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Occurrence and movement of total and tylosin-resistant enterococci, erm genes and tylosin in tile-drained agricultural fields receiving swine manure application

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Occurrence and movement of total and tylosin-resistant enterococci, *erm* genes and tylosin in tile-drained agricultural fields receiving swine manure application

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LIST OF ABBREVIATIONS

Abbreviation	Full Name
APHIS	Animal and Plant Health Inspection Service
ARB	Antibiotic Resistant Bacteria
ARG	Antibiotic Resistant Gene
ARS	Agricultural Research Service
CFU	Colony Forming Unit
CP	Chisel Plow
DNA	Deoxyribonucleic Acid
EPA	Environmental Protection Agency
erm	Erythromycin ribosome methylation
ERS	Economic Research Service
FDA	Food and Drug Administration
GC-MS	Gas Chromatography-Mass Spectrometry
HLB	Hydrophilic Lipophilic Balanced
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification
MIC	Minimum Inhibitory Concentration
MLS	Macrolide-Lincosamide-Streptogramin
NADA	New Animal Drug Information
NAHMS	National Animal Health Monitoring System
NT	No Till
PCR	Polymerase Chain Reaction
ppb	Parts per Billion
ppm	Parts per Million
PSA	Plot Set A

Abbreviation	Full Name
PSB	Plot Set B
qPCR	Quantitative Polymerase Chain Reaction
RBBC	Repeated Bead Beating Plus Column Extraction
SDS	Sodium Dodecyl Sulfate
SPE	Solid Phase Extraction
tet	*tetracycline*
TMDL	Total Maximum Daily Loads
UAN	Urea and Ammonium Nitrate
USDA	The United States Department of Agriculture
WHO	World Health Organization

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ABSTRACT

The use of tylosin at subtherapeutic levels by the swine industry provides selective pressure for the development of antibiotic resistance in gastrointestinal bacteria. The land application of swine manure to drained agricultural fields might introduce elevated levels of total and tylosin-resistant enterococci, *erm* genes and tylosin. The goal of this study was to develop an understanding of the occurrence and transport of total and tylosin-resistant enterococci, *erm* genes and tylosin in tile-drained chisel plow and no-till agricultural fields that have received multi-year application of liquid swine manure through injection over two growing seasons.

Resistance to tylosin in manure, soil and water samples was investigated at the field scale level using phenotypic based (membrane filtration) and genotypic based (qPCR) methods and compared with samples from control plots treated with urea and ammonium nitrate (UAN). Tylosin was quantified using LC-MS/MS. Plots in a corn-soybean rotation were identified for sampling from 2010-2012. Soil samples were collected from each manure plot, from both the direct area of injection and from the area between the manure bands and from control plots. Each one-acre plot is drained separately and tile water samples were collected directly from the discharge tile line weekly while the tiles were flowing. The results of this study suggest that tylosin usage has increased the short-term occurrence of total and tylosin-resistant enterococci, *erm* genes, and tylosin in soils, but has had minimal effect on tile drainage water quality under dryer than average conditions.

CHAPTER 1: GENERAL INTRODUCTION

1.1 Introduction

Antibiotic resistance is becoming a major concern on a global scale, even leading some members of the European Union to ban the use of agricultural antibiotics for growth promotion (Aarestrup et al., 2001). Both agricultural and human use of antibiotics potentially contributes to a population of antibiotic resistant organisms that might spread resistance to pathogenic bacteria. The practice of land application of swine manure provides large-scale introduction of antibiotics into the environment. Once released, the antibiotics are transported via soil particles and potentially into groundwater (Campagnolo et al., 2002; Chee-Sanford et al., 2009).

The antibiotic tylosin is a macrolide antibiotic used by the swine industry at therapeutic levels for disease treatment and at sub-therapeutic levels as prophylactic and growth promoting agents. This use provides selective pressure for the development of antibiotic resistance in gastrointestinal bacteria. The land application of swine manure to drained agricultural fields might accelerate the transport of tylosin-resistant enterococci and erythromycin ribosomal methylase (*erm*) genes. Tylosin is not completely metabolized in the pigs, and metabolites are excreted in manure (Teeter and Meyerhoff, 2003), which is typically applied to agricultural fields as fertilizer (Chee-Sanford et al., 2009). The presence of tylosin in waste pits and in manure amended soil can lead to selective pressures on antibiotic resistant bacteria (ARB). These selective pressures confer resistance on antibiotic resistant genes (ARG). Antibiotic resistant genes are most commonly carried on mobile genetic elements, which disseminate between microorganisms (Mazel and Davies, 1999; de la Cruz and Davies, 2000; Roberts, 2004). However, ARG are also mobilized by the processes of transduction and transformation (Ochman et al., 2000). Recent studies have suggested that swine manure can contribute significantly to the antibiotic resistance in the environment (Chee-Sanford et al., 2009; Chen et al., 2010; Koike et al., 2010).

