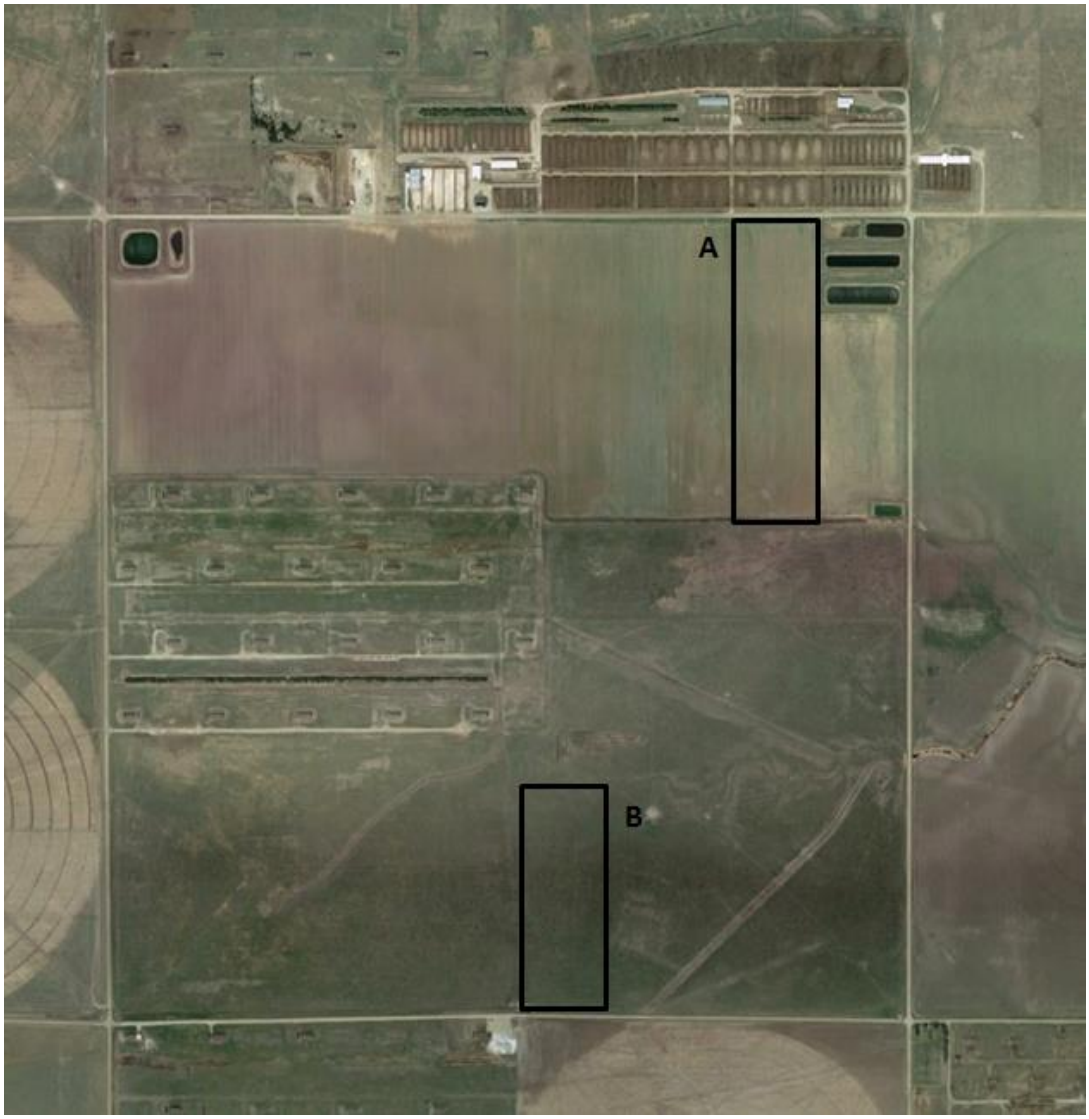

Figure B.2 Aerial View of Project Site near Clay Center, Nebraska

This is an aerial image of the US Meat Animal Research Center. The runoff-amended field is labeled (A), and the cool-season pasture is labeled (B).

Figure B.2 Beef Cattle Feedlot and Runoff Holding Pond

This is the first runoff holding pond located directly south of the east boundary of the feedlot.

