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Fate and transport of antibiotic resistant bacteria and resistance genes in artificially drained agricultural fields receiving swine manure application

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Fate and transport of antibiotic resistant bacteria and resistance genes in artificially drained agricultural fields receiving swine manure application

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ABSTRACT

The growing numbers of swine receiving antimicrobial additives in feed at sub-therapeutic levels as a prophylactic and growth promoter has led to increasing concerns regarding levels of antibiotics and antibiotic resistant bacteria in their excrement. Application of swine manure to agricultural fields as fertilizer creates a pathway for antibiotic resistant bacteria and their associated resistance genes to enter the environment. This study monitored enterococci, tylosin resistant enterococci and four genes known to confer macrolide antibiotic resistance (*ermB, ermC, ermF* and *msrA*) in soil and subsurface artificial drainage water. Manure concentrations for *ermB, ermC* and *ermF* were all $>10^9$ copy g^{-1}. *MsrA* was not detected in manure, soil or water. The average enterococci concentration in manure was 1.76×10^5 $CFUg^{-1}$, with 83% resistant to tylosin. The next highest concentrations of enterococci and tylosin resistant enterococci were located in soil from the manure injection band which contained median concentrations >200 $CFUg^{-1}$ soil. Gene abundances of *ermB, ermC* and *ermF* in manured soil returned to levels identified in non-manured control plots by the spring following manure application. While enterococci and tylosin resistant enterococci concentrations in drainage water samples showed no trends between treatments, resistance genes *ermB* and *erm*F were found at significantly higher concentrations ($p<0.01$) in drainage water from manured plots when compared to non-manured plots gene concentrations. *ErmB* was found in 78% of drainage water samples from plots with manure treatment. *ErmF* was detectable in 44% of drainage water samples from manure amended plots. No significant differences ($p>0.10$) were identified due to tillage treatments for any of the genes detected. Although *ermC* was detected at the highest concentrations of the three genes in drainage water, concentrations in water from manure treated plots were not significantly greater ($p>0.10$) than the control plot concentrations. These results suggest a short-term increase in antibiotic resistant bacteria and resistance genes in soil from manure application. Additionally, this study is the first to report significant increases in resistance gene abundances in agricultural drainage water from soils receiving manure application.

CHAPTER I: GENERAL INTRODUCTION

1.1 Introduction

Swine production is an economic cornerstone in the Midwestern United States and provides a substantial portion of the region's gross farm income. More than 66 million swine were produced in the United States in 2012, with over 67% grown in feeding operations containing over 5000 pigs (USDA 2014). Many farmers use a variety of antimicrobial additives in swine feed at sub-therapeutic levels as a prophylactic and growth promoter. Research has documented the positive effects of antibiotics in swine feed at subtherapeutic levels in a variety of contexts, including: improvement of growth rates, increased feeding efficiencies, reduced mortality rates and heightened reproductive rates (Hays 1981, Cast 1981, Zimmerman 1986, Cromwell 1991). These improvements coupled with declining prices has led to approximately 90% of starter feeds, 75% of grower feeds and 50% of finisher feeds incorporating antibiotics (Cromwell 2002). The most frequently used antimicrobials in the swine industry include: tetracyclines, tylosin, and sulfamethazine or other sulfas (McEwen & Fedora-Cray 2002). Apley et al. (2012) estimated an annual use of 533,973 kg of chlortetracycline, 165,803 kg of tylosin and 154,973 kg of oxytetracycline in swine feed in the United States using data from the National Animal Health Monitoring System (NAHMS) and a 2009 survey of swine-exclusive practitioners.

Macrolide antibiotics, such as erythromycin and tylosin, obstruct protein synthesis through stimulating the release of the peptidyl-tRNA molecule from the ribosome during protein elongation. The release causes stoppage of protein synthesis by creating a premature chain termination (Weisblum 1995 & 1998). Antibiotic resistance genes are capable of reducing the effectiveness of antibiotics through a variety of mechanisms including: altered antibiotic target sites, decreased uptake or efflux, "bypass" pathways and enzymatic inactivation or modification (Hawkey 1998). Erythromycin ribosome methylation (*erm*) genes are responsible for coding for methyltransferase enzymes, which add one or two methyl groups to a single adenine (A2058) (Weisblum 1998). The methyl groups reduce the ability of erythromycin and tylosin to bind to the 50S ribosomal subunit, therefore hindering the effectiveness of the antibiotic. Furthermore,

the binding site for erythromycin overlaps binding sites for other macrolides, lincosamides and streptogramin B antibiotics (MLS_B) (Leclercq & Courvalin 1991). Therefore, resistance encoded by *erm* genes may cause cross resistance in the MLS_B family of antibiotics. In addition to macrolide resistance being conferred by alteration of target sites, other classes of genes which code for antibiotic efflux systems have been identified. The *msr* gene family has been classified as a predecessor for proteins which are part of the ABC transporter superfamily (Roberts 1999). ABC transporter proteins utilize energy stemming from adenosine triphosphate binding and hydrolysis to translocate substances across membranes. Antibiotic resistance is a major threat to public health due to the growing demands for new antibiotics in order to keep up with the wide variety of resistance mechanism identified.

The growing number of animals receiving antibiotics have led to concerns over the increased abundance of antibiotic resistant bacteria inside the animals and excreted their manures (Khachatourians 2008). Koike et al. (2010) found *erm* genes present in 100% of manure samples taken from confined animal feeding operations known to administer antibiotics. Additionally, Chen et al. (2010) identified genes conferring erythromycin (*erm*) and tetracycline (*tet*) resistance persisting in in swine manure post biofilter treatment. Prior studies have identified elevated levels of antibiotics, antibiotic resistant bacteria and antibiotic resistance genes in ground and surface water surrounding confined animal feeding operations (Campagnelo 2002, Chee-Sanford et al. 2009, Heuer et al. 2011). The potential for antibiotics, antibiotic resistant bacteria and antibiotic resistance genes leaching into the environment is becoming of greater concern, with approximately 9.2 million hectares of farmland receiving manure annually (Dolliver & Gupta 2007).

Approximately one third of Iowa cropland utilizes subsurface drainage systems (Zucker and Brown, 1998). Farmlands equipped with artificial drainage systems have shown relationships between precipitation, drainage flow rates and nutrient export (Kanwar et al. 1999, Bakhsh et al. 2005, and Lawlor et al. 2011). While there is significant knowledge regarding the release of nutrients from agricultural fields, less is known regarded the export of bacteria. Rainfall simulations on tile drained, swine manure treated plots by Hoang et al. (2013) identified peak concentrations of enterococci and tylosin resistant enterococci following hydrograph peaks.

Previously, Garder et al. (2014) quantified antibiotic resistant bacteria and resistance genes in tile drained agricultural fields receiving swine manure application. Elevated levels of antibiotic resistant bacteria and resistance genes were found in manure injections bands in soil following swine manure application, but these genes returned to levels equivalent to control plot concentrations one year after application. Tile drainage samples from the same plots maintained under different tillage and manure treatments did not show significant differences in antibiotic resistant bacteria and resistance gene concentrations. The authors suggested that below average precipitation and cumulative tile drainage flow may have contributed to the lack of statistically significant differences. The objective of this study is to identify the effects of tillage and manure treatments on antibiotic resistant bacteria and resistance gene levels in soil and tile drainage and determine tile flow impacts.

1.2 Specific Objectives:

The specific objectives of this study were to:

1. Using a standardized system, quantify in liquid swine manure, soil and subsurface drainage:
    a. Enterococci and tylosin resistant enterococci concentrations
    b. Resistance gene concentrations: *ermB, ermC*, *ermF* and *msrA*
2. Determine if levels of enterococci, tylosin resistant enterococci and resistance genes significantly differ between plots receiving swine manure and nonorganic fertilizer under no-till and chisel plow regimes
3. Identify persistence of enterococci, tylosin resistant enterococci and resistance genes in soil and tile drainage following manure application

1.3 Hypotheses:

This study identified and assessed the following hypotheses:

1. Concentrations of enterococci, tylosin resistant enterococci and resistance genes in tile drainage will be higher in plots receiving swine manure than in plots receiving nonorganic fertilizer

2. Concentrations of enterococci, tylosin resistant enterococci and resistance genes in tile drainage will be greater in no-till plots than chisel plow tillage regimes
3. Enterococci, tylosin resistant enterococci and resistance gene levels will decrease in soil following manure application over the two year crop rotation
4. Enterococci, tylosin resistant enterococci and resistance gene levels in tile drainage will be greater in samples taken during or immediately after rainfall events than under dry weather flow conditions

CHAPTER 2: LITERATURE REVIEW

2.1 Antibiotics in Agriculture

On a global basis, 50% of all antimicrobials produced are administered for veterinary purposes (Teuber 2001). Antimicrobials are administered to food animals in the United States for therapeutic and non-therapeutic treatments. Therapeutic doses of antimicrobials are given to animals which are already diseased. Treatment may be administered to individual animals, but are commonly given to entire groups through the addition in feed or water in order to increase efficiency. Non-therapeutic treatments are administered at subtherapeutic levels to promote growth and improve feed efficiency of the animals (McEwen & Fedorka-Cray 2002).

2.1.1 Antibiotics in Swine Production

Research has documented the positive effects of antibiotics in swine feed at subtherapeutic levels in a variety of contexts, including: improvement of growth rates, increased feeding efficiencies, reduced mortality rates and heightened reproductive rates. These noted improvements coupled with declining prices has led to approximately 90% of starter feeds, 75% of grower feeds and 50% of finisher feeds incorporating antibiotics (Cromwell 2002). Apley et al. (2012) estimated an annual use of 533,973 kg of chlortetracycline, 165,803 kg of tylosin and 154,973 kg of oxytetracycline in swine feed in the United States using data from the National Animal Health Monitoring System (NAHMS) and a 2009 survey of swine-exclusive practitioners.

2.2 Antibiotic Structures and Mechanisms of Action

Antibiotics are compounds produced by organisms which impede the growth of other organisms. The compounds hinder bacterial growth and survival through a variety of inhibition mechanisms. Growth impediments result from the inhibition of bacterial cell wall synthesis, inhibition of protein synthesis or inhibition of DNA function (Morley et al. 2005). Antibiotics are classified by either their mechanism of action or chemical structure. Major

groups of antibiotics include: aminoglycosides, β-lactams, quinolones, tetracyclines, macrolides, oxazolidinones, and sulfonamides (Kümmerer, 2009).

2.2.1 Macrolide Antibiotics

Macrolides are naturally occurring secondary metabolites that are biosynthesized in a stepwise manner from 2-, 3-, and 4-carbon building blocks by actinomycete bacteria. The metabolites have shown to possess antimicrobial, antifungal, antiparasitic, antitumor or agrochemical properties (Poehlsgaard & Douthwaite 2005). Antimicrobial macrolides consist of a central lactone ring between 14 and 16 atoms to which amino and/or neutral sugars are held by glycosidic bonds (Roberts et al. 1999). Macrolides obstruct protein synthesis through stimulating the release of the peptidyl-tRNA molecule from the ribosome during elongation. The release causes stoppage of protein synthesis by creating a premature chain termination (Weisblum 1995 & 1998).

The inhibitory action of the 14-member-ring macrolide erythromycin takes effect in the early stages of protein synthesis by halting growth of nascent peptide chains in the ribosome (Andersson & Kurlan 1997). Additionally, erythromycin and other 14-member-ring macrolides inhibit growth by preventing assembly of new large ribosomal subunits, which results in gradual depletion of functional ribosomes within a cell (Chittum & Chapney 1994). Peptide bond formation on the large ribosomal subunit is associated with the central loop in domain V of 23S rRNA (Cundliffe 1990). Chemical footprinting has mapped interactions of macrolides and other MLS_B antibiotics to this domain (Douthwaite 1992). Additionally, erythromycin interactions have also been mapped to hairpin 35 in domain II of the rRNA. It is believed that these two regions are folded close together in the 23S rRNA tertiary structure, creating a binding pocket for macrolides (Hansen et al 1999).

Tylosin is a wide-spectrum antibiotic produced by the fermentation of select Streptomyces strains (McGuire et al. 1961). The 16-member-ring macrolide tylosin binds to the same area of the large subunit as erythromycin, but inhibits peptide bond formation directly by interfering with nucleotides in the peptidyl transferase loop. Recent evidence supports that tylosin also binds to the central loop in domain V and hairpin 35 in domain II of the 23S rRNA (Vester & Douthwaite 2001). Tylosin molecules that bind to these locations

cause premature dissociation of the peptidyl-tRNA from the ribosome, which in turn halts peptide formation (Menninger 1995).

2.3 Tylosin Detection in the Environment

The large amount of antibiotics used in food animal production has led to growing concerns of potential antibiotic reservoirs of s in the environment. In a study performed by Feinman and Matheson (1978), up to 67% of tylosin orally administered to feedlot animals was excreted in feces. This information coupled with industry moving towards CAFO's creates possible contamination risks from manure leachate in storage facilities and over application when applied as fertilizer to surrounding cropland.

2.3.1 Tylosin Concentrations in Manure

Over a three year study, Dolliver and Gupta (2007) reported tylosin concentrations in swine manure ranging from 47-775 g ha^{-1} for application rates between 65,478 and 130,955 L ha^{-1}. Tylosin degradation rates in manure have been investigated under different storage conditions. Kolz et al. (2005) compared tylosin dissipation in swine manure lagoon slurry under aerobic and anaerobic conditions. Tylosin was less persistent in aerated manure, with 90% disappearance occurring within 12 to 26 hours, while 90% disappearance time under anaerobic conditions took to between 30 to 130 hours. Both sets of samples still contained residual concentrations of tylosin after eight months of incubation. Garder et al. (2014) detected mean tylosin concentrations in swine manure ranging from 17 to 128 μg kg^{-1}. Possible explanations for the range of tylosin concentrations reported in the manure include: dissimilar time frames for antibiotic administration in regards to manure collection, different levels of tylosin administered in feed and non-uniform storage times prior to field application.