Pathogens and pathogen indicators persist in the environment and provide a medium in which antibiotic resistance can reside. Enterococci are one such bacterium found in the intestinal tract of warm-blooded animals and in humans. There are two main pathogenic strains: *Enterococcus faecalis* and *Enterococcus faecium* (Franz et al., 1999; Shepard and

Gilmore, 2002). Enterococci are gram positive bacteria which are targeted by macrolides like tylosin. Enterococci play an important role as indicators of pathogens, for their use in regulatory standards (USEPA, 1986), and in past studies on bacteria transport (Soupir et al., 2006; Sapkota et al., 2007). Pappas et al. (2008) found higher concentrations of enterococci in tile water than *E. coli* or fecal coliform in a central Iowa study and Hoang (2010) reported high incidences in tile water.

Tylosin is structurally related to erythromycin, which is the most commonly prescribed macrolide-lincosamide-streptogramin (MLS) antibiotic in humans (Stephenson et al., 1997; Portillo et al., 2000). Both tylosin and erythromycin inhibit protein synthesis by binding to the 23S ribosomal RNA of the 50S subunit (Roberts, 2004). *Erm* genes are responsible for tylosin-resistance in bacteria and the *erm* class of genes is among the most commonly acquired genes conferring resistance to MLS antibiotics (Chen et al., 2007). In enterococci, MLS resistance is most commonly mediated by the *erm*B gene (Portillo et al., 2000). Tylosin use in animal production has been shown to lead to increased levels of erythromycin resistance in enterococci isolated from swine (Jackson et al., 2004b). Various e*rm* genes have been found in swine waste lagoons (Chen et al., 2007; Koike et al., 2010). Additionally, a wide variety of resistance genes are found naturally in soils, even in the absence of manure application (Schmitt et al., 2006; Allen et al., 2010). Tylosin has previously been detected in tile flow (Dolliver and Gupta, 2008a). However, no study has looked at the comprehensive release of enterococci, tylosin-resistant enterococci, *erm* genes, and tylosin from tile-drained fields receiving swine-manure application.

Land application of swine manure introduces excess enterococci, tylosin-resistant enterococci, *erm* genes, and tylosin into the environment. Between 25-35% of cropland in Iowa is artificially drained (Zucker and Brown, 1998). However, these highly developed drainage systems may facilitate pollutant transport to downstream water bodies. Additionally, high concentrations of confined swine operations resulted in the common use of swine manure in corn production. Antibiotics and bacteria move through the matrix, but can move faster in no-till fields versus chisel-plow plots via transport through macropores (Cullum, 2009). The quality of tile drainage water in highly drained areas, such as Iowa, is an important concern. Studies by Kanwar et al. (1999), Bakhsh et al. (2005), Malone et al.

(2007), and Lawlor et al. (2011), have found a strong correlation between nitrate transport, precipitation patterns and tile drain flow. Therefore, it is possible that antibiotics and antibiotic-resistant bacteria will also be related to precipitation and hydrology. Concentrations of pathogens reaching tile drainage during high flows have been reported (Dean and Foran, 1992; Joy et al., 1998; Hunter et al., 2000). Field experiments in drained conditions, combined with an assessment of tillage practices and crop rotation will address the issue of ARB, ARG and antibiotic transport.

Presently, there is insufficient information on antibiotic and resistance gene transport to tile waters under natural conditions. Previously, Hoang (2010) used PCR and membrane filtration to quantify tylosin resistance in *Enterococcus* from liquid swine manure, treated soil and tile drainage water under an artificial rainfall simulation. This study aims to extend the initial analysis by Hoang (2010) by performing a similar analysis under natural conditions over two study years and includes quantitative PCR and analysis of tylosin.