Figure B.3 Furrow Irrigation by Gated Pipe from the Runoff Holding Pond

This shows the irrigation management on the runoff-amended field. Water leaves the pipe and runs down (right in the relation to the image) the field by gravity. This image also shows the north boundary of the runoff-amended field.

Figure B.4 Apparent Electrical Conductivity Apparatus used to Det*erm*ine Manure Accumulation Patterns on Both Fields

This image shows the 1 meter soil conductivity probe attached with a non-metallic sled used to generate and receive the electrical signal in the soil. GPS with correction was used to relate each data point with a precise latitude and longitude coordinate. Data was collected at 5 points per second while driving in a serpentine pattern across the surface of each field.

Figure B.5 Hydraulic Soil Probe used to Extract Intact Soil Cores

This shows the apparatus used to extract the soil cores in plastic sleeves. This image shows the extraction of a 2 meter core on the cool-season pasture.

Figure B.6 Soil Core Segmenting

The 2 meter cores were segmented into 20 centimeters segments using a reciprocating saw. The saw blade was sterilized using 100% ethanol solution.

Figure B.7 Soil Samples Prepared for Analysis

The final processed soil samples were contained in bags, labeled, and stored in -80°C until further processing. There were 72 soil samples from each field representing 10 different depths and six different EMI values.

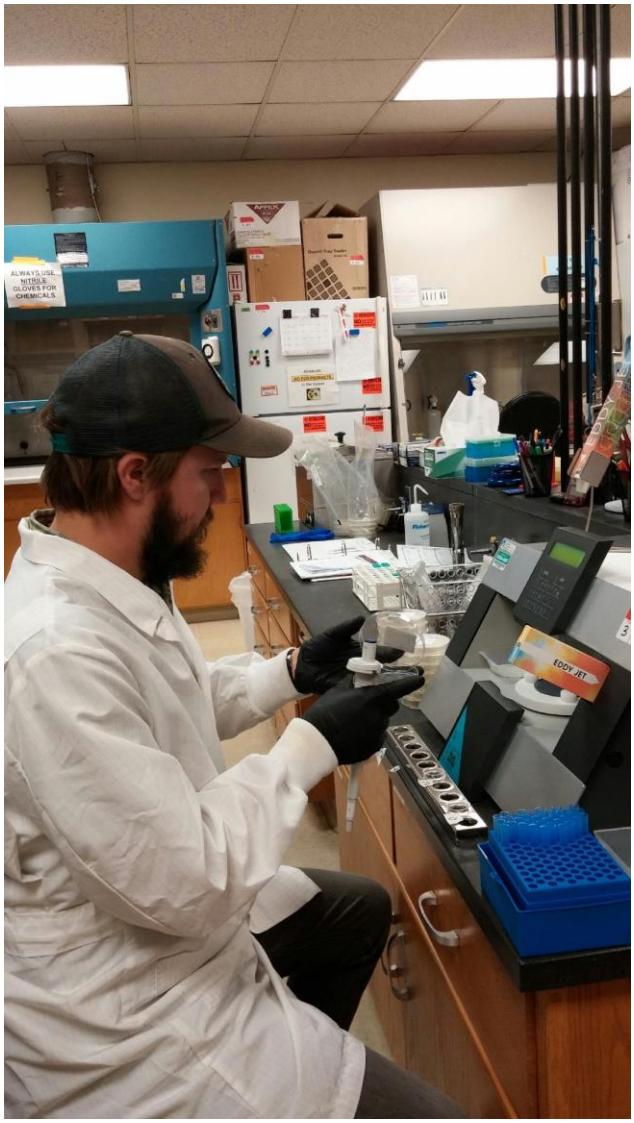

Figure B.8 Spiral Plating for Antibiotic Resistant Bacteria Analysis

This image shows the laboratory methods used in the analysis of antibiotic resistant bacteria. Samples were process within 24 hours of collection at the USDA-ARS laboratory located on the University of Nebraska-Lincoln's east campus. Samples were processed in duplicate and at two different dilutions.