2.3.2 Tylosin in Concentrations in Soil

Carlson & Mabury (2006) found tylosin dissipation half-lives to be significantly shorter in manure amended plots (4.5 days) than manure free plots (6.1 days). This suggests an increased rate of biodegradation in the manure amended plot due to the introduction of manure microbial communities. Tylosin was not detected in soil samples from plots

amended with swine manure containing a predicted tylosin concentration of 117.94 mg L^{-1} (Kay et al. 2004). These results contrast findings by Halling-Sorensen et al. (2005) who detected tylosin in soil samples continuously over a 155 day experimental period. However, tylosin levels rapidly declined from original concentrations of 30 and 50 µg kg^{-1} of manured soil to 1 and 5 µg kg^{-1}. Garder et al. (2014) did not find statistically significant differences in tylosin concentrations when comparing soil swine manure injection bands, spacing between bands and control plots, indicating rapid loss of tylosin after manure injection.

2.3.3 Tylosin Concentrations in Runoff Water

Previous studies have found low levels of tylosin in waters surrounding confined animal feeding operations (CAFO's) and manure amended fields. A recent year-long study by Song et al. (2010) did not detect tylosin in tile drainage located near a CAFO in Lansing, Michigan, but occasionally identified the antibiotic in low concentrations in samples taken from stagnant ditch water surrounding the operation. Identification of antibiotics persisting in the environment near CAFO's led to studies focused on antibiotic prevalence in runoff resulting from the application of swine manure to agricultural fields as fertilizer. A three year field study conducted at the University of Wisconsin Agricultural Research Station found tylosin present in leachate and surface runoff samples in manure amended fields. Tylosin was detected in 19% of surface runoff samples with a maximum concentration of 6.0 µg L^{-1} and 8% of leachate samples containing a maximum of 1.2 µg L^{-1}. The majority of tylosin losses in leachate (97%) and runoff (89%) in the study were identified during non-growing season sampling (Dolliver & Gupta 2007). Kay et al. (2004) failed to detect tylosin in water samples (0.35 µg L^{-1} limit of quantification) derived from automatically collected subsurface drain flow samples underlying plots amended with swine manure). A more recent study conducted by Garder et al. detected tylosin in numerous samples obtained from spring subsurface drainage following fall manure application, but no sample exceeded a concentration of 1 µg L^{-1} (2013).

2.4 Antibiotic Resistance

Strains of bacteria may be intrinsically resistant to antibiotics or acquire the ability to resist the mode of action of a specific antibiotic from the environment. Intrinsic resistance stems from the ability of a bacterial species to resist the mode of action of an antimicrobial due to its structural or functional characteristics. Bacterial species may be insensitive to the antimicrobial for a variety of reasons including: lack of affinity of the drug for the bacterial target site, inaccessibility of the drug into the bacterial cell, extrusion of the drug from the cell by export systems and production of enzymes which inactivate the drug (Forbes et al. 1998). Acquired resistance takes place when a particular organism attains the ability to resist the mechanism of a specific antibiotic. Mutations of preexisting genes or acquisition of genetic material from foreign microorganisms are both pathways for resistance acquisition (Gillespie 2001).

2.4.1 Antibiotic Resistance Mechanisms

Mechanisms by which bacteria exhibit antibiotic resistance can be classified into three major catagories: altered antibiotic target sites, decreased uptake or efflux and enzymatic inactivation or modification (Hawkey 1998). Resistance mechanisms can occur naturally in certain types of bacteria or be aquired through a variety of genetic means (Morley et al 2005). Resistance attained through enzymatic inactivization is achieved by preventing the antibiotic from reaching its associated target site (Hawkey 1998). A classic example of this type of resisance is β-lactamase enzymes hydrolyzing the amide bond of the four-membered β-lactam ring in β-lactam based drugs (Wilke et al 2006). Numerous cases of macrolide antibiotic resistance reported in clinical strains are tied to substitutions of particular nucleotides in the 23S rRNA within the 50S subunit of bacterial ribosomes (Vester and Douthwaite 2001). Erythromycin methyltransferase is responsible for catalyzing the methylation of a single adenine (A2058). Modification of this nucleotide reduces the binding ability of tylosin and erythromycin in the 50S ribosomal subunit (Weisblum 1998). The final mechanism, efflux systems, work by using genes which code for transport proteins to pump the antibiotic out of the cell or cellular membrane. This allows for keeping intracellular antibiotic concentrations low, therefore limiting opportunities for target site interaction (Roberts et al, 1999).

2.4.1.1 Macrolide Target Alterations

The first method of macrolide resistance identified was attributable to the posttranscriptional modification of the 23S rRNA by the adenine-N^6 methlytransferase. The methlytransferase enzyme adds one or two methyl groups to a single adenine (A2058). Over the last 30 years genes encoding such enzymes have been titled *erm*, which stands for erythromycin ribosome methylation (Weisblum 1998). The binding of the methyl groups reduce the ability of erythromycin to bind to the 50S ribosomal subunit by altering the antibiotic's attachment site, therefore hindering the effectiveness of the antibiotic. Additionally, the binding site for erythromycin overlaps binding sites for other macrolides, lincosamides and streptogramin B antibiotics (MLS_B) (Leclercq & Courvalin 1991). Therefore, resistance encoded by *erm* genes may cause cross resistance in the MLS_B family of antibiotics. *Erm* genes have been identified in a wide variety of both gram posititve and negative bacteria. The family of genes tend to be associated with conjugative or transposition in chromosomal DNA, but have also been identified in plasmids (Roberts 1999).

2.4.1.2 Macrolide Efflux Systems

In addition to macrolide resistance being conferred by alteration of target sites, other classes of genes which promote the establishment of antibiotic efflux systems have been identified. The *mef* and *lmr* family of genes have been classified as predecessors for proteins in major facilitator superfamily (MFS). Proteins in MFS facilitate movement of solutes across cell membranes in response to chemiosmotic ion gradients. Other gene families which confer macrolide resistance through antibiotic exportation include: *car, msr, ole, smr* and *vga*. These genes are part of the ABC transporter superfamily (Roberts 1999). ABC transporter proteins utilize energy stemming from adenosine triphosphate binding and hydrolysis to translocate substances across membranes.

2.5 Resistance in the Environment

While previous studies have documented levels of antibiotics in the environment resulting from the application of swine manure as organic fertilizer, less is known about the persistence and spread of antibiotic resistant bacteria and their associated families of genes.

Administration of tylosin at sub-therapeutic levels to swine is capable of altering the intestinal flora by selecting for bacteria resistant to macrolides (Aarestrup and Carstensen 1998). Previous studies have indicated spikes in antibiotic resistant indicator organisms in manure generated from swine fed antibiotics when compared to manure collected from organic farms (Angulo et al, 2004, Jindal et al. 2006). Additionally studies identifying the presence of resistance genes in the environment are largely derived from enterococcal and *E coli* isolate collections, while little research has been conducted on the quantification of entire resistance gene pools.

2.5.1 Antibiotic Resistance Isolated from Swine Production Waste

High levels of *erm* genes have been detected in samples derived from carcasses and waste products from swine receiving antibiotics in feed. Chen et al. (2007) indicated *ermB* as the most prevalent (72% of total *erm* copies) of the *erm* family of genes (A, B, C, F, T and X) in swine manure. *ErmT* was the next most prevalent, containing approximately one quarter of the resistance genes. *ErmA*, *ermF* and *ermT* together comprised the remaining 3% of resistance copies enumerated, while *ermC* was not detected. Fifty macrolide resistant enterococci isolates were retrieved from tonsillar and colon swabs from a set of pork carcasses from four slaughter houses as part of a study conducted in Belgium. PCR results from DNA extracted from the isolates tested indicated positive identification for *ermB* (De Leener et al. 2004). Similar results were reported by Jackson et al. (2004). Enterococci were isolated from swine fecal samples from three farms. Approximately 59% were resistant to tylosin from a farm where tylosin was administered for growth promotion, while 28.5% of isolates were resistant where tylosin was given for disease prevention and only 2.4% were resistant from samples taken from a farm where tylosin was not incorporated in feed. Of the isolates resistant to erythromycin, 96% contained *ermB*. *ErmA* and *ermC* were not identified in any of the isolates tested (Jackson et al 2004).

2.5.2 Resistance Genes in the Environment

While numerous studies have identified increased concentrations of antibiotic resistant bacteria and antibiotic resistance genes in ground and surface water surrounding confined animal feeding operations (Campagnelo 2002, Chee-Sanford et al. 2009, Heuer et

al. 2011), less is known regarding transport capabilities of the resistant bacteria through the environment. Rainfall simulation experiments performed by Hoang et al. (2013) detected enterococci in soil samples after manure application and prior to rainfall ranging from $6.3x10^3$ to $1.3x10^4$ with over 75% resistant to tylosin. Over 69% of isolates collected from tylosin resistant enterococci during the experiment contained either *ermB, ermF* or *msrA,*while 10% or less of the isolates contained either *ermC* or *ermT*. A more recent study by Garder et al. (2014) at the same field site enumerated resistance gene levels persisting in the soil and also in subsurface drainage samples in the year following manure application. *ErmB* and *ermF* were detected both soil and water samples, while *ermT* was not present. The research was conducted on plots in a two year corn-soybean rotation with manure applied every other year. The abundance of genes detected in soil immediately after manure injection dropped down to similar levels of the genes idenfied in the control plots after a full year. Both *ermB* and *ermF* were detected in numerous tile drainage samples, but a signficant different was not seen between manured and control plots or differences in tillage practices. While *erm* gene concentrations seen by Garder et al. (2014) decreased to background levels idenfied in soil a year after manure application, a study analyzing archived soils from the Netherlands for resistance gene levels by indicated an increase in levels since the 1970's (Knapp et al. 2010). Compared to levels quantified in the 1970's, beta-lactamases showed the largest relative increase, followed by tetracyclines and erythromycin. Previous studies attempting to quantify antibiotics, antibiotic bacteria and antibiotic resistance genes in the environment resulting from the administration of antibiotics at sub-therapeutic levels to swine, have yet to identify consistent trends regarding frequency of detections and mean concentrations. The variety of antibiotic combinations and concentrations available for subtherapuetic use make it difficult to identify which resistance genes are selected for in swine's intestinal tract.

CHAPTER 3: MATERIALS & METHODS

3.1 Study Site

Four plots were used for this study at Iowa State's Northeast Research and Demonstration Farm, near Nashua, IA (43.0° N, 92.5° W). The soils at the site consist of moderately well to poorly drained Floyd loam, Kenyon silty-clay loam and Readlyn loam overtop of glacial till, with slopes ranging from 1 to 3% (Bakhsh et al. 2000). The plots were chosen based on combinations of tillage practices, crop rotation and nitrogen application history as described in Table 1. All four plots are maintained as two year corn-soybean rotations, with nitrogen application in the form of swine manure or urea and ammonium nitrate (UAN) only prior to the corn growing season. Manure has not been applied to the control plots (Plots receiving UAN application) since 1978, while the manure plots have been under various manure application rates since 1993. Manure was last injected as bands 10 to 15 cm below the soils surface by shanks on October 31, 2012. UAN was injected into the control plots in late April of 2013.

Table 1: Iowa State Northeast Research and Demonstration Farm plot descriptions.

Plot	Tillage	Nitrogen Management
23	Chisel plow*	2012 Fall inject swine manure at 168 kg N ha^{-1}
24	Chisel plow	Spring preplant spoke inject UAN at 168 kg N ha^{-1}
25	No-till	2012 Fall inject swine manure at 168 kg N ha^{-1}
34	No-till	Spring preplant spoke inject UAN at 168 kg N ha^{-1} with Cover Crop

*Tilled to a depth of 20 cm within two weeks of manure application

Each 4047 m^2 plot is individually drained by a 10 cm diameter subsurface drain located 1.2 m below the plot's surface. Border drains are located around the edge of each plot to prevent cross flow between plots. Connected to each plot's drain is a sump furnished with an effluent pump and a Neptune T-10 1" diameter flowmeter. Subsurface flow of individual plots has been monitored at the research site since 1988.

3.2 Sample Collection

The manure used in this study was obtained from a commercial swine operation, which incorporates tylosin into feed for sub-therapeutic rates (facility manager, personal communications, 2012). Manure samples were collected directly from the injector on the day of application. Samples were stored in a 4°C refrigerator overnight before being transported back to Iowa State in a cooler on ice. After subsamples were removed for enterococci analysis, the remaining samples were frozen at -20°C for DNA extractions to be performed within three months.

Soil samples were collected the day after manure application (November 1, 2012) and the following spring prior to field seeding on May 7, 2013. The process was repeated in the second year of the rotation with samples collected on November 15, 2013 and April 17, 2014. Three composite samples were collected from both the band injection location and interband locations on the two plots which received manure. Three composite samples were also collected from each non-manured control plot. Each composite sample consisted of three 15 cm cores collected along parallel transects. Soil probes were cleaned with 70% ethanol between manure band, interband and control plot sample collections. Each composite sample was placed in a one gallon plastic bag and transported back to Iowa State University in coolers containing ice. Prior to removing subsamples for enterococci and tylosin resistant enterococci analysis, composite samples were sieved through 8 mm soil sieve to increase the homogeneity of the sample. Additional subsamples were removed within 24 hours of collection for moisture content analysis. The remaining soil was frozen at -20°C for DNA extraction within three months.

Tile water samples were collected directly from tile discharge in each plot's sump. Samples were collected on a weekly basis following the beginning of tile flow on April 15, 2013 till flow ceased July 15, 2013. Grab samples were also collected following rainstorms to ensure a range of flows were represented. A total of volume 2000 mL was collected in two 1 L plastic bottles which were transported back to the Water Quality Research Lab at Iowa State University on ice. Flow meter readings were recorded at each sampling. Samples were analyzed for enterococci and tylosin resistant enterococci within 24 hours. Samples

were also filtered for DNA extraction within 24 hours then processed immediately or frozen at -20°C.