1.2 Goal and objectives

This study was developed to provide information vital to the pork producers of the United States on the fate of antibiotics and antibiotic resistance in surface water as a result of manure applied to drained agricultural fields. This information comes in light of recent suggestions that manure from animals treated with antibiotics to promote growth application is introducing unsafe levels of antibiotics and therefore increasing persistence of antibiotic resistance in the natural environment. The goal of this research project is to further understand the occurrence and movement of total and tylosin-resistant enterococci, *erm* genes and tylosin in tile-drained agricultural fields that have received multi-year application of liquid swine manure through injection.

The specific objectives of this study were to:

1. In liquid swine manure, soil and tile drainage water
    a. Quantify the occurrence of total and tylosin-resistant enterococci
    b. Quantify the occurrence of *erm*B, *erm*F and *erm*T
    c. Quantify tylosin

2. Compare occurrence of total and tylosin-resistant enterococci, *erm*B, *erm*F, *erm*T, and tylosin between no-till and chisel plow fields relative to controls.
3. Compare the quantity of total and tylosin-resistant enterococci, *erm*B, *erm*F, *erm*T, and tylosin in liquid swine manure, soil and tile drainage water over two years

1.3 Hypothesis

The following hypothesis were evaluated and tested during the course of this study:

1. The highest concentrations of total and tylosin-resistant enterococci, *erm* genes, and tylosin will be present in highest concentrations in the manure samples, followed by the soil samples and drainage water samples with the lowest concentration.
2. There will be higher concentrations of total and tylosin-resistant enterococci, *erm* genes, and tylosin in the fields with manure application when compared to the control plots without manure.
3. The tile drainage water samples will have a decreased concentration of total and tylosin-resistant enterococci, *erm* genes, and tylosin over the course of the drainage season.
4. Tile water from the chisel plow plots will have lower concentrations of total and tylosin-resistant enterococci, *erm* genes, and tylosin enterococci than the no-till plots.

1.4 Thesis organization

The objectives of this research project were met by the analysis of field samples in the laboratory. Chapter 2 of this document presents a literature review on the uses of antibiotics, the development of resistance, composition of the antibiotics, methods for detection and quantification, and presents results on previous findings on this topic. Chapter 3 presents a paper written for submission to a peer-reviewed journal. Chapter 4 provides general conclusions for this research project and includes recommendations for future research in this field of study.

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CHAPTER 2: LITERATURE REVIEW

The study of antibiotic-resistant bacteria and antibiotic resistance genes plays an important role in agriculture and in human health. Antibiotic use in swine increases the persistence of antibiotic resistance in the animal, and resistance excreted in the manure. When the manure is applied as a fertilizer, these antibiotic residues, antibiotic resistant genes, and/or antibiotic resistant bacteria may move from the liquid swine manure, through the soil and into subsurface drainage systems. Therefore, it is important to understand the uses of antibiotics in agriculture, important bacteria, the development of resistance in the environment, the composition of the antibiotics, the transport of antibiotic resistance, and the methods for detection and quantification. This literature review will provide an overview of these topics and how they relate to the present study.

2.1 Antibiotic resistance

Antibiotic resistance is the acquired ability of a microorganism to grow in the presence of an antibiotic to which the microorganism is typically sensitive. Antibiotic resistance is becoming a greater concern on a global scale, even leading some members of the European Union to ban the use of agricultural antibiotics (Aarestrup et al., 2001). Both agricultural and human use of antibiotics is potentially contributing to a population of antibiotic resistant organisms that might spread resistance to pathogenic bacteria. In agriculture, antibiotics are used in both subtherapeutic and therapeutic levels to promote growth and to treat infection respectively in livestock.

The introduction of antibiotics to the intestinal system of animals provides selective pressures for the development of extensive resistance, which is excreted in manure. Some levels of resistant bacteria occur naturally in manure from organic operations (Jindal et al., 2006) and in manure amended soil (Onan and LaPara, 2003). One of the greatest issues today in the use of antibiotics is not in the treatment of humans or animals for disease, but rather the use of antibiotics at a subtherapeutic level to promote growth and prevent disease (Jackson et al., 2004b; Dolliver and Gupta, 2008a; Heuer et al., 2011). These antibiotics and metabolites pass through without being completely metabolized, and are frequently detected feces from pigs (Mackie et al., 2006) and other animals such cattle. Antibiotic resistant

bacteria have been found in pigs from organic farms not administrating antibiotics; however, those under the administration of antibiotics discharge up to 70% more resistant organisms (Langlois et al., 1986). Kolpin (2002) reported that 50% of surface waters in the United States tested positive for some sort of antibiotic, which Dolliver and Gupta (2008a) attributed to the combination of both agricultural and human sources.