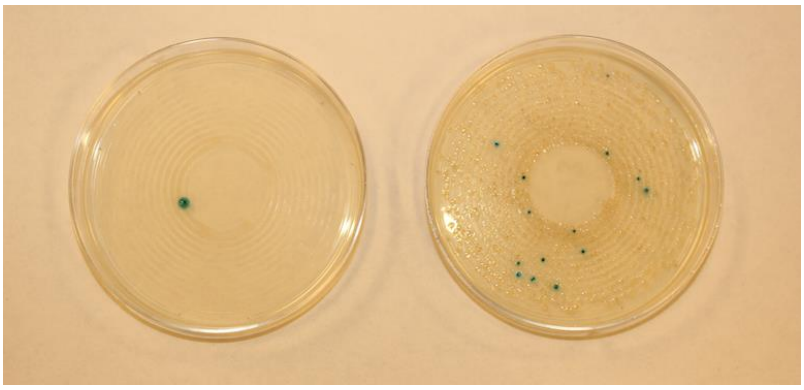

Figure B.9 Example of Selective Media for Fecal Indicator Bacteria

This is an image of the cultured based methods used. The green dots represent *E. coli* bacteria cultured from the soil. Bacterial populations were determined by counting the entire plate or counting a fraction of a plate and calculated using an equation.

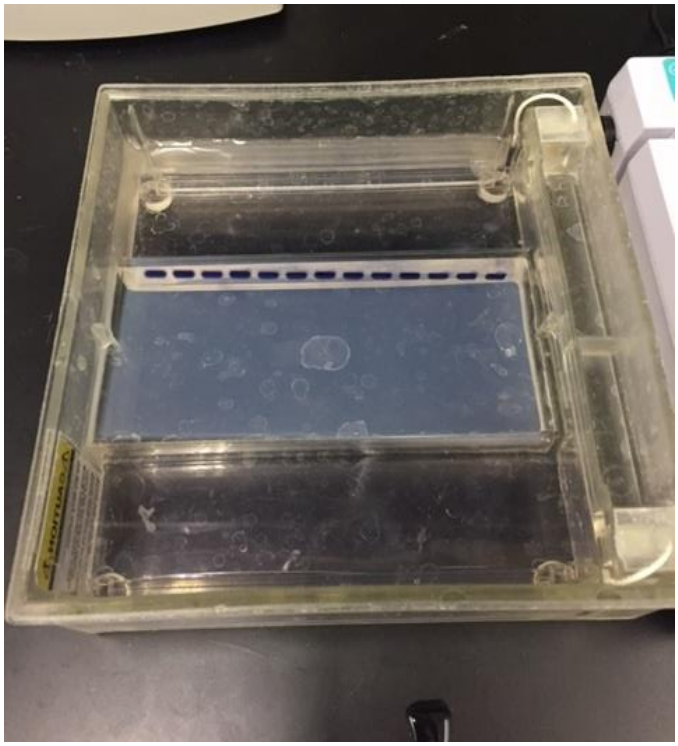

Figure B.10 Gel Electrophoresis Apparatus

Gel electrophoresis was used to validate successful amplification of the 16S rRNA gene as well as many of the antibiotic resistance genes tested.

Appendix C. Supporting Information

Table C.1 Coordinates and Relevant Information on Sampling Locations

Core	Latitude	Longitude	Collection Date	Data Point No.	PRP (mS m^{-1})	HCP (mS m^{-1})	Relative Classification[1]
Runoff-Amended Field							
Core 1	40.553009	-98.168424	June 19, 2015	11837	46.7	82.8	High
Core 2	40.551991	-98.167777	June 19, 2015	2232	42.7	84.1	High
Core 3	40.550853	-98.168144	June 19, 2015	7448	38.5	73.3	Moderate
Core 4	40.549815	-98.168723	June 19, 2015	15310	24.6	54.3	Low
Core 5	40.549832	-98.167785	June 19, 2015	2631	33.0	73.3	Moderate
Core 6	40.548759	-98.168441	June 19, 2015	10998	32.4	65.4	Low
Cool-Season Pasture							
Core 1	40.540156	-98.174647	June 16, 2015	973	12.3	34.2	Low
Core 2	40.541083	-98.174690	June 16, 2015	809	13.5	40.2	Low
Core 3	40.541963	-98.174587	June 16, 2015	1498	18.8	45.1	Moderate
Core 4	40.541776	-98.173777	June 16, 2015	7330	21.6	42.0	High
Core 5	40.540643	-98.173729	June 16, 2015	7056	34.3	63.1	High
Core 6	40.539972	-98.173917	June 16, 2015	5780	24.2	49.8	Moderate

[1] Relative classification is based on the perpendicular (PRP) electrical conductivity only.