3.3. Enterococci and Tylosin Resistant Enterococci Enumeration

Manure, soil and tile water samples were analyzed for enterococci and tylosin resistant enterococci through membrane filtration as described by APHA (1998) with 0.45 micron filters comprised of mixed esters of cellulose (Millipore, Billerica, MA). Samples were analyzed in triplicate within 24 hours of collection. Soil and manure samples were diluted prior to filtration. After filtration the membranes were placed on mEnterococcus agar (Difco, Detroit Michigan) or mEnterococcus agar infused with 35 mg L^{-1} tylosin (Sigma-Aldrich, St. Louis, MO). Tylosin concentrations in mEnterococcus agar were set slightly higher than the tylosin resistance breakpoint for enterococci established by the Clinical and Laboratory Standards Institute (2011). After placement of filters on each respective agar, the plates were enumerated after incubating at 35 ± 0.5°C for 48 hours. Results for water samples were reported as colony forming units (cfu) per 100 mL and per gram of manure and soil on a dry weight basis. CFU's counted on mE agar accounted for total enterococci per sample, while enterococci counts on mE infused with tylosin indicated levels of tylosin resistant enterococci.

3.4 DNA extraction

Tile water samples (250 mL) were filtered through 22 µm sterile filters. Mo Bio Power Water DNA kits were used to extract DNA from the filters. Filters were processed for extraction within 24 hours of tile water collection or frozen in bead tubes for extraction on a later date. DNA was extracted by using MoBio Power Soil DNA kits. Soil cores were frozen after collection and subsamples (10 g) we thawed at a later date for DNA extraction. In order to maximize the yield and purity of manure DNA extracts, the repeated bead beading plus column extraction method (RBBC) was used (Yu and Morrison 2004). The RBBC method combines bead beating with a lysis buffer containing sodium dodecyl sulfate and EDTA.

3.5 qPCR Protocols

Quantitative PCR was performed on a MJ Research Opticon2 qPCR instrument operated in the 96-well format. Each gene was analyzed separately. Each individual reaction had cumulative volume of 25 µL, consisting of: 2.5 µL of DNA, 5 µL each of forward and reverse primer and 12.5 µL of Qiagen SYBR Green Master Mix. Conditions and primer sequences defined by Garder et al. (2014) were used for *ermB* and *ermF*. *ErmC* qPCR protocols and primer sequences were adapted from Koike et al. (2010). Temperature gradients resulted in an optimal annealing temperature of 51.4 °C for *ermC*. *MsrA* PCR primers and protocols described by Sutcliffe et al. (1996) were adapted for this study. The optimal annealing temperature for *msrA* was 54 °C. Additionally, the molarities of each primer used in reactions were optimized by combining forward and reverse primers at various concentrations. Quantitative PCR standards were created by inserting amplified qPCR product into pCR-4TOPO in *E coli* using TOPO TA cloning kits (Invitrogen Corp., Carlsbad, CA). DNA from transformed *E coli* was extracted using a 5 Prime FastPlasmid Mini Kit. *ErmB* and *ermC* product were derived from *Enterococcus* isolate Man T1-C, described by Hoang et al. (2010). *ErmF* product originated from a reference *E coli* strain purchased from M. C. Roberts's lab (University of Washington). *MsrA* product originated from plasmid pAT10 inside *S. aureus* strain RN4220, which was also purchased from M. C. Roberts's lab. Blanks and negative controls were included in each qPCR assay. Negative controls consisted of PCR grade water and *Pseudomonas stutzeri* genomic DNA (ATCC 14405).

3.6 qPCR Value Standardization

Multiple 96-well qPCR plate runs were necessary due to the number of samples analyzed in this study. Limits of quantification and detection were set to minimize variability in quantitation between plates for each gene. All samples were run in triplicate wells. The difference in copies per reaction well between each of the triplicates was calculated. The average copies per reaction and standard deviation was calculated for the two samples with the smallest difference. If the third value did not fall within three standard deviations of the average value between the two with the smallest difference, the value was considered an outlier and discarded. A single limit of quantification (LOQ) and limit of

detection (LOD) was used for each gene. The LOQ copy number per reaction well for each 96-well plate was calculated from the most dilute DNA standard before Ct values deviated from the linear range of the standard curve or from the average Ct of a false positive (amplification above Ct in wells with water as template or *P stutzeri* genomic DNA) noted in a single run. Once all qPCR runs for a specific gene were complete, the LOQ was set as the highest copies per reaction identified from standard curve analysis or false positive copies per well from the set of plates. The LOD was set as smallest copies per reaction identified from standard curve analysis or false positive copies per well from the set of plates. Only values above the LOQ were enumerated. Values between the LOQ and LOD were reported as detected, but unquantifiable.

3.7 Statistical Analysis

Statistical analysis was performed with JMP®, Version 10.0.2. (SAS Institute Inc., Cary, NC, 1989-2007). Water samples analyzed for resistance genes below the specified LOQ and above the LOD were assigned the average of the LOQ and LOD for analysis. Additionally, samples below the LOD were assigned a value of zero for analysis. The non-parametric Wilcoxon ranked sum test was used to determine if resistance gene concentrations in tile drainage from different plots were significantly different. Wilcoxon ranked sum test was also performed on enterococci present in tile drainage. Resistant enterococci concentrations were not analyzed due to a lack of positive samples.

CHAPTER 4: RESULTS

4.1 Enterococci and Tylosin Resistant Enterococci

Total enterococci concentrations followed the expected trends in relative concentrations with the greatest levels found in manure followed by soil and water (Table 2 & Table 3). The average enterococci concentration in manure was 176,053 CFU g^{-1} manure with 83% resistant to tylosin.

Enterococci concentrations were greatest in soil samples collected from the soil band location immediately following manure application. The average enterococci concentrations in the band locations for both no-till and chisel plow plots decreased to background concentrations as defined by the concentrations in control plots by the time samples were collected the following spring (Table 2). Band locations were unidentifiable during soil sample collection in the second year following manure application. Concentrations of enterococci in the manured plots were similar in the second year to levels identified the in the control plots. Tylosin resistant enterococci concentrations were detected at the same order of magnitude as total enterococci in the band location immediately following manure application. The resistant enterococci levels dropped two orders of magnitude in band samples collected the following spring. No tylosin resistant enterococci were detected in interband or control plot samples in the year following manure application or any of the soil samples collected during the second year of the crop rotation.

Table 2: Enterococci and tylosin resistant enterococci concentrations in soil manure band and interband locations and no-manure control plots under no-till and chisel plow tillage.

Indicator	Treatment	Location	Fall 2012	Spring 2013	Fall 2013	Spring 2014
Median Enterococci CFU g^{-1} soil	No-Till Manure	Band	210	8	0*	4*
		Interband	0	0		
	No-Till Control	Composite	16	0	4	4
	Chisel Plow Manure	Band	268	8	0	0
		Interband	0	0		
	Chisel Plow Control	Composite	4	4	4	8
Median Tylosin Resistant Enterococci CFU g^{-1} soil	No-Till Manure	Band	219	4	0	0
		Interband	0	0		
	No-Till Control	Composite	0	0	0	0
	Chisel Plow Manure	Band	249	4	0	0
		Interband	0	0		
	Chisel Plow Control	Composite	0	0	0	0

*Manure bands were no longer visible one year after manure application

Enterococci levels in drainage water were highly variable in all four plots (Table 3). No significant differences ($p>0.10$) in enterococci concentrations were detected between tillage practices or manure application using the Wilcoxon Ranked Sum Test. Enterococci was frequently detected in drainage samples from all four plots. The geometric mean for enterococci in recreational waterbodies of 33 CFU 100 mL^{-1} (USEPA 1986) was exceeded in 8 of 64 samples (Figure 1). There was not a significant relationship between time after application or instantaneous flow rate (data not shown) and enterococci concentrations ($p>0.10$). Cumulative tile drainage for each plot was above the 10-year average (Table 4).

Table 3: Enterococci and tylosin resistant enterococci in tile drainage from plots receiving manure application under no-till and chisel plow conditions.

Indicator	Quantification	No Till Manure	No Till Control	Chisel Plow Manure	Chisel Plow Control
Enterococci CFU 100 mL^{-1}	Mean[a]	22	9	16	19
	% of Non-Detects	7%	20%	25%	20%
Tylosin Resistant Enterococci CFU 100 mL^{-1}	Mean[a]	<1	N/A[b]	16	<1
	% of Non-Detects	88%	100%	94%	93%

[a] Means were calculated excluding the samples where enterococci were not detected.
[b] Tylosin resistant enterococci was not detected in drainage samples derived from the no-till control plot.

Table 4: Cumulative tile drainage from plots receiving manure application under no-till and chisel plow tillage regimes.

Treatment	2013 (m^3)	10-year Average 2003-2012 (m^3)
No-Till Manure	413.0	286.0
No-Till Control	465.0	192.1
Chisel Plow Manure	375.5	337.7
Chisel Plow Control	370.0	161.2

Tylosin resistant enterococci were rarely detected and concentrations were not significantly different ($p>0.10$) between manure or tillage treatments using Wilcoxon Ranked Sum Test (Table 3). Mean tylosin resistant enterococci concentrations in drainage were less than one in two plots and not detected in a third. Tylosin resistant enterococci were only detected in three samples from plots with histories of manure application.

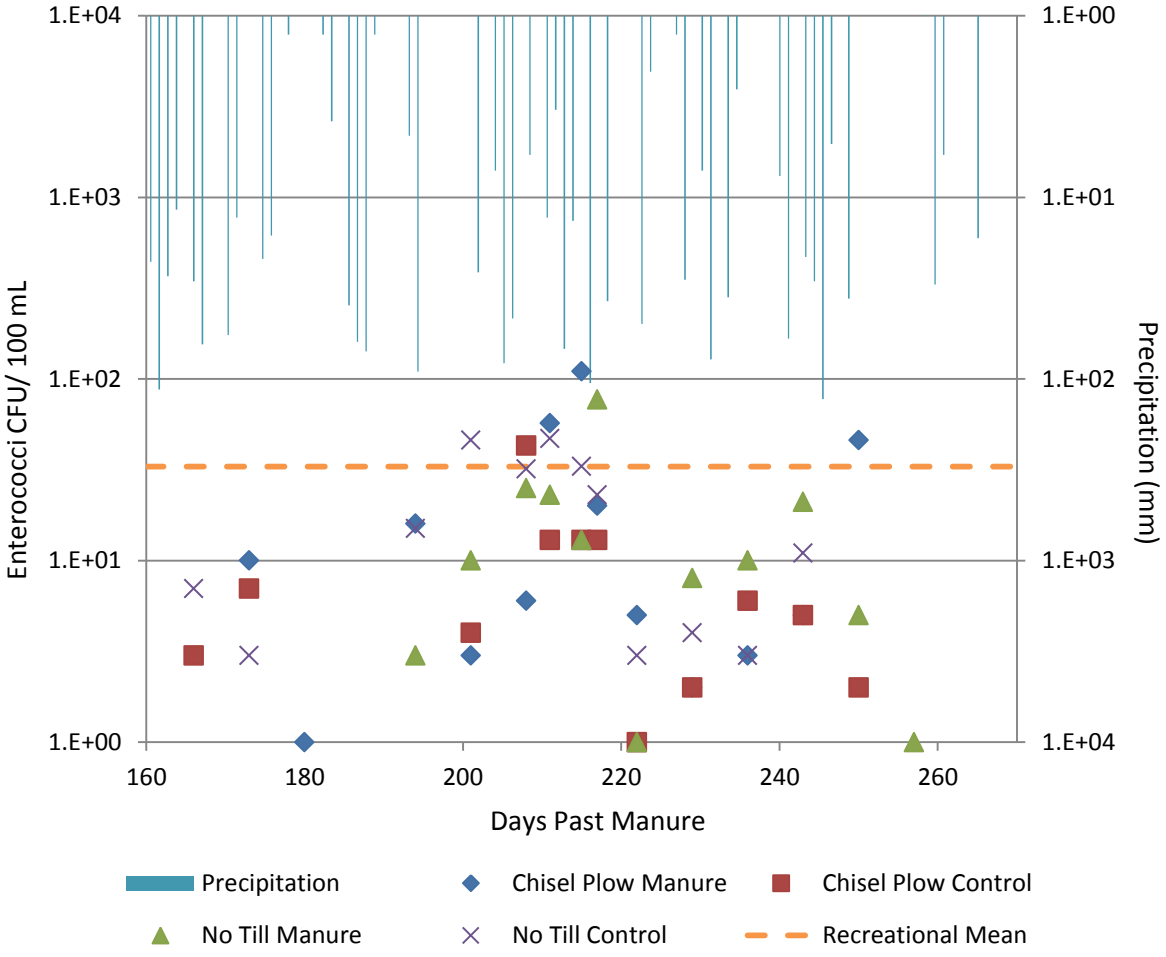

Figure 1: Enterococci concentrations in tile drainage samples and precipitation following manure application in plots under no-till and chisel plow tillage regimes. The USEPA geometric mean for enterococci in recreational waters (33 CFU 100 mL $^{-1}$) is represented by the dashed line.

4.2 Antibiotic Resistance Genes

The highest concentrations of *erm* genes were found in manure samples. *MsrA* was not detected in the manure samples. *ErmB* was present at the highest concentrations, with an average concentration of 7.29 x 10^9 copies g^{-1} manure. Average *ermC* and *ermF* concentrations were 2.44 x 10^7 copies g^{-1} manure and 1.26 x 10^8 copies g^{-1} manure, respectively.

The highest soil concentrations for all *erm* genes were detected in soil manure bands immediately following manure application, and only *msrA* was not found in quantities above the specified LOD (Table 5). Each gene was identified at $>10^6$ copies g^{-1} soil in manure bands (Table 6), except for *ermC* in the chisel plowed plot. Gene concentrations in soils collected from the interband location of manured plots and control plots immediately after manure application were below detection limits for each *erm* gene. Gene concentrations in both the chisel plow and no-till soil bands the following spring were approximately an order of magnitude lower than the previous fall. *ErmB* was detected in 75% of soil samples from manure treated plots in the second year after manure application. *ErmF* was only detected in one soil sample in the second year of the crop rotation, while *ermC* was not detected.

Table 5: Limits of quantification (LOQ) and limits of detection (LOD) for qPCR amplification for manure, soil and water.