2.2 Antibiotic use in swine production

Antibiotics are commonly used to treat infections in humans and animals and are also used as a feed additive to promote growth in animals. Antibiotics have been used for treating infections in the medical practice for over 70 years, since the first clinical use of penicillin (Hawkey, 2000). The US Food and Drug Administration (FDA) approved the practice of adding small amounts of antibiotics to feedstock in the 1950's to allow producers to "grow animals faster on less feed" (Shea, 2003). This allows a shortened growth period by increasing growth rates and prevents disease in animals before they become severely ill, which increases the profitability for the producer (Shea, 2003). It is also considered to improve the overall quality of the meat with higher protein and lower fat content (Cromwell, 2002). This practice may also introduce antibiotics to surface water that are similar in chemical structure to those utilized in humans. This is a major issue because some of antibiotics used to treat infections and resistance genes in humans are naturally derived (Martinez, 2008). In itself, natural derivation is only a small part of the problem, as cross resistance is the underlying issue. As antibiotics become more prevalent in the environment, there becomes an increased chance that humans will be in direct contact with more antibiotics and antibiotic resistant pathogens, thus decreasing the effectiveness of antibiotics used to treat human illness. This is a serious health concern that stems from the use of antibiotics in agriculture.

In 2000, the World Health Organization (WHO) made the recommendation that antimicrobials currently used or are under development for human treatment be phased out as growth promoters in animals (WHO, 2000). In the United States, there are three categories of antibiotics used in agriculture: 1) feed antibiotics, 2) over-the-counter drugs, and 3) veterinary prescription drugs (Barton, 2000). By limiting the types and usage of antibiotics,

the goal set by WHO might be attainable. However, certain antibiotic types are chemically related, such that a ban of cross-usage will not explicitly solve this problem.

2.2.1 Antibiotic types and classifications

Antibiotics are antimicrobial compounds, derived either naturally or synthetically, that inhibits growth or kills bacteria or microorganisms. Most antimicrobials are produced by the genus *Streptomyces*, a microorganism found frequently in soil (Clewell, 2008). Antibiotics and antimicrobials differ in that some antimicrobials kill viruses. Antibiotics can be classified by either their chemical structure or the mechanism of action (Kümmerer, 2009). There are many subgroups of antibiotics including but not limited to aminoglycosides, beta-lactams, quinolones, tetracyclines, macrolides, oxazolidinones, and sulfonamides (Kümmerer, 2009).

Antibiotics, as defined by Morley, are "compounds produced by living organisms that impede the growth of other organisms" (2005). Antibiotics work by targeting the growth processes of the cell. The most selective antibiotics are those which affect the cell wall or the chemical structure of the cell. Most antibiotics are effective at treating only gram-positive (i.e. enterococci) or gram-negative bacteria (*E. coli*), but not both. Therefore, there is an inherent need to have an indicator organism match the type of the antibiotic in question. Antibiotics are separated into categories, or classes, based on their target, shown in Figure 1. The specific categories include 1) inhibition of cell wall synthesis; 2) inhibition of folic acid metabolism; 3) alteration of the cytoplasmic membrane structure; 4) inhibition of DNA gyrase; 5) inhibition of DNA-dependent RNA polymerase; 6) inhibition of protein synthesis.