Table C.2 Percent Moisture from each Soil Sample[1]

Depth (cm)	Runoff-Amended Field						Cool-Season Pasture					
	Core 1	Core 2	Core 3	Core 4	Core 5	Core 6	Core 1	Core 2	Core 3	Core 4	Core 5	Core 6
0 - 20	2.47	1.98	1.97	2.02	1.97	2.16	1.71	1.66	2.11	2.03	2.34	2.14
20 - 40	2.39	2.45	2.00	2.43	2.34	2.45	1.65	2.52	2.19	2.40	3.41	2.38
40 - 60	2.37	2.41	2.59	1.82	2.49	1.83	1.31	1.49	1.90	1.90	2.10	2.03
60 - 80	2.10	1.97	1.90	1.54	2.04	1.43	1.14	1.28	1.33	1.11	2.26	1.52
80 - 100	2.78	1.94	1.60	1.27	2.25	1.30	0.98	1.16	1.10	1.06	2.15	1.41
100 - 120	2.42	2.28	1.31	1.24	2.09	1.28	1.12	1.28	1.45	1.05	1.50	1.06
120 - 140	2.38	2.05	1.30	1.20	1.45	1.30	1.08	1.27	1.30	1.10	1.11	1.22
140 - 160	2.18	1.76	1.23	1.23	1.26	1.25	1.20	1.40	1.40	1.11	1.07	1.21
160 - 180	2.42	1.53	1.28	1.18	1.29	1.35	1.32	1.40	1.44	1.08	1.18	1.28
180 - 200	2.32	1.36	1.17	1.14	1.26	1.26	1.38	1.52	n/a	1.13	1.26	1.26
200 - 220	n/a	0.87	1.21	1.15	1.16	1.28	1.35	1.57	n/a	n/a	n/a	1.36
220 - 240	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

[1] Moisture content calculated on a dry basis from ASABE standards.

Table C.3 Relevant Information used in Disc Diffusion Analysis

Antibiotic Agent	Drug Class	Dose (µg)	Thresholds (mm)[1]		Animal Use
			Resistant	Susceptible	
Ceftriaxone	Cephalosporin	30	≤ 19	> 19	Cattle
Co-trimoxazole	Cephalosporin	25	≤ 10	> 10	Humans/Cattle
Erythromycin	Cephalosporin	12	≤ 13	> 13	Humans/Cattle
Gentamicin	Macrolide	10	≤ 12	> 12	Humans/Cattle
Nalidixic Acid	Tetracycline	30	≤ 13	> 13	Humans/Cattle
Tetracycline	Antifolate	30	≤ 11	> 11	Humans/Cattle
Ciprofloxacin	Quinolone	5	≤ 15	> 15	Humans
Streptomycin	Quinolone	10	≤ 11	> 11	Humans/Cattle
Chloramephenicol	Aminoglycoside	30	≤ 12	> 12	Humans
Cefoxitin	Aminoglycoside	30	≤ 14	> 14	Cattle
Meropenem	Carbapenems	10	≤ 19	> 19	Humans
Amplicillin	Penicillin	10	≤ 13	> 13	Humans

[1] Defined from the Clinical Laboratory Standards Institute (CLSI). Intermediate classification was considered resistant in this study.