	Copies g^{-1} manure		Copies g^{-1} soil		Copies 100mL $^{-1}$	
Gene	LOQ	LOD	LOQ	LOD	LOQ	LOD
ErmB	4800	480	6400	640	480	48
ErmC	7.52×10^4	N/A*	1.00×10^5	N/A	7520	N/A
ErmF	6880	2240	9170	2990	688	224
msrA	7.92×10^4	N/A	1.06×10^5	N/A	7920	N/A

*No LOD was established; copies per reaction identified from lowest dilution of the standard curve used for LOQ were uniform across all plates and negative controls were not amplified.

Table 6: *Erm* gene concentrations in soil following manure application in plots under no-till and chisel plow management.

Gene	Treatment	Location	Fall 2012	Spring 2013	Fall 2013	Spring 2014
ErmB Copies g^{-1}	No Till Manure	Band	5.46×10^7	2.66×10^5	<LOD+	1.59×10^5
		Interband	<LOQ*	<LOD		
	No Till Control	Composite	<LOD	6.61E+04	<LOD	<LOD
	Chisel Plow Manure	Band	1.73×10^6	5.77×10^5	2.45×10^4	4.18×10^4
		Interband	<LOD	<LOD		
	Chisel Plow Control	Composite	<LOD	<LOD	2.42×10^4	<LOD
ErmC Copies g^{-1}	No Till Manure	Band	1.53×10^6	3.24×10^5	<LOD	<LOD
		Interband	<LOD	<LOD		
	No Till Control	Composite	<LOD	<LOD	<LOD	<LOD
	Chisel Plow Manure	Band	<LOD	5.77×10^5	<LOD	<LOD
		Interband	<LOD	<LOD		
	Chisel Plow Control	Composite	<LOD	2.88×10^5	<LOD	<LOD
ErmF Copies g^{-1}	No Till Manure	Band	2.58×10^6	2.28×10^5	<LOD	5.16×10^4
		Interband	<LOD	<LOD		
	No Till Control	Composite	<LOD	6.14×10^4	<LOD	<LOD
	Chisel Plow Manure	Band	1.29×10^7	8.75×10^4	<LOD	<LOD
		Interband	<LOD	<LOD		
	Chisel Plow Control	Composite	<LOD	<LOD	<LOD	<LOD

+Less than limit of detection for specified gene (Table 5).
*Less than limit of quantification, greater than limit of detection for specified gene (Table 5).

Quantitative PCR detected *ermB*, *ermC*, and *ermF* in tile drainage water grab samples, while levels of *msrA* were not above the limit of detection (Table 5). Fifteen to 17 drainage samples were collected from each plot. Five of the samples from each plot were collected during or immediately after rainfall events.

ErmB was detected in 82% of the water samples collected from the no-till, manure treated plot, with 59% above the LOQ (Table 7). This was followed by the manure treated, chisel plow plot in which *ermB* was detected in 73%, with 33% above the LOQ. Only one drainage sample in each control plot was above the limit of quantification for *ermB*. However, similar percentages of samples from all plots were above the limit of detection and below quantification (24-44%). Mean concentrations of *ermB* in samples above the limit of quantification were similar in chisel plow and no till plots receiving manure application. The Wilcoxon Ranked Sum Test did not identify significant differences ($p>0.10$) in *ermB* concentrations between the no-till and chisel plow treatments for both the manured and

control plots and therefore were combined for further analysis. After data for the two tillage regimes were combined, concentrations of *ermB* in water from the manure treated plots were significantly greater ($p<0.01$) than those from the control plots using the Wilcoxon Ranked Sum Test. *ErmB* was detected in all five no-till, manure treated samples and in all but one sample from the chisel plow, manure treated samples collected during rainfall events. Additionally, the 61% of quantifiable *ermB* samples were from the first half of the sampling period (Figure 2).

Table 7: *Erm* gene concentrations in tile drainage following manure application in plots under no-till and chisel plow tillage regimes.

		No Till Manure	No Till Control	Chisel Plow Manure	Chisel Plow Control
ErmB copies 100 mL^{-1}	Mean >LOQ	4670	3170	3940	636
	% <LOQ, >LOD	24%	31%	40%	44%
	% <LOD	18%	56%	33%	50%
ErmC copies 100 mL^{-1}	Mean >LOQ	1.79×10^4	6.35×10^4	9.71×10^4	1.36×10^4
	% <LOQ, >LOD	0 %	0%	0%	0%
	% <LOD	71%	69%	73%	75%
ErmF copies 100 mL^{-1}	Mean >LOQ	1810	N/A*	1230	N/A*
	% <LOQ, >LOD	6%	6%	27%	0%
	% <LOD	56%	94%	67%	100%

*No drainage samples in control plots contained concentrations of *ermF* above the specified LOQ.

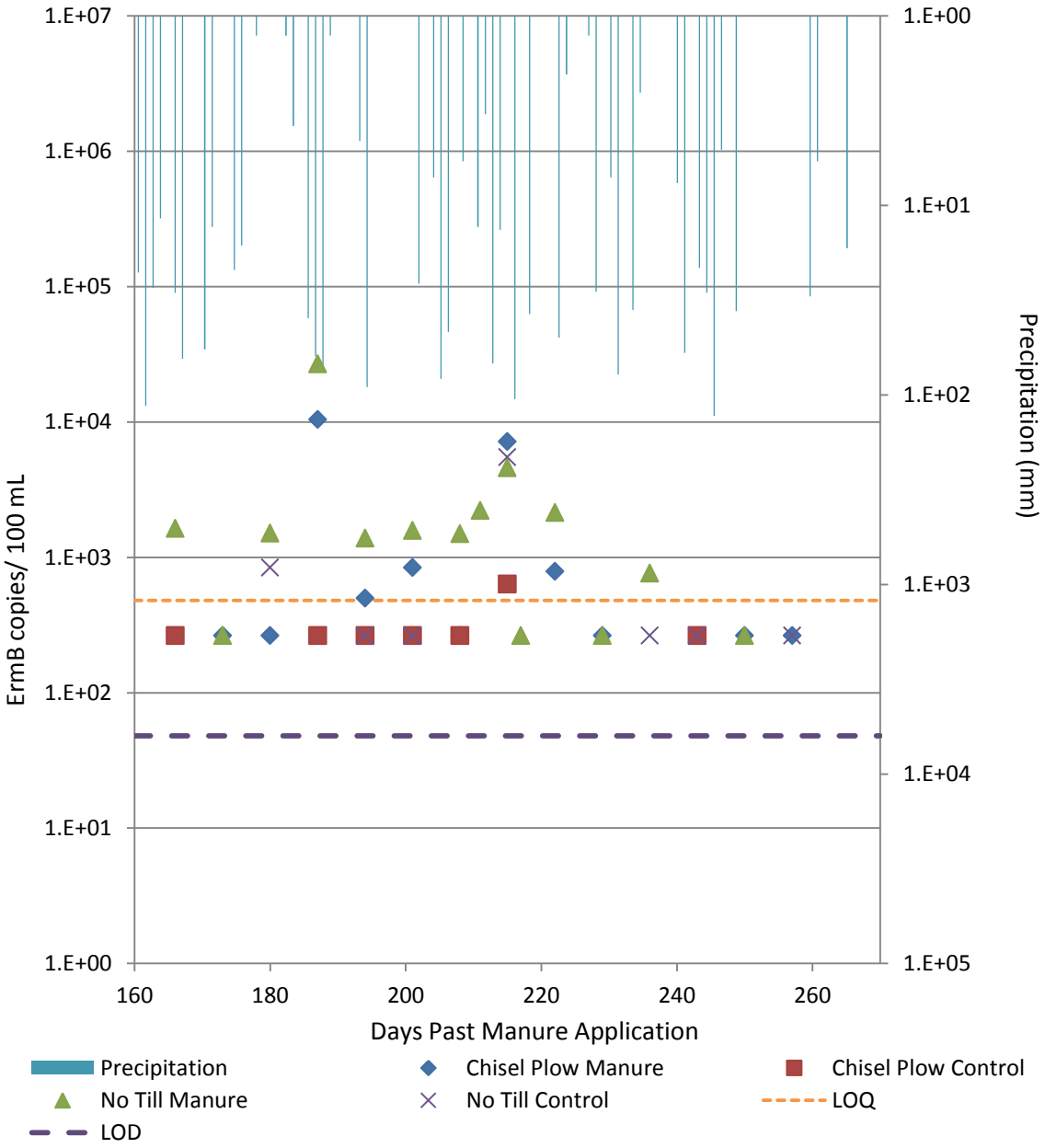

Figure 2: *ErmB* concentrations in tile drainage following manure application in plots under no-till and chisel plow regimes with LOQ and LOD. Concentrations less than the LOQ and greater than LOD were assigned the average value of the LOQ and LOD for visualization.

ErmC was detected in drainage water from all four plots. Although *ermC* had the greatest average concentration in samples above the limit of quantification between the three genes detected (Table 7), levels in water from the manure treated plots were not significantly

different (p>0.10) from water draining from the control plots using the Wilcoxon Ranked Sum Test. The frequencies of detection for the four plots were quite similar, ranging from 25% to 33%. The majority of *ermC* detections were from samples collected during the second half of the sampling season (Figure 3).

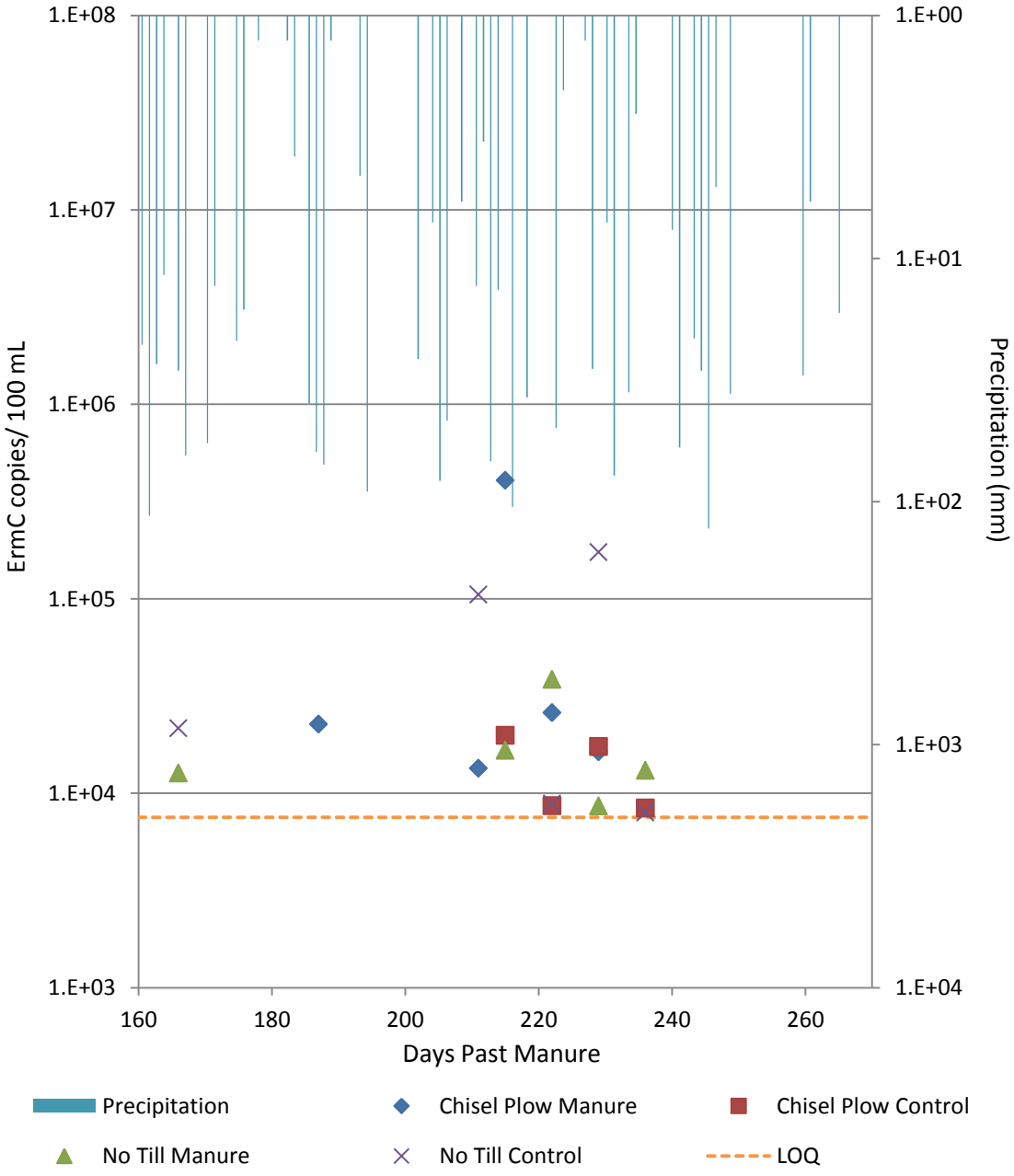

Figure 3: *ErmC* concentrations in tile drainage following manure application in plots under no-till and chisel plow regimes with LOQ.