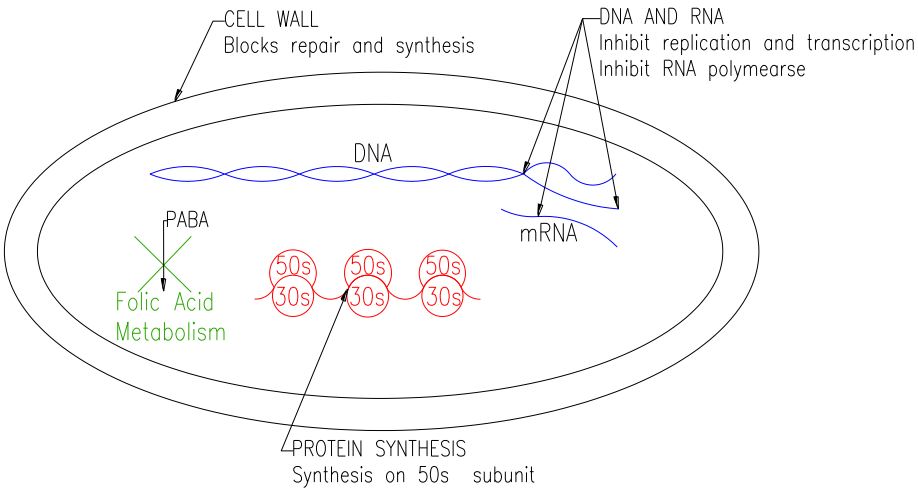


Figure 1: Mode of action of major antimicrobial agents modified after Madigan et al. (2000)

The macrolide antimicrobial class is one of the most common used in swine production and are also used in humans. Macrolides fall under the larger superfamily of antibiotics macrolide-lincosamide-streptogramin (MLS). Macrolides are either bacteriostatic (halts bacterial reproduction) or bactericidal (destroys bacteria). Macrolide antibiotics, such as erythromycin, tylosin, kitasamycin, josamycin, oleandomycin, spiramycin are effective by inhibiting protein synthesis in Gram-positive bacteria (Barton, 2000). They work by inhibiting protein synthesis by binding to sites on the 23S subunit which is a component of the 50S submit of the 70s ribosome of bacteria (Ōmura, 2002).

For many years, tylosin (in animals) and erythromycin (humans) have been used to treat staphylococcal and streptococcal infections (Gilmore, 2002). Due to the close genetic properties between tylosin and erythromycin, resistant *Staphlococcus aureus* cells, after being exposed to a low concentration of erythromycin, for example, show resistance to other macrolides (Ōmura, 2002). This is a major cause for concern for human health as this cross-resistance between macrolides used in swine and other food animals may also have major resistance implications in humans. Macrolides are an alternative for use in patients with a penicillin allergy and are used in humans to treat pneumonia and *Legionella*. Most gram-negative bacteria are intrinsically resistant to macrolides (Ōmura, 2002).

Kümmerer and Henninger (2003) reported that there were nearly 250 different chemical compounds authorized for use in human and veterinary medicine. Estimates from the mid 1990's show that more than 500 metric tons of antimicrobials were produced each year, with 11% being macrolides (Madigan et al., 2000). Other annual estimates have the value on the range of 100-200 metric tons (Wise, 2002). Since the 1940's, estimates have indicated that over 1 million metric tons of antibiotics have been produced (Andersson and Hughes, 2010). While antibiotics have saved countless lives, such large quantities are bound to lead towards selective pressure, which will have a major impact on society.

2.2.2 Antibiotic use in swine

Starting in the 1940's, it was realized that antibiotics could be administered in animal production to treat diseases and promote growth (Aarestrup and Carstensen, 1998). Antibiotics are administered at a therapeutic level to treat disease in sick animals by injection or by adding an appropriate dose to the feed or the water. Antibiotics can also be used to promote growth, increase feed efficiency and ward off disease when used at a subtherapeutic levels (Cromwell, 2002).

There are four main types of antibiotic usage in swine production. Those types include therapeutic, metaphylactic, prophylactic, and subtherapeutic (McEwen and Fedorka-Cray, 2002). Therapeutic antibiotics are used to treat disease in individual animals or groups via injection, in feed, or in water. Metaphylactic use is for mass medication of a group of swine to eliminate or minimize an expected spread of disease and can also be administered through injection, feed, or in water. Prophylactics are given in feed to prevent disease. Finally, subtherapeutic antibiotics are used to facilitate growth promotion, improve feed efficiency and minimize disease within the herd (McEwen and Fedorka-Cray, 2002).