Table C.4 Microbial Populations from the Runoff-Amended Field in CFU g_{dw}^{-1}

Core and Segment[1]		Soiling Dwelling Bacteria				*Enterococcus*				*Escherichia coli*			
		Total Population	Cefotaxime Resistant	Erythromycin Resistant	Tetracycline Resistant	Total Population	Cefotaxime Resistant	Erythromycin Resistant	Tetracycline Resistant	Total Population	Cefotaxime Resistant	Erythromycin Resistant	Tetracycline Resistant
Core 1	Top	1.11E+7	3.50E+6	2.05E+6	4.77E+4	-	-	-	-	-	-	-	-
	Middle	1.78E+6	4.70E+5	9.42E+4	4.01E+3	9.79E+1	-	-	-	1.96E+2	-	-	1.96E+2
	Bottom	1.43E+6	1.19E+4	3.60E+4	-	-	-	-	-	-	-	-	-
Core 2	Top	3.67E+7	1.04E+7	3.57E+6	9.15E+4	1.08E+3	-	-	-	2.65E+3	-	3.43E+3	8.82E+2
	Middle	6.08E+6	1.87E+6	7.35E+5	8.28E+4	9.80E+1	-	1.96E+2	-	7.84E+2	-	1.37E+3	3.92E+2
	Bottom	2.48E+6	3.55E+5	4.42E+5	1.08E+3	-	-	-	-	-	-	-	-
Core 3	Top	1.13E+7	1.33E+5	9.70E+5	3.45E+6	-	-	-	-	-	-	9.80E+1	-
	Middle	1.12E+6	6.87E+5	1.72E+5	3.57E+4	-	-	-	-	-	-	9.81E+1	-
	Bottom	1.04E+6	9.41E+4	6.02E+3	7.90E+2	-	-	-	-	-	-	-	-
Core 4	Top	2.59E+7	1.03E+7	1.48E+6	1.30E+5	7.84E+2	-	-	-	1.96E+2	-	9.80E+1	3.92E+2
	Middle	1.91E+6	8.76E+5	4.73E+5	1.28E+3	-	-	-	-	1.97E+2	-	1.97E+2	-
	Bottom	6.70E+5	5.01E+4	1.19E+3	-	-	-	-	-	-	-	-	-
Core 5	Top	1.23E+7	5.82E+6	4.71E+5	1.62E+4	2.94E+2	-	-	-	2.94E+2	-	6.86E+2	9.80E+1
	Middle	6.27E+5	2.95E+5	8.66E+4	5.09E+3	-	-	-	-	9.80E+1	-	9.80E+1	9.80E+1
	Bottom	4.44E+5	2.36E+5	2.09E+4	3.95E+2	-	-	-	-	-	-	-	-
Core 6	Top	1.17E+7	1.00E+7	1.03E+5	1.04E+5	-	-	-	-	-	-	-	-
	Middle	5.42E+6	1.43E+6	1.78E+5	1.38E+3	-	-	-	-	-	-	-	-
	Bottom	2.57E+5	3.48E+4	2.96E+2	-	-	-	-	-	-	-	-	-

- = Not detected

[1] Top = 0 – 20 cm; Middle = 60 – 80 cm; Bottom = 160 – 180 cm

Table C.5 Microbial Populations from the Cool-Season Pasture in CFU g_{dw}^{-1}

Core and Segment[1]		Soiling Dwelling Bacteria				*Enterococcus*				*Escherichia coli*			
		Total Population	Cefotaxime Resistant	Erythromycin Resistant	Tetracycline Resistant	Total Population	Cefotaxime Resistant	Erythromycin Resistant	Tetracycline Resistant	Total Population	Cefotaxime Resistant	Erythromycin Resistant	Tetracycline Resistant
Core 1	Top	3.34E+6	2.39E+6	6.19E+4	6.18E+4	-	-	-	-	-	-	-	-
	Middle	1.04E+6	5.44E+5	4.15E+3	6.03E+3	-	-	-	-	-	-	-	-
	Bottom	7.37E+4	2.00E+4	6.91E+2	1.97E+2	-	-	-	-	-	-	-	-
Core 2	Top	6.69E+6	4.65E+6	7.77E+5	3.51E+5	-	-	-	-	-	-	-	-
	Middle	1.01E+6	5.03E+5	4.68E+4	7.80E+3	-	-	-	-	-	-	-	-
	Bottom	1.39E+6	1.22E+5	6.90E+2	2.96E+2	-	-	-	-	-	-	-	-
Core 3	Top	7.24E+6	3.69E+6	5.87E+5	1.23E+5	-	-	-	-	-	-	-	-
	Middle	1.74E+6	6.71E+5	5.82E+3	1.43E+4	-	-	-	-	-	-	-	-
	Bottom	2.59E+5	1.32E+5	3.84E+3	6.90E+2	-	-	-	-	-	-	-	-
Core 4	Top	5.98E+6	4.28E+6	4.02E+5	3.25E+4	-	-	-	-	-	-	-	-
	Middle	2.12E+6	9.39E+5	1.09E+4	4.05E+3	-	-	-	-	-	-	-	-
	Bottom	6.33E+4	1.80E+4	1.29E+3	6.92E+4	-	-	-	-	-	-	-	-
Core 5	Top	7.29E+7	3.67E+7	9.57E+5	8.98E+5	-	-	-	-	1.95E+2	-	9.77E+1	-
	Middle	1.04E+6	7.14E+5	4.48E+4	3.13E+3	-	-	-	-	-	-	-	-
	Bottom	1.51E+5	1.72E+4	1.28E+3	4.94E+2	-	-	-	-	-	-	-	-
Core 6	Top	8.71E+6	3.93E+6	4.60E+5	9.23E+4	-	-	-	-	-	-	-	-
	Middle	3.77E+6	1.16E+6	7.18E+4	1.38E+3	-	-	-	-	-	-	-	-
	Bottom	7.90E+4	1.83E+4	1.97E+2	9.87E+1	-	-	-	-	-	-	-	-