ErmF was detected in 44% of tile drainage samples from the manure applied, no-till plot and 33% of samples from the manure applied, chisel plow plot. However, the majority of samples collected from the manure applied no-till plot were above the specified LOQ, while bulk of detects in the manure applied chisel plow plot were below the LOQ (Table 7). *ErmF* was not detected in any water samples from the chisel plow, control plot, and only one sample collected from the no-till, control plot. Wilcoxon Ranked Sum Test results did not identify significant differences ($p>0.10$) in *ermF* concentrations between the no-till and chisel plow treatments for both the manure applied and control plots and were therefore combined for further analysis. After data for the two tillage regimes were combined, concentrations of *ermF* in the drainage from manure treated plot were significantly greater ($P<0.01$) than those in drainage from the control plots using the Wilcoxon Ranked Sum Test. The majority of water samples containing *ermF* concentrations above the LOQ were collected during the first half of the sampling season (Figure 4), similar to *ermB.*

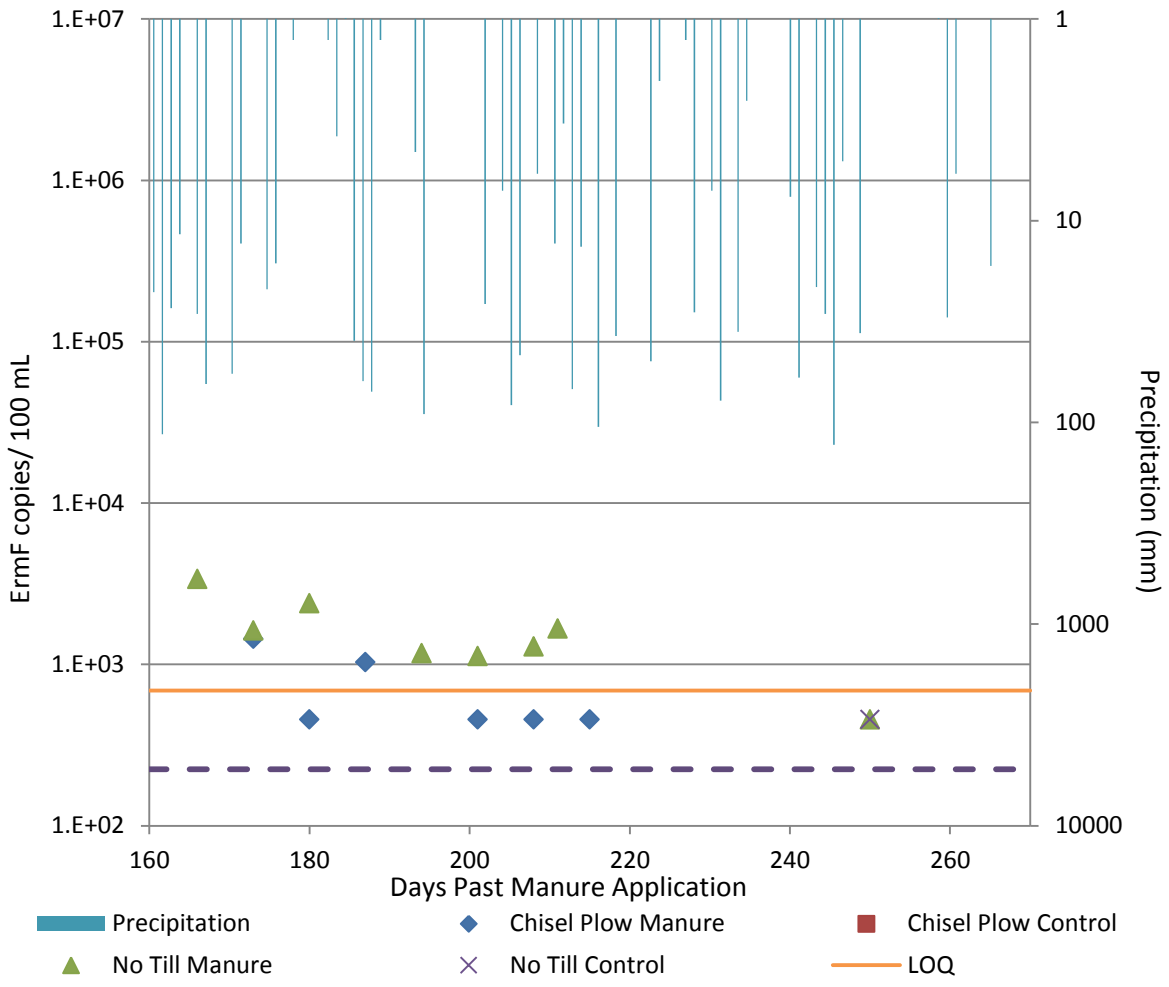

Figure 4: *ErmF* concentrations in tile drainage following manure application in plots under no-till and chisel plow regimes with LOQ and LOD. Concentrations less than the LOQ and greater than LOD were assigned the average value of the LOQ and LOD for visualization.

CHAPTER 5: DISCUSSION

Enterococci concentrations present in liquid swine manure were similar to levels reported by Garder et al. (2014) in samples processed immediately after application. Tylosin resistant enterococci in swine manure (65%-100%) were similar to levels previously identified (Garder et al. 2014, Trang et al. 2013, Onan et al. 2003). The fractions of tylosin resistant enterococci from the soil manure band immediately following application (93%-100%) were comparable to percentages of tylosin resistant enterococci in the manure injected in the no-till and chisel plow plots.

Concentrations of total and tylosin resistant enterococci in manure bands following application were approximately an order of magnitude lower than reported by Hoang et al. (2013) and Garder et al. (2014). Additionally, enterococci concentrations in band locations dropped to background concentrations within six months, while Garder et al. (2014) reported concentrations above those reported in non-manured control plots after the same time lapse. Prior studies have reported percentages of tylosin resistant enterococci in manure treated soils ranging from 5%-100% (Garder et al. 2014, Halling-Sorensen et al. 2005, Onan et al. 2003). The large range tylosin resistant enterococci in soils noted in previous studies likely stems from variable initial concentrations in manure. Levels of antibiotics administered in feed vary depending on the growth cycle of the swine, which affect concentrations of resistant bacteria excreted in manure.

Erm genes concentrations in soil followed a similar pattern to enterococci concentrations during the first year of the study. *Erm* genes were greatest in the soil band samples taken immediately after manure application. However, concentrations of *ermB* and *ermF* were both at least two orders of magnitude lower than concentrations previously reported by Garder et al. (2014). Additionally, *ermB* was only identified in one interband sample in the first year of the crop rotation, while Garder et al. (2014) detected *ermB* and *ermF* at quantifiable levels in every interband sample in the two previous years (fall 2010 – spring 2012). The drought conditions witnessed during the summer of 2012 in Northeast

Iowa may have caused additional duress to the bacteria hosting the resistance genes and therefore hastened the return of overall concentrations found in control plots.

Enterococci concentrations in tile drainage were not significantly different ($p>0.10$) across tillage or manure treatments. These finding are consistent with levels from the same sampling location in previous years when below average precipitation was recorded (Garder et al. 2014). Furthermore, enterococci concentrations were not correlated with time after application or instantaneous flow rates. These results, observed in a year with greater than average cumulative tile flow, may refute the notion by Garder et al. (2014) that reduced macropore flow contributed to a lack of differences seen in enterococci concentrations across tillage and manure treatments. Additionally, antibiotic resistant enterococci only account for a small percentage of the bacterial populations in soil and water samples. Therefore, non-significant differences noted in concentrations between treatments in this study may not be indicative of overall bacterial transport into soil and water from manure application.

Precipitation totals for April through June in 2011 and 2012 at the study site were 31.8 cm and 26.5 cm, respectively. These totals were nearly doubled in 2013, with 62.4 cm of precipitation from April through June. Additionally, total drainage from plots in 2011 and 2012 were below the ten year average, while flows from the same plots in 2013 exceeded the average. Hoang et al. (2013) identified correlations between enterococci concentrations in tile flow and total suspended solids during rainfall simulations immediately following manure application. Sediment concentrations in tile drainage have been shown to increase following rainfall events (Ball Coelho et al. 2012). Therefore, above average precipitation witnessed in spring of 2013 may have created additional opportunities for bacteria harboring resistance genes to be transported from soil to drainage water.

Although none of the genes analyzed showed significant differences ($P>0.10$) due to tillage treatments, *ermB* and *ermF* mean concentrations were slightly greater in drainage water from the no-till manure treated plot than the manure treated chisel plowed plot. When tillage treatments were combined, both *ermB* and *ermF* concentrations in tile drainage were significantly greater ($P<0.01$) in plots with manure application than their control plot counterparts. Garder et al. (2014) did not detect any significant differences in gene concentrations in drainage water due to tillage or manure treatments during 2011 and 2012 at

the same study site. Additionally, Garder et al. (2014) used a unique LOQ for each 96-well qPCR run. Therefore, samples in this study lower than the specified LOQ, but greater than the LOD would have been classified as quantifiable by Garder et al. (2014). Setting a conservative LOQ in this study allowed for greater confidence of enumerated samples, but affected the sensitivity of the Wilcoxon Ranked Sum analysis by assigning a uniform rank for samples below the LOQ and LOD.

ErmB was the most frequently detected gene in water, with the majority of drainage samples from manure treated plots containing concentrations above the limit of detection. *ErmF* was the next most prevalent gene in manure treated plots with 44% of samples above the limit of detection. The detection frequency and magnitude for *ermB* and *ermF* were consistent with results obtained by Garder et al. (2014), however, their detection frequencies were not specific to a particular treatment type. Koike et al. (2010) detected *ermB* in 87% of samples and *ermF* in 40% of samples taken from wells near swine lagoons, which were previously identified as being contaminated by swine lagoon leachate.

ErmC was detected at the highest concentrations of the three genes which were identified, but detection frequency and concentrations were comparable across all treatments. Additionally, the majority of the positive samples were from later in the sampling season, as opposed to *ermB* and *ermF*, which were mainly detected during the first portion. Hoang et al. (2013), using PCR, only detected *ermC* in 9% of enterococci isolates which were phenotypically resistant to tylosin. Phylogenic analysis performed on resistance genes by Koike et al. (2010) concluded that RNA methylases can be organized into two major clusters: bacteria containing high-G + C contents, such as streptomyces, and bacteria containing low-G + C contents, which include commensal, pathogenic and environmental bacteria. Isolates containing *ermC* were identified in subsets by Koike et al. (2010). *ErmC* was not detected by Liang et al. (2013) in water or soil samples collected from wastewater trenches exporting waste from a swine farm. *ErmC* was detected in hog house effluent by Chen et al. (2007), but less frequently than the five other *erm* genes screened for in the study. *ErmC* concentrations in water samples from this study are likely from naturally occurring bacterial communities in soil, due to similar concentrations in manured and control plot drainage and

the majority of quantifiable concentrations occurring towards the end of the tile drainage period.

MsrA was not detected in any samples, including manure; however, Hoang et al. (2013) detected *msrA* in 97% of tylosin resistant enterococci isolated from manure, soil and water samples. While *erm* genes confer resistance by target site modification, *msrA* is responsible for encoding a transport protein containing two ATP-binding domains. The ATP-binding domains are part of an efflux system which works to translocate macrolides across cell membranes (Ross et al. 1995). This mode of resistance may be less prevalent in the environment due to the transport systems having to utilize energy to export the antibiotic across the membrane. Although Hoang et al. (2013) identified *msrA* in nearly 100% of enterococci isolates phenotypically resistant to tylosin, the proportion of extracted enterococci DNA to total DNA extracted in an environmental sample may be quite small.

Currently, water quality standards do not exist for antibiotic resistant bacteria or resistance genes. Attempting to create a numerical standard would be an arduous task due to background levels of resistance naturally occurring in the environment. However, relative concentrations of resistant bacteria and genes may be monitored in order to identify the effects of anthropogenic activities on microbial communities in soil and water. Increased levels of antibiotic resistant bacteria in the environment are of great concern due to their associated public health risks. This study utilized two methods to quantify antibiotic resistance in water and soil: phenotypic resistance to tylosin demonstrated by enterococci and enumeration of macrolide antibiotic resistance genes through qPCR. Although enterococci is used commonly as an indicator organism for fecal contamination in surface waters (USEPA 1986), results from this study have indicated that concentrations of antibiotic resistance enterococci do not accurately portray total concentrations of resistance genes found in soil and water microbial communities. Koike et al. (2009) identified numerous macrolide resistance genes in a wide range of bacteria genera. In order to more accurately represent antibiotic resistance in environmental samples, additional research is needed to help identify the bacteria harboring the majority of resistance gene copies.

CHAPTER 6: CONCLUSIONS

This study was the first to report significantly higher levels of resistance genes *ermB* and *ermF* in subsurface water draining from manured plots when compared against non-manured control plots. Previous work by Garder et al. (2014) at the same location identified concentrations of *ermB* and *ermF* of the same magnitude as reported in this study, but found no significant differences between manure treatments. Although enterococci is commonly used as an indicator in for fecal pollution, results from this study prove total and tylosin resistant enterococci do not accurately portray levels of antibiotic resistance genes in drainage stemming from manure amended plots. Enterococci concentrations in drainage water samples were not significantly different between manure and tillage treatments, while tylosin resistant enterococci were rarely detected. The above average precipitation recorded in the spring of 2013 likely induced the transport of bacteria harboring these genes from manure into subsurface drainage. While water quality standards do not currently exist for antibiotic resistant bacteria or resistance genes, transport of resistant bacteria into the environment raises concerns regarding public health. Artificial subsurface drainage incorporated in agricultural fields increases the rate at which shallow groundwater enters surrounding recreational surface waters.

Although mean *ermC* concentrations in drainage water were the greatest of the three resistance genes detected, concentrations were not significantly different between drainage water from the manured land from the non manured land. These results indicated that *ermC* concentrations detected in tile drainage were from bacteria naturally residing in the soil. While the majority of bacteria harboring ermC in drainage water are not likely to have stemmed from manure application, horizontal transfer of their genes to pathogenic bacteria found in swine manure may be of concern to the public. Further research is needed to determine the rates at which antibiotic resistance genes may spread through horizontal gene transfer.