The use of antibiotics in swine growth promotion includes 90% of starter feeds, 75% of grower feeds, and 50% of finisher feeds because of the immense economic value to the producer (Cromwell, 2002). Antibiotics improved the growth rate in the swine by an average of 16.4% in young pigs while improving the feed efficiency by nearly 7% (Cromwell, 2002). For larger hogs, growth rate increased by 10.6% while improving the feed efficiency by 2% (Cromwell, 2002). The same study also evaluated the long term effects of administering antibiotics by comparing the effective growth benefit for the first time period with the

second. Data was collected and compared from 1950-1977 and from 1978-1985. The results shows that there was no overall loss in effectiveness during the 35 years (Cromwell, 2002).

Estimates from the late 1990's indicated that nearly 10.4 million pounds of antibiotics were used subtherapeutically in roughly 90 million swine per year (Mellon et al., 2001). Of that 10.4 million pounds, 12.1% were used in starter feeds, 17% in feeding and 70.3% in the finishing stages (Mellon et al., 2001). The Center for Disease Control found that antibiotics in feed for both livestock and poultry was connected to antibiotic resistant bacteria in humans (Holmberg et al., 1984). This was one of the first studies to report on the link between subtherapeutic antibiotic use and human health concerns. Since then, hundreds of studies report on findings of antibiotic resistance in water, with one of the main sources being from agricultural waste.

Table 1 lists some common antibiotics approved by the FDA and modeled after Cromwell (2002) and Hoang (2010).

The United States Department of Agriculture (USDA) Animal and Plant Health Inspection Service (APHIS) National Animal Health Monitoring System (NAHMS) conducted an extensive survey of swine producers in 2006. It was estimated that 55% of finishing operations administered an antimicrobial or feed additive for growth promotion with the most common products being chlortetracycline (52%), tylosin (44%), bacitracin (29%), and ractopamine (28%) (APHIS, 2007). This estimate is lower than estimates by Mellon (2001). The recommended dosage for treatment of swine dysentery from the NADA (New Animal Drug Application) is 250 mg tylosin tartrate/gallon of water for 3-10 days followed by 40-100 g/ton feed for 2-6 weeks (NADA, 2008). Similarly, subtherapeutic rates ranging from 7-140 g/ton feed have been reported (Khachatourians, 1998). The FDA (2006) maximum concentration in the uncooked edible tissue of poultry, cattle & swine is 0.2 ppm.

Table 1: Antibiotics approved for uses in swine feed by FDA

Antimicrobial	Class	Subtherapeutic rate	Trade name
		Antibiotics	
Apramycin	Aminoglycoside	150 g/ton	Apramycin
Bacitracin Methylene Disalicylate	Bacitracin	45-90 g/ton	Coban, BMD
Bacitracin Zinc	Bacitracin	10-50 g/ton	Albac
Bambermycin	Bambermycin	2-4 g/ton	Flavomycin
Chlortetracycline	Tetracycline	10-50 g/ton	Aureomycin
Lincomycin	Lincosamide	20 g/ton	Lincomix
Neomycin	Aminoglycoside	NA	Neomycin
Oxytetracycline	Tetracycline	10-50 g/ton	Terramycin
Penicillin	B-lactam	10-50 g/ton	Penicillin
Tiamulin	Diterpene	10 g/ton	Tiamutin
Tylosin	Macrolide	20-100 g/ton (starter)	Tylan
		20-40 g/ton (grower)	Tylan
		10-20 g/ton (finisher)	Tylan
Virginiamycin	Streptogrammin	5-10 g/ton	Stafac
		Chemotherapeutics	
Arsanilic acid	Arsenical	10-30 g/ton	
Carbadox	Quinoxaline	10-25 g/ton	Mecadox
Roxarsone	Arsenical	22.7-34.1 g/ton	3-Nitro
Sulfamethazine	Sulfonamide	100 g/ton	Sulfamethazine
Sulfathiazole	Sulfonamide	100 g/ton in combination with chlortetracycline	Sulfathiazole

Source: Modeled after Hoang (2010) and Cromwell (2002))

2.2.3 Economics of antibiotic ban in swine

While antibiotic use has significant benefits to productivity and health of swine in growth operations, following the lead of some European nations, some are calling for a ban on subtherapeutic dosing of antibiotics. The European Union has prohibited in agricultural use, subtherapeutic antibiotics that are also key in human medicine, such as penicillin, tetracycline, and streptogramin (Mellon et al., 2001). Estimates have shown that there are over 13 million pounds of antibiotics used in the United States in cattle, swine, and poultry each year that are currently banned in Europe (Mellon et al., 2001). These include

chlortetracycline, bacitracin, tylosin, oxytetracycline, sulfathiazole, sulfamethazine, penicillin, lincomycin, and apramycin. While some beleive that a similar ban in the United States is necessary to preserve the quality of food products and the environment, there are significant economic repercussions to such a ban.