- = Not detected

[1] Top = 0 – 20 cm; Middle = 60 – 80 cm; Bottom = 160 – 180 cm

Table C.6 Means of Soil Dwelling Bacteria in log CFU g_{dw}^{-1}

Depth	Bacterial Culture	Runoff-Amended Field	Cool-Season Pasture	*p-value*
Pooled Depths	Total Population	**6.49**	**6.12**	**0.0256**
	Cefotaxime Resistant	5.78	5.71	0.6779
	Erythromycin Resistant	**5.08**	**4.28**	**0.0226**
	Tetracycline Resistant	3.92	3.89	0.8284
Top (0 - 20 cm)	Total Population	7.21	6.97	0.2862
	Cefotaxime Resistant	6.58	6.73	0.5697
	Erythromycin Resistant	5.95	5.62	0.5166
	Tetracycline Resistant	5.09	5.14	0.7359
Middle (60 - 80 cm)	Total Population	6.32	6.20	0.5887
	Cefotaxime Resistant	5.89	5.86	0.9013
	Erythromycin Resistant	**5.32**	**4.27**	**0.0124**
	Tetracycline Resistant	3.84	3.68	0.6045
Bottom (160 - 180 cm)	Total Population	**5.91**	**5.22**	**0.0040**
	Cefotaxime Resistant	4.87	4.54	0.2438
	Erythromycin Resistant	**3.97**	**2.96**	**0.0395**
	Tetracycline Resistant	2.84	2.86	0.6130

Means in **bold** if significantly different ($\alpha < 0.05$)

Table C.7 PCR Conditions

Target Gene	Primer	Sequence (5' - 3')	Annealing Temperature (°C)	Linear Range	R^2	Efficiency	Reference
16S	FW	CGG TGA ATA CGT TCG ACT T	56	10^2 - 10^8	≥ 0.987	91% - 110%	Suzuki et al., 2000
	RV	GGW TAC CTT GTT AC					
tet(X)	FW	CAA TAA TTG GTG GTG GAC CC	60	10^0 - 10^8	≥ 0.992	101% - 108%	Ng et al., 2001
	RV	TTC TTA CCT TGG ACA TCC CG					
tet(Q)	FW	TTA TAC TTC CTC CGG CAT CG	63	10^1 - 10^6	≥ 0.981	113% - 115%	Ng et al., 2001
	RV	ATC GGT TCG AGA ATG TCC AC					
erm(A)	FW	AGT CAG GCT AAA TAT AGC TAT C	63	10^2 - 10^7	≥ 0.986	74% - 86%	Koike et al., 2009
	RV	CAA GAA CAA TCA ATA CAG AGT CTA C					
erm(C)	FW	AAT CGT GGA ATA CGG GTT TGC	63	10^2 - 10^7	≥ 0.987	91% - 93%	Koike et al., 2009
	RV	CGT CAA TTC CTG CAT GTT TTA AGG					