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APPENDIX A

DATA

Enterococci and tylosin resistant enterococci CFU/ 100 mL

Plot	Date	Grab ID	mE			mE+TYL		
24	4/15/2013	GW2	10	0	0	0	0	0
25	4/15/2013	GW2	0	0	0	0	0	0
34	4/15/2013	GW2	20	0	0	0	0	0
23	4/22/2013	GW3	10	0	20	0	0	0
24	4/22/2013	GW3	0	0	20	0	0	0
25	4/22/2013	GW3	0	0	0	0	0	0
34	4/22/2013	GW3	0	0	10	0	0	0
23	4/29/2013	GW4	2	2	0	0	0	0
24	4/29/2013	GW4	0	0	1	0	0	0
25	4/29/2013	GW4	0	0	0	0	0	0
34	4/29/2013	GW4	0	1	0	0	0	0
23	5/6/2013	GW5	0	0	0	0	0	0
24	5/6/2013	GW5	0	0	0	0	0	0
25	5/6/2013	GW5	0	0	0	0	0	0
34	5/6/2013	GW5	0	0	0	0	0	0
23	5/13/2013	GW6	39	4	4	0	0	0
24	5/13/2013	GW6	0	0	0	0	0	0
25	5/13/2013	GW6	1	5	2	0	0	0
34	5/13/2013	GW6	27	4	14	0	0	0
19	5/20/2013	GW7/E2	41	37	56	0	0	0
20	5/20/2013	GW7/E2	108	147	128	0	0	0
29	5/20/2013	GW7/E2	2	4	6	0	0	0
30	5/20/2013	GW7/E2	271	266	270	4	5	3
23	5/20/2013	GW7/E2	5	2	3	0	0	0
24	5/20/2013	GW7/E2	2	6	4	0	0	0
25	5/20/2013	GW7/E2	8	14	9	0	0	0
34	5/20/2013	GW7/E2	62	46	29	0	0	0
23	5/27/2013	GW8/E3	5	8	6	0	0	0
24	5/27/2013	GW8/E3	49	36	43	0	0	0
25	5/27/2013	GW8/E3	29	18	29	0	0	0
34	5/27/2013	GW8/E3	33	34	28	0	0	0
23	5/30/2013	GE4	40	80	50	0	0	0
24	5/30/2013	GE4	0	40	0	0	0	0
25	5/30/2013	GE4	40	0	30	0	1	0
34	5/30/2013	GE4	60	40	40	0	0	1

23	6/3/2013	GW9	100	130	100	0	0	0
24	6/3/2013	GW9	10	20	10	0	0	0
25	6/3/2013	GW9	30	10	0	0	0	0
34	6/3/2013	GW9	20	50	30	0	0	0
23	6/5/2013	GE5	0	40	20	0	0	0
24	6/5/2013	GE5	40	0	0	0	0	0
25	6/5/2013	GE5	30	100	100	1	0	0
34	6/5/2013	GE5	20	10	40	0	0	0
23	6/10/2013	GW10	5	4	5	0	0	0
24	6/10/2013	GW10	3	1	0	0	0	0
25	6/10/2013	GW10	1	1	0	0	0	0
34	6/10/2013	GW10	4	2	2	0	0	0
23	6/17/2013	GW11	2	3	1	0	0	0
24	6/17/2013	GW11	1	2	2	0	0	0
25	6/17/2013	GW11	8	8	7	0	0	0
34	6/17/2013	GW11	4	5	4	0	0	0
23	6/24/2013	GW12	1	5	4	0	0	0
24	6/24/2013	GW12	8	4	6	0	0	0
25	6/24/2013	GW12	11	10	8	0	0	0
34	6/24/2013	GW12	3	1	4	0	0	0
23	7/1/2013	GW13	7	4	4	0	0	0
24	7/1/2013	GW13	4	5	7	0	0	0
25	7/1/2013	GW13	33	14	15	0	0	0
34	7/1/2013	GW13	13	8	11	0	0	0
23	7/8/2013	GW14	50	71	17	16	16	16
24	7/8/2013	GW14	1	2	2	0	0	0
25	7/8/2013	GW14	5	4	7	0	0	0
25	7/15/2013	GW15	2	1	0	0	0	0

Soil moisture contents

Sample Period	Plot	Plot type	Band	Tray Wt (g)	Tray+ moist soil (g)	Tray+ dry soil (g)	MC (%)	ww/dw
Fall 2012	23	Band	A	0.991	2.037	1.862	16.73	1.201
Fall 2012	23	Band	B	1.024	2.076	1.898	16.92	1.204
Fall 2012	23	Band	C	1.004	2.075	1.892	17.09	1.206
Fall 2012	23	NO Band	A	1.012	2.01	1.859	15.13	1.178
Fall 2012	23	NO Band	B	0.999	2.007	1.846	15.97	1.19
Fall 2012	23	NO Band	C	1.004	2.083	1.903	16.68	1.2
Fall 2012	24	Control	A	1.005	2.137	1.975	14.31	1.167
Fall 2012	24	Control	B	1.008	2.281	1.989	22.94	1.298
Fall 2012	24	Control	C	1.005	2.121	1.954	14.96	1.176
Fall 2012	25	Band	A	1.033	2.012	1.825	19.1	1.236
Fall 2012	25	Band	B	1.013	2.037	1.854	17.87	1.218
Fall 2012	25	Band	C	1.01	2.001	1.866	13.62	1.158
Fall 2012	25	NO Band	A	1.022	2.057	1.908	14.4	1.168
Fall 2012	25	NO Band	B	1.025	2.032	1.885	14.6	1.171
Fall 2012	25	NO Band	C	1.017	2.002	1.841	16.35	1.195
Fall 2012	34	Control	A	0.985	2.01	1.854	15.22	1.18
Fall 2012	34	Control	B	1.001	2.039	1.866	16.67	1.2
Fall 2012	34	Control	C	1.012	2.006	1.822	18.51	1.227
Spring 2013	23	Band	A	0.991	15.552	12.883	18.33	1.224
Spring 2013	23	Band	B	1.011	16.874	14.031	17.92	1.218
Spring 2013	23	Band	C	1.026	16.981	13.984	18.78	1.231
Spring 2013	23	NO Band	A	1.028	32.891	26.649	19.59	1.244
Spring 2013	23	NO Band	B	0.999	16.894	14.005	18.18	1.222
Spring 2013	23	NO Band	C	0.995	15.242	12.751	17.48	1.212
Spring 2013	24	Control	A	0.998	16.791	13.873	18.48	1.227

Spring 2013	24	Control	B	1.022	13.515	11.293	17.79	1.216
Spring 2013	24	Control	C	1.019	16.456	13.603	18.48	1.227
Spring 2013	25	Band	A	0.997	17.621	14.063	21.4	1.272
Spring 2013	25	Band	B	1.005	15.418	12.732	18.64	1.229
Spring 2013	25	Band	C	1.017	19.103	15.649	19.1	1.236
Spring 2013	25	NO Band	A	1.005	18.963	15.726	18.03	1.22
Spring 2013	25	NO Band	B	1.027	17.836	14.823	17.92	1.218
Spring 2013	25	NO Band	C	1.041	17.191	14.074	19.3	1.239
Spring 2013	34	Control	A	1	12.817	10.851	16.64	1.2
Spring 2013	34	Control	B	1	14.314	12.127	16.43	1.197
Spring 2013	34	Control	C	0.998	13.762	11.585	17.06	1.206
Fall 2013	23	NO Band	A	1.018	17.909	15.242	15.79	1.188
Fall 2013	23	NO Band	B	0.98	15.617	15.28	2.3	1.024
Fall 2013	23	NO Band	C	0.983	14.947	12.05	20.75	1.262
Fall 2013	24	Control	A	1.009	17.214	14.412	17.29	1.209
Fall 2013	24	Control	B	1.015	16.071	13.681	15.87	1.189
Fall 2013	24	Control	C	0.991	16.334	13.759	16.78	1.202
Fall 2013	25	NO Band	A	1.008	15.609	13.09	17.25	1.208
Fall 2013	25	NO Band	B	1.011	18.222	15.204	17.54	1.213
Fall 2013	25	NO Band	C	1.002	15.958	13.302	17.76	1.216
Fall 2013	34	Control	A	0.98	14.616	12.455	15.85	1.188
Fall 2013	34	Control	B	1.022	15.252	12.948	16.19	1.193
Fall 2013	34	Control	C	0.998	15.878	13.029	19.15	1.237
Spring 2014	23	NO Band	A	0.988	6.132	5.234	17.46	1.211
Spring 2014	23	NO Band	B	1.034	6.314	5.335	18.54	1.228
Spring	23	NO	C	1.001	6.142	5.221	17.91	1.218

2014		Band						
Spring 2014	24	Control	A	0.982	6.208	5.318	17.03	1.205
Spring 2014	24	Control	B	1.012	6.343	5.388	17.91	1.218
Spring 2014	24	Control	C	1.005	6.137	5.258	17.13	1.207
Spring 2014	30	Band	A	1.009	6.259	5.365	17.03	1.205
Spring 2014	30	Band	B	1.009	6.672	5.523	20.29	1.255
Spring 2014	30	Band	C	1.025	6.842	5.704	19.56	1.243
Spring 2014	25	NO Band	A	1.002	6.49	5.535	17.4	1.211
Spring 2014	25	NO Band	B	0.985	6.413	5.475	17.28	1.209
Spring 2014	25	NO Band	C	1.017	6.463	5.47	18.23	1.223
Spring 2014	34	Control	A	1.019	6.334	5.389	17.78	1.216
Spring 2014	34	Control	B	1.03	6.267	5.368	17.17	1.207
Spring 2014	34	Control	C	1.027	6.443	5.432	18.67	1.23

Enterococci and tylosin resistant soil CFU

Sample Period	Plot	Plot type	Band	ent avgs (10^-1)	ent avgs (ww)	ent avgs (dw)	ent + tyl avgs (10^-1)	ent+tyl avgs (ww)	ent+tyl avgs (dw)
Fall 2012	23	Band	A	22.33	223.33	268.21	15.00	150.00	180.14
Fall 2012	23	Band	B	18.33	183.33	220.67	20.67	206.67	248.76
Fall 2012	23	Band	C	22.67	226.67	273.38	20.67	206.67	249.26
Fall 2012	23	NO Band	A	0.00	0.00	0.00	0.00	0.00	0.00
Fall 2012	23	NO Band	B	0.33	3.33	3.97	0.00	0.00	0.00
Fall 2012	23	NO Band	C	0.00	0.00	0.00	0.00	0.00	0.00
Fall 2012	24	Control	A	0.00	0.00	0.00	0.00	0.00	0.00
Fall 2012	24	Control	B	0.33	3.33	4.33	0.00	0.00	0.00
Fall 2012	24	Control	C	3.00	30.00	35.28	0.00	0.00	0.00
Fall 2012	25	Band	A	17.00	170.00	210.14	11.67	116.67	144.21
Fall 2012	25	Band	B	43.67	436.67	531.68	49.33	493.33	600.68
Fall 2012	25	Band	C	17.33	173.33	200.67	19.00	190.00	219.96
Fall 2012	25	NO Band	A	0.67	6.67	7.79	0.00	0.00	0.00
Fall 2012	25	NO Band	B	0.00	0.00	0.00	0.00	0.00	0.00
Fall 2012	25	NO Band	C	0.00	0.00	0.00	0.00	0.00	0.00
Fall 2012	34	Control	A	0.00	0.00	0.00	0.00	0.00	0.00
Fall 2012	34	Control	B	1.33	13.33	16.00	0.00	0.00	0.00
Fall 2012	34	Control	C	5.00	50.00	61.36	0.00	0.00	0.00
Spring 2013	23	Band	A	0.33	3.33	4.08	0.00	0.00	0.00
Spring 2013	23	Band	B	0.67	6.67	8.12	0.33	3.33	4.06
Spring 2013	23	Band	C	0.67	6.67	8.21	1.33	13.33	16.42
Spring 2013	23	NO Band	A	0.00	0.00	0.00	0.00	0.00	0.00
Spring 2013	23	NO Band	B	0.00	0.00	0.00	0.00	0.00	0.00
Spring 2013	23	NO Band	C	0.00	0.00	0.00	0.00	0.00	0.00
Spring 2013	24	Control	A	0.67	6.67	8.18	0.00	0.00	0.00
Spring 2013	24	Control	B	0.33	3.33	4.05	0.33	3.33	4.05
Spring 2013	24	Control	C	0.33	3.33	4.09	0.00	0.00	0.00
Spring 2013	25	Band	A	0.00	0.00	0.00	0.33	3.33	4.24
Spring 2013	25	Band	B	1.33	13.33	16.39	1.67	16.67	20.48
Spring 2013	25	Band	C	0.67	6.67	8.24	0.33	3.33	4.12
Spring 2013	25	NO Band	A	0.00	0.00	0.00	0.67	6.67	8.13
Spring 2013	25	NO Band	B	0.00	0.00	0.00	0.00	0.00	0.00
Spring 2013	25	NO Band	C	0.67	6.67	8.26	0.00	0.00	0.00
Spring 2013	34	Control	A	3.33	33.33	39.99	0.00	0.00	0.00
Spring 2013	34	Control	B	0.00	0.00	0.00	0.00	0.00	0.00

Spring 2013	34	Control	C	0.00	0.00	0.00	0.00	0.00	0.00
Fall 2013	23	NO Band	A	0.00	0.00	0.00	0.00	0.00	0.00
Fall 2013	23	NO Band	B	0.00	0.00	0.00	0.00	0.00	0.00
Fall 2013	23	NO Band	C	0.00	0.00	0.00	0.00	0.00	0.00
Fall 2013	24	Control	A	0.33	3.33	4.03	0.00	0.00	0.00
Fall 2013	24	Control	B	0.33	3.33	3.96	0.00	0.00	0.00
Fall 2013	24	Control	C	0.33	3.33	4.01	0.00	0.00	0.00
Fall 2013	25	NO Band	A	0.00	0.00	0.00	0.00	0.00	0.00
Fall 2013	25	NO Band	B	0.33	3.33	4.04	0.00	0.00	0.00
Fall 2013	25	NO Band	C	0.00	0.00	0.00	0.00	0.00	0.00
Fall 2013	34	Control	A	0.33	3.33	3.96	0.00	0.00	0.00
Fall 2013	34	Control	B	0.33	3.33	3.98	0.00	0.00	0.00
Fall 2013	34	Control	C	1.00	10.00	12.37	0.00	0.00	0.00
Spring 2014	23	NO Band	A	0.00	0.00	0.00	0.00	0.00	0.00
Spring 2014	23	NO Band	B	0.00	0.00	0.00	0.00	0.00	0.00
Spring 2014	23	NO Band	C	0.00	0.00	0.00	0.00	0.00	0.00
Spring 2014	24	Control	A	5.33	53.33	64.28	0.00	0.00	0.00
Spring 2014	24	Control	B	0.00	0.00	0.00	0.00	0.00	0.00
Spring 2014	24	Control	C	0.67	6.67	8.04	0.00	0.00	0.00
Spring 2014	30	Band	A	3.33	33.33	40.17	1.33	13.33	16.07
Spring 2014	30	Band	B	17.00	170.00	213.27	14.33	143.33	179.82
Spring 2014	30	Band	C	121.33	1213.33	1508.43	25.67	256.67	319.09
Spring 2014	25	NO Band	A	0.00	0.00	0.00	0.00	0.00	0.00
Spring 2014	25	NO Band	B	0.33	3.33	4.03	0.00	0.00	0.00
Spring 2014	25	NO Band	C	0.67	6.67	8.15	0.00	0.00	0.00
Spring 2014	34	Control	A	0.67	6.67	8.11	0.00	0.00	0.00
Spring 2014	34	Control	B	0.33	3.33	4.02	0.00	0.00	0.00
Spring 2014	34	Control	C	0.33	3.33	4.10	0.00	0.00	0.00

Manure moisture contents

Plot	tin (g)	Moist manure + tin (g)	dry manure+tin (g)	MC	ww/dw
23	62.777	246.99	65.772	0.983742	61.50684
25	59.165	232.103	69.908	0.937879	16.09774

Manure enterococcus and tylosin resistant enterococcus

	Plot 23 avg dilutions		Plot 23 avg ww		Plot 23 avg dw	
Dilution	ent	ent+ tyl	ent	ent+ tyl	ent	ent+ tyl
10^-1	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
10^-2	35.00	52.67	3500.00	5266.67	215274	323936
10^-3	3.67	4.67	3666.67	4666.67	225525	287032
10^-4	0.33	0.00	3333.33	0.00	205023	0
10^-5	0.00	0.00	0.00	0.00	0	0

	Plot 25 avg dilutions		Plot 25 avg ww		Plot 25 avg dw	
Dilution	ent	ent+ tyl	ent	ent+ tyl	ent	ent+ tyl
10^-1	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
10^-2	85	55	8500	5500	136831	88538
10^-3	10	7.33333333	10000	7333.3333	160977	118050
10^-4	2.333333	2.66666667	23333.33	26666.667	375614	429273
10^-5	0.333333	0.33333333	33333.33	33333.333	536591	536591

Resistance genes in drainage water

Gene	Date	Grab	Days since manure application	Plot 23	Plot 24	Plot 25	Plot 34
ErmB	4/15/2013	GW2	166		264	1636	<LOD
ErmB	4/22/2013	GW3	173	264	<LOD	264	<LOD
ErmB	4/29/2013	GW4	180	264	264	1512	840
ErmB	5/3/2013	E1	184	10434	264	26820	<LOD
ErmB	5/6/2013	GW5	187	500	264	1390	264
ErmB	5/13/2013	GW6	194	840	264	1581	264
ErmB	5/20/2013	GW7/E2	201	264	264	1497	<LOD
ErmB	5/27/2013	GW8/E3	208	<LOD	<LOD	2224	<LOD
ErmB	5/30/2013	E4	211	7148	636	4586	5499
ErmB	6/3/2013	GW9	215	<LOD	<LOD	264	<LOD
ErmB	6/5/2013	E5	217	788	<LOD	2146	<LOD
ErmB	6/10/2013	GW10	222	264	<LOD	264	<LOD
ErmB	6/17/2013	GW11	229	<LOD	<LOD	766	264
ErmB	6/24/2013	GW12	236	<LOD	264	<LOD	264
ErmB	7/1/2013	GW13	243	264	<LOD	264	<LOD
ErmB	7/8/2013	GW14	250	264	<LOD	<LOD	264
ErmB	7/15/2013	GW15	257			<LOD	

ErmC	4/15/2013	GW2	166		<LOD	12696	21618
ErmC	4/22/2013	GW3	173	<LOD	<LOD	<LOD	<LOD
ErmC	4/29/2013	GW4	180	<LOD	<LOD	<LOD	<LOD
ErmC	5/3/2013	E1	184	22716	<LOD	<LOD	<LOD
ErmC	5/6/2013	GW5	187	<LOD	<LOD	<LOD	<LOD
ErmC	5/13/2013	GW6	194	<LOD	<LOD	<LOD	<LOD
ErmC	5/20/2013	GW7/E2	201	<LOD	<LOD	<LOD	<LOD
ErmC	5/27/2013	GW8/E3	208	13448	<LOD	<LOD	105024
ErmC	5/30/2013	E4	211	407190	19887	16606	<LOD
ErmC	6/3/2013	GW9	215	<LOD	<LOD	<LOD	<LOD
ErmC	6/5/2013	E5	217	25992	8648	38479	8857
ErmC	6/10/2013	GW10	222	16323	17383	8611	173836
ErmC	6/17/2013	GW11	229	<LOD	8390	13099	7965
ErmC	6/24/2013	GW12	236	<LOD	<LOD	<LOD	<LOD
ErmC	7/1/2013	GW13	243	<LOD	<LOD	<LOD	<LOD
ErmC	7/8/2013	GW14	250	<LOD	<LOD	<LOD	<LOD
ErmC	7/15/2013	GW15	257			<LOD	
ErmF	4/15/2013	GW2	166		<LOD	3380	<LOD
ErmF	4/22/2013	GW3	173	1439	<LOD	1618	<LOD
ErmF	4/29/2013	GW4	180	456	<LOD	2388	<LOD
ErmF	5/3/2013	E1	184	1030	<LOD	<LOD	<LOD
ErmF	5/6/2013	GW5	187	<LOD	<LOD	1172	<LOD
ErmF	5/13/2013	GW6	194	456	<LOD	1127	<LOD
ErmF	5/20/2013	GW7/E2	201	456	<LOD	1289	<LOD
ErmF	5/27/2013	GW8/E3	208	N/A	<LOD	1668	<LOD
ErmF	5/30/2013	E4	211	456	<LOD	<LOD	<LOD
ErmF	6/3/2013	GW9	215	<LOD	<LOD	<LOD	<LOD
ErmF	6/5/2013	E5	217	N/A	<LOD	<LOD	<LOD
ErmF	6/10/2013	GW10	222	N/A	<LOD	<LOD	<LOD
ErmF	6/17/2013	GW11	229	N/A	<LOD	<LOD	<LOD
ErmF	6/24/2013	GW12	236	N/A	<LOD	<LOD	<LOD
ErmF	7/1/2013	GW13	243	N/A	<LOD	456	456
ErmF	7/8/2013	GW14	250	N/A	<LOD	<LOD	<LOD
ErmF	7/15/2013	GW15	257			<LOD	

Resistance genes in soil

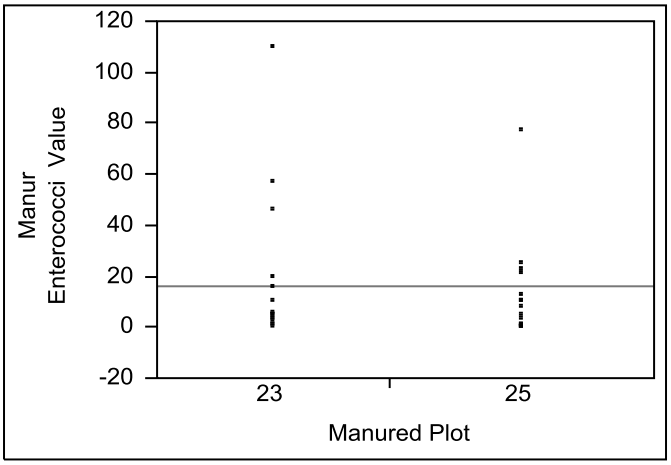
Sampling Period	Location	ErmB	ErmC	ErmF
Fall 2012	23 band A	63865	N/A	251351
Fall 2012	23 band B	54612607	1531372	2583092
Fall 2012	23 band C	123906622766	2929941	18807624
Fall 2012	23 interband A	3300	N/A	N/A
Fall 2012	23 interband B	64117	N/A	N/A
Fall 2012	23 interband C	3300	N/A	N/A
Fall 2012	24A	N/A	N/A	N/A
Fall 2012	24B	N/A	N/A	N/A
Fall 2012	24C	N/A	N/A	N/A
Fall 2012	25 band A	N/A	1185388	14116397
Fall 2012	25 band B	1730329	N/A	12939590
Fall 2012	25 band C	5932241	N/A	8788736
Fall 2012	25 interband A	N/A	N/A	40653
Fall 2012	25 interband B	N/A	N/A	N/A
Fall 2012	25 interband C	N/A	N/A	N/A
Fall 2012	34 A	N/A	N/A	N/A
Fall 2012	34 B	N/A	N/A	N/A
Fall 2012	34 C	N/A	N/A	N/A
Spring 2013	23 band A	66343	3488058	50608
Spring 2013	23 band B	266082	323872	366346
Spring 2013	23 band C	1366597	N/A	228082
Spring 2013	23 interband A	N/A	N/A	N/A
Spring 2013	23 interband B	3300	N/A	N/A
Spring 2013	23 interband C	N/A	N/A	N/A
Spring 2013	24A	98584	N/A	N/A
Spring 2013	24B	66050	N/A	61406
Spring 2013	24C	N/A	N/A	191251
Spring 2013	25 band A	183074	509722	13096
Spring 2013	25 band B	321942	577488	87479
Spring 2013	25 band C	385923	977551	181054
Spring 2013	25 interband A	N/A	N/A	228966
Spring 2013	25 interband B	N/A	N/A	N/A
Spring 2013	25 interband C	3300	180907	N/A
Spring 2013	34 A	96414	N/A	N/A
Spring 2013	34 B	N/A	1626420	N/A
Spring 2013	34 C	N/A	288272	N/A
Fall 2013	23 A	N/A	N/A	N/A
Fall 2013	23 B	N/A	N/A	N/A

Fall 2013	23 C	N/A	N/A	N/A
Fall 2013	24A	N/A	N/A	N/A
Fall 2013	24B	N/A	N/A	N/A
Fall 2013	24C	N/A	N/A	N/A
Fall 2013	25 A	57173	N/A	N/A
Fall 2013	25 B	22688	N/A	N/A
Fall 2013	25 C	24533	N/A	N/A
Fall 2013	34 A	69417	N/A	15761
Fall 2013	34 B	24214	N/A	N/A
Fall 2013	34 C	20529	N/A	N/A
Spring 2014	23 A	88617	N/A	N/A
Spring 2014	23 B	271526	N/A	143802
Spring 2014	23 C	158557	N/A	51571
Spring 2014	24A	N/A	N/A	N/A
Spring 2014	24B	N/A	N/A	N/A
Spring 2014	24C	N/A	N/A	N/A
Spring 2014	25 A	41817	N/A	N/A
Spring 2014	25 B	139273	N/A	33796
Spring 2014	25 C	3300	N/A	N/A
Spring 2014	34 A	N/A	N/A	N/A
Spring 2014	34 B	N/A	N/A	N/A
Spring 2014	34 C	N/A	N/A	N/A

APPENDIX B

STATISTICAL ANALYSIS

Enterococci No Till Manure verse Chisel Plow Manure



Missing Rows
30

Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

Level	Count	Score Sum	Expected Score	Score Mean	(Mean-Mean0)/Std0
23	14	234.000	217.000	16.7143	0.689
25	16	231.000	248.000	14.4375	-0.689

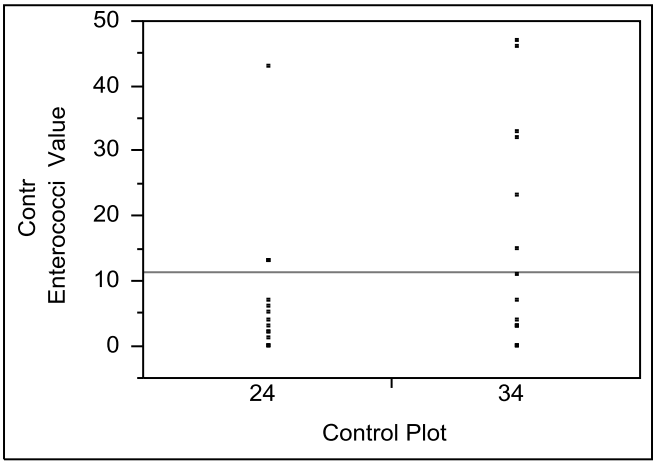
2-Sample Test, Normal Approximation

S	Z	Prob>|Z|
234	0.68868	0.4910

1-way Test, ChiSquare Approximation

ChiSquare	DF	Prob>ChiSq
0.5035	1	0.4780

Enterococci No Till Control verse Chisel Plow Control



Missing Rows
30

Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

Level	Count	Score Sum	Expected Score	Score Mean	(Mean-Mean0)/Std0
24	15	206.000	232.500	13.7333	-1.085
34	15	259.000	232.500	17.2667	1.085

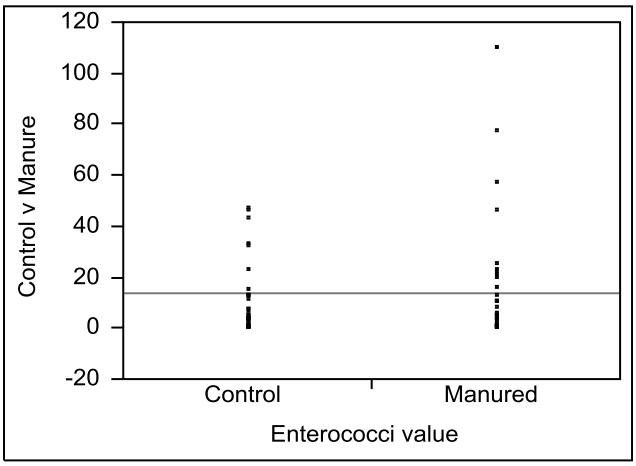
2-Sample Test, Normal Approximation

S	Z	Prob>|Z|
259	1.08472	0.2780

1-way Test, ChiSquare Approximation

ChiSquare	DF	Prob>ChiSq
1.2223	1	0.2689

Enterococci Combined Tillage Treatments, Manured verse Control Plots



Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

Level	Count	Score Sum	Expected Score	Score Mean	(Mean-Mean0)/Std0
Control	30	889.000	915.000	29.6333	-0.379
Manured	30	941.000	915.000	31.3667	0.379