Table C.8 Copy Numbers of ARGs

Core	EMI (mS m-1)	Depth (cm)	16S	*erm*(C)		*tet*(Q)	
			Absolute Abundance[1]	Absolute Abundance[1]	Relative Abundance[2]	Absolute Abundance[1]	Relative Abundance[2]
Core 1	46.7	0-20	9.42E+09	3.66E+04	4.04E-06	3.47E+05	3.83E-05
		20-40	3.48E+09	ND	ND	< MDL	< MDL
		40-60	1.90E+07	ND	ND	ND	ND
		60-80	1.98E+08	ND	ND	< MDL	< MDL
		100-120	2.39E+08	ND	ND	< MDL	< MDL
		160-180	1.90E+08	ND	ND	< MDL	< MDL
Core 2	42.7	0-20	4.22E+09	< MDL	< MDL	4.09E+08	9.71E-02
		20-40	2.15E+09	ND	ND	< MDL	< MDL
		40-60	4.45E+08	< MDL	< MDL	< MDL	< MDL
		60-80	1.15E+09	5.73E+04	5.00E-05	< MDL	< MDL
		100-120	1.05E+09	< MDL	< MDL	< MDL	< MDL
		160-180	3.36E+08	< MDL	< MDL	< MDL	< MDL
Core 3	38.5	0-20	1.89E+10	8.94E+04	4.73E-06	< MDL	< MDL
		20-40	1.83E+09	ND	ND	< MDL	< MDL
		40-60	4.27E+08	ND	ND	< MDL	< MDL
		60-80	9.19E+07	ND	ND	ND	ND
		100-120	9.10E+06	ND	ND	ND	ND
		160-180	6.96E+06	ND	ND	ND	ND
Core 4	24.6	0-20	1.72E+10	ND	ND	3.33E+08	1.93E-02
		20-40	1.21E+08	ND	ND	ND	ND
		40-60	2.29E+07	ND	ND	< MDL	< MDL
		60-80	3.63E+08	8.76E+04	2.41E-04	< MDL	< MDL
		100-120	2.75E+07	ND	ND	ND	ND
		160-180	1.84E+07	ND	ND	ND	ND
Core 5	33.0	0-20	1.22E+10	5.35E+04	4.39E-06	< MDL	< MDL
		20-40	2.04E+09	ND	ND	< MDL	< MDL
		40-60	5.07E+07	ND	ND	ND	ND
		60-80	9.10E+07	ND	ND	ND	< MDL
		100-120	4.96E+08	4.71E+04	9.50E-05	< MDL	ND
		160-180	1.27E+07	ND	ND	ND	ND
Core 6	32.4	0-20	6.47E+09	6.34E+04	9.80E-06	ND	ND
		20-40	4.66E+08	ND	ND	ND	ND
		40-60	4.94E+08	ND	ND	ND	ND
		60-80	6.56E+07	ND	ND	ND	ND
		100-120	1.82E+07	ND	ND	ND	ND
		160-180	2.02E+06	ND	ND	< MDL	< MDL

[1] Absolute abundance: number of copies per gram of dry soil

[2] Relative Abundance: number of copies per 16S rRNA

ND = Not Detected

Appendix D. List of Abbreviations

AHI, The Animal Health Institute

AR, antibiotic resistance

ARB, antibiotic resistant bacteria

ARDB, Antibiotic Resistance Gene Database

ARG, antibiotic resistance genes

BMP, best management practice

CARD, Comprehensive Antibiotic Resistance Database

CEC, ChromAgar E. Coli

CEC+c, ChromAgar E. Coli with 4 µg mL^{-1} cefotaxime

CEC+e ChromAgar E. Coli with 10 µg mL^{-1} erythromycin

CEC+t ChromAgar E. Coli with 16 µg mL^{-1} tetracycline

CAFO, confined animal feeding operation

CFU, colony forming units

CLSI, Clinical Laboratory Standards Institute

CTC, chlortetracycline

CWA, Clean Water Act

DEQ, Department of Environmental Quality

DNR, Department of Natural Resources

EC_a, apparent electrical conductivity

ELG, effluent limit guidelines

EMI, electromagnetic Induction

FARAD, Food Animal Residue Avoidance Database

FIB, fecal indicator bacteria

GPS, global positioning system

HCP, horizontal coplanar

LC/MS/MS, liquid chromatography-tandem mass spectrometry

ME, m-Enterococcus

ME+c, m-Enterococcus with 4 µg mL^{-1} cefotaxime

ME+e m-Enterococcus with 10 µg mL^{-1} erythromycin

ME+t m-Enterococcus with 16 µg mL^{-1} tetracycline

MIC, minimum inhibitory concentrations

NPDES, National Pollution Discharge Elimination System

OD, optical density

PBS, phosphate buffered saline

PCR, polymerase chain reaction

PRP, perpendicular

qPCR, qualitative polymerase chain reaction

R2A+c, R2A with cefotaxime

R2A+e R2A with erythromycin

R2A+t R2A with tetracycline

RSSD, response surface sampling design

RTK, real-time kinematic

SPE, solid phase extraction

TSB, trypic soy broth

USDA, United States Department of Agriculture

USDA-ARS, United States Department of Agriculture – Agricultural Research Service

USEPA, United States Environmental Protection Agency

USMARC, United States Meat Animal Research Center

WHO, World Health Organization