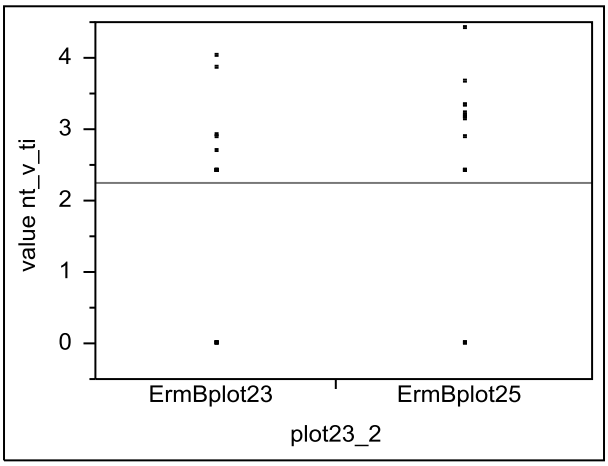
2-Sample Test, Normal Approximation

S	Z	Prob>|Z|
941	0.37868	0.7049

1-way Test, ChiSquare Approximation

ChiSquare	DF	Prob>ChiSq
0.1491	1	0.6994

ErmB No Till Manure verse Chisel Plow Manure



Missing Rows
32

Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

Level	Count	Score Sum	Expected Score	Score Mean	(Mean-Mean0)/Std0
ErmBplot23	16	228.500	272.000	14.2813	-1.582
ErmBplot25	17	332.500	289.000	19.5588	1.582

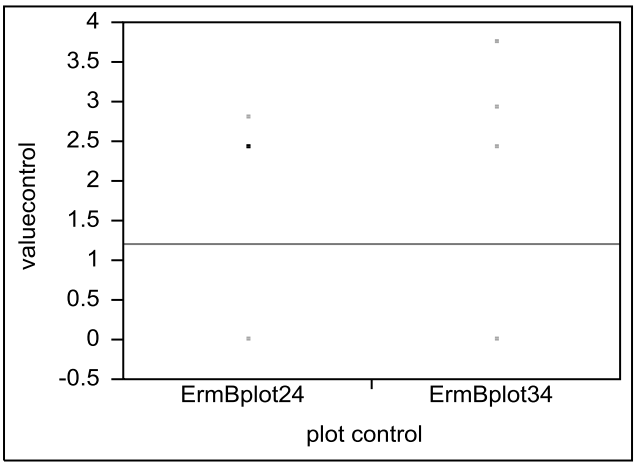
2-Sample Test, Normal Approximation

S	Z	Prob>|Z|
228.5	-1.58221	0.1136

1-way Test, ChiSquare Approximation

ChiSquare	DF	Prob>ChiSq
2.5619	1	0.1095

ErmB No Till Control verse Chisel Plow Control



Missing Rows
33

Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

Level	Count	Score Sum	Expected Score	Score Mean	(Mean-Mean0)/Std0
ErmBplot24	16	266.500	264.000	16.6563	0.084
ErmBplot34	16	261.500	264.000	16.3438	-0.084

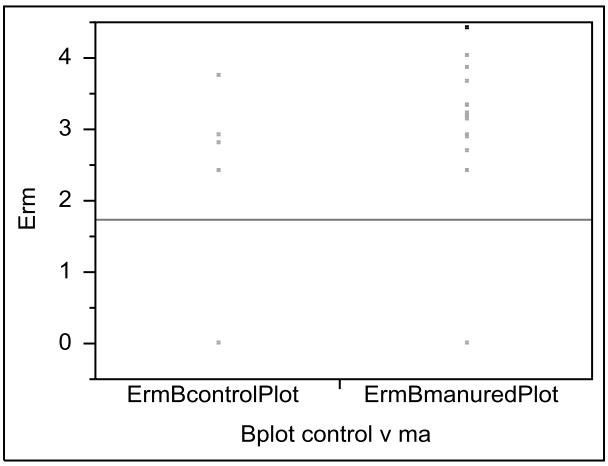
2-Sample Test, Normal Approximation

S	Z	Prob>|Z|
261.5	-0.08438	0.9328

1-way Test, ChiSquare Approximation

ChiSquare	DF	Prob>ChiSq
0.0111	1	0.9160

ErmB Combined Tillage Treatments, Manured verse Control Plots



Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

Level	Count	Score Sum	Expected Score	Score Mean	(Mean-Mean0)/Std0
ErmBcontrolPlot	32	823.000	1056.00	25.7188	-3.208
ErmBmanuredPlot	33	1322.00	1089.00	40.0606	3.208

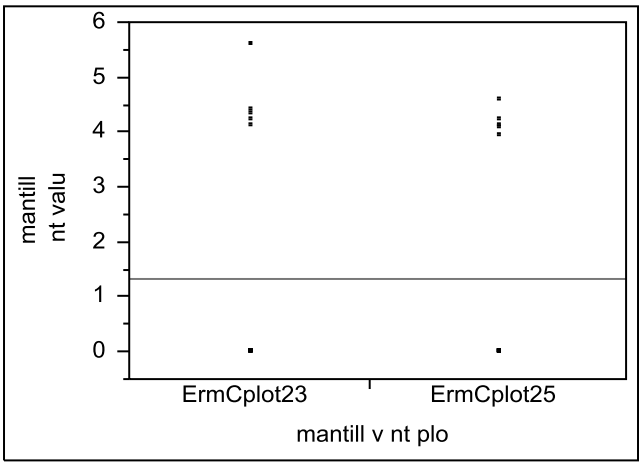
2-Sample Test, Normal Approximation

S	Z	Prob>|Z|
823	-3.20782	0.0013*

1-way Test, ChiSquare Approximation

ChiSquare	DF	Prob>ChiSq
10.3344	1	0.0013*

ErmC No Till Manure verse Chisel Plow Manure



Missing Rows
32

Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

Level	Count	Score Sum	Expected Score	Score Mean	(Mean-Mean0)/Std0
ErmCplot23	16	281.000	272.000	17.5625	0.376
ErmCplot25	17	280.000	289.000	16.4706	-0.376

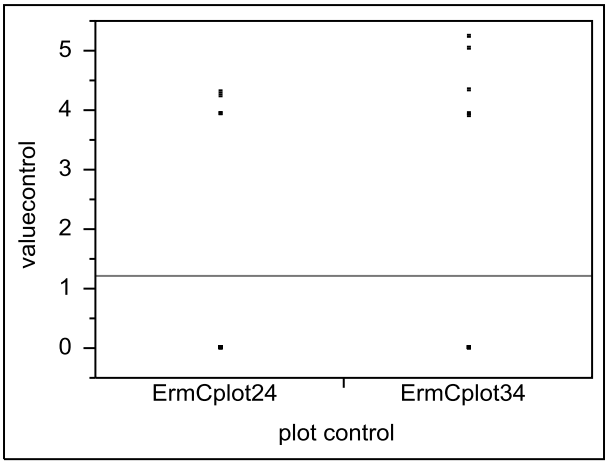
2-Sample Test, Normal Approximation

S	Z	Prob>|Z|
281	0.37639	0.7066

1-way Test, ChiSquare Approximation

ChiSquare	DF	Prob>ChiSq
0.1588	1	0.6902

ErmC No Till Control verse Chisel Plow Control



Missing Rows
33

Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

Level	Count	Score Sum	Expected Score	Score Mean	(Mean-Mean0)/Std0
ErmCplot24	16	252.000	264.000	15.7500	-0.546
ErmCplot34	16	276.000	264.000	17.2500	0.546

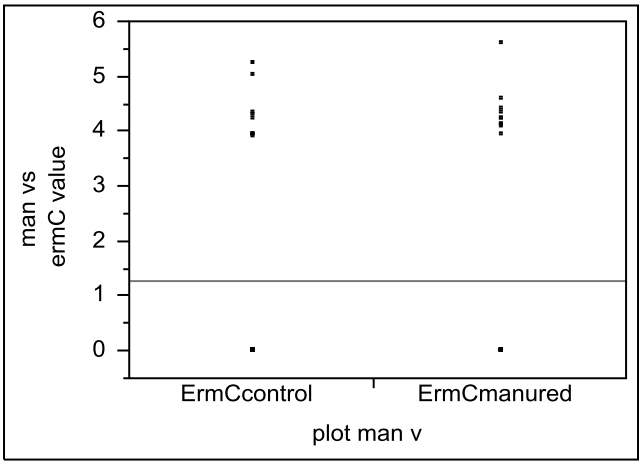
2-Sample Test, Normal Approximation

S	Z	Prob>|Z|
276	0.54648	0.5847

1-way Test, ChiSquare Approximation

ChiSquare	DF	Prob>ChiSq
0.3252	1	0.5685

ErmC Combined Tillage Treatments, Manured verse Control Plots



Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

Level	Count	Score Sum	Expected Score	Score Mean	(Mean-Mean0)/Std0
ErmCcontrol	32	1037.50	1056.00	32.4219	-0.294
ErmCmanured	33	1107.50	1089.00	33.5606	0.294

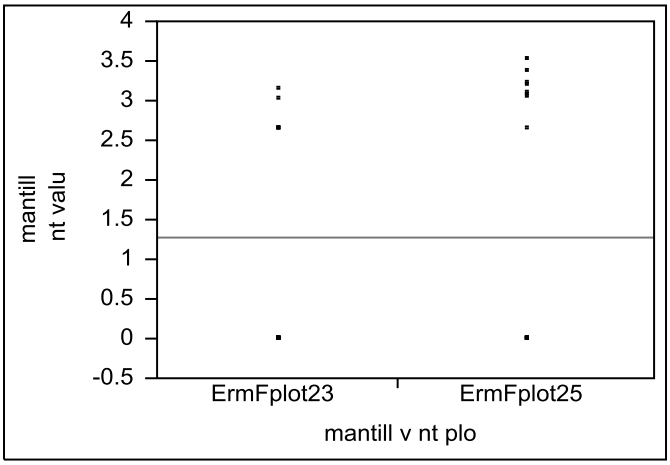
2-Sample Test, Normal Approximation

S	Z	Prob>|Z|
1037.5	-0.29394	0.7688

1-way Test, ChiSquare Approximation

ChiSquare	DF	Prob>ChiSq
0.0913	1	0.7626

ErmF No Till Manure verse Chisel Plow Manure



Missing Rows
32

Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

Level	Count	Score Sum	Expected Score	Score Mean	(Mean-Mean0)/Std0
ErmFplot23	16	242.000	272.000	15.1250	-1.184
ErmFplot25	17	319.000	289.000	18.7647	1.184

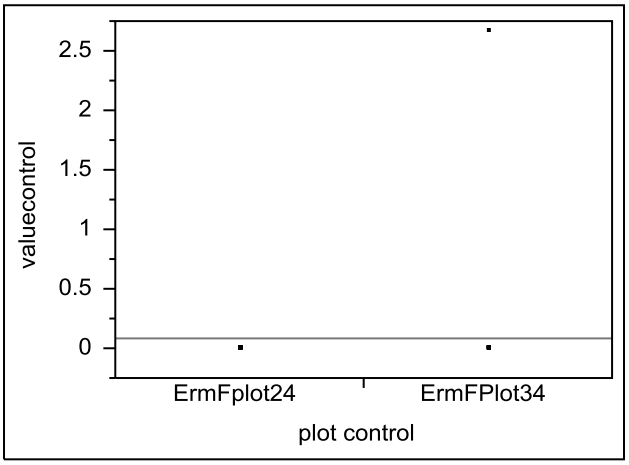
2-Sample Test, Normal Approximation

S	Z	Prob>|Z|
242	-1.18353	0.2366

1-way Test, ChiSquare Approximation

ChiSquare	DF	Prob>ChiSq
1.4486	1	0.2287

ErmF No Till Control verse Chisel Plow Control



Missing Rows
33

Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

Level	Count	Score Sum	Expected Score	Score Mean	(Mean-Mean0)/Std0
ErmFplot24	16	256.000	264.000	16.0000	-0.938
ErmFPlot34	16	272.000	264.000	17.0000	0.938

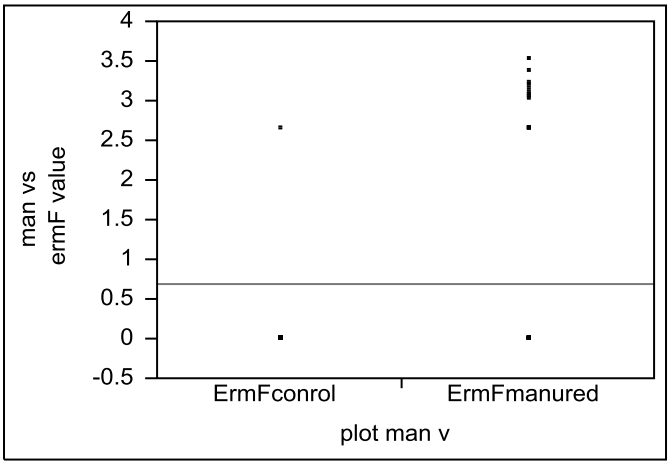
2-Sample Test, Normal Approximation

S	Z	Prob>|Z|
272	0.93750	0.3485

1-way Test, ChiSquare Approximation

ChiSquare	DF	Prob>ChiSq
1.0000	1	0.3173

ErmF Combined Tillage Treatments, Manured verse Control Plots



Wilcoxon / Kruskal-Wallis Tests (Rank Sums)

Level	Count	Score Sum	Expected Score	Score Mean	(Mean-Mean0)/Std0
ErmFconrol	32	844.000	1056.00	26.3750	-3.762
ErmFmanured	33	1301.00	1089.00	39.4242	3.762

2-Sample Test, Normal Approximation

S	Z	Prob>|Z|
844	-3.76219	0.0002*

1-way Test, ChiSquare Approximation

ChiSquare	DF	Prob>ChiSq
14.2211	1	0.0002*