Studies have evaluated the economic impact of banning the use of antibiotics and the effect it would have on the consumer. The USDA Economic Research Service (ERS) lead investigator McBride noted that there was an economic impact of $4.50 per head, or about a 4.5% increase in the cost of production in the first year after a ban (2006). A similar study also estimated the increase in cost to be $4.50 per head, or a total of $700 million over ten years for the entire US pork industry (Hayes and Jensen, 2003). An increase in net return of nearly $3 per pig was estimated when antibiotics were used for growth promotion (Cromwell, 2002). Brorsen (2002) noted that a ban would cost over $240 million annually, impacting both the consumers and the producers (2002). Brorsen's data was based on the cost difference in the 5% improvement in the Feed:Gain (F:G) ratio at a commercial farm and 4.57% improvement in the F:G at a dirt lot facility using tylosin as a growth promoter.

European studies may provide some insight following the ban of antibiotics in the late 1990's. The reduction of vancomycin/apramycin resistance levels in enterococci reduced only slightly immediately following the ban (Aarestrup et al., 2001), and resistance determinants have been found to persist at detectable levels in the 12 years following (Johnsen et al., 2009). While current US practices allow the use of antibiotics, it is likely that even in the event of a ban, levels of antibiotics, antibiotic resistant bacteria and antibiotic resistant genes are expected to be found in the environment in elevated levels for years following.

2.3 Enterococci

Enterococci are commensal bacteria found in the intestinal tract of humans and warm blooded animals. They are used as indicators of pathogens in regulatory standards, and are widely used in studies on bacteria transport and survival. Enterococci are gram-positive cocci found in pairs and in short chains (Gilmore, 2002). The most frequently isolated species are *Enterococcus faecalis* and *Enterococcus faecium* (Gilmore and Ferretti, 2003). *E. faecalis* is considered to be more prevalent as a pathogen than *E. faecium* (Amyes, 2007). Enterococci

are more numerous in the intestinal tract during early life, and play a more minor, yet important role in adult humans (Gilmore, 2002) by helping with food digestion. Enterococci grow in a broad range of temperatures (10-60°C), media with high salinity (6.5% sodium chloride), and in broad pH ranges (neutral up to 10) (Gilmore, 2002). Enterococci are facultative anaerobes and have an optimum growth temperature of 35°C but can grow within the range of 10-45°C (Gilmore, 2002). Cools (2001) showed that enterococci are hardy and persistent in the soil. Enterococci were more persistent than *E. coli*, in soil, except for in sandy soil at 25°C; however, enterococci survived best in the loamy soil (Cools et al., 2001). Enterococci are used in cheese making and are also a probiotic that can be used to assist with the microbial balance in the human or animal intestine (Franz et al., 1999).

Recently, studies have evaluated the concentration on enterococci in environmental samples. Enterococci have been reported in surface water (35 CFU/100 mL up gradient, 610 CFU/100 mL down gradient) and in groundwater (18 CFU/100 mL up gradient, 85 CFU/100 mL down gradient) located close to swine confinement operations (Sapkota et al., 2007). Enterococci have also been found in pastureland runoff receiving manure application of up to 1.19×10^5 CFU/100 mL (Soupir et al., 2006). The species of enterococci found from over 1,400 isolates from urban runoff, bay, ocean and sewage water samples was determined in California (Moore et al., 2008). The five main species isolated found were *E. faecalis*, *E. faecium*, *E. hirae*, *E. casseliflavus*, and *E. mundtii* with a frequency ranging from 7-36% (Moore et al., 2008). Enterococci can also be found in the environment from municipal wastewater discharges, leachate from septic tanks and wildlife deposition.

2.3.1 Indicator bacteria

Indicator bacteria have been used since the start of the twentieth century to assess fecal contamination from warm-blooded animals (Maier et al., 2009) since it would be both costly and inefficient to look for all pathogens. These bacteria must be easily isolated and quantified using simple laboratory methods. The detection of indicator bacteria suggests that fecal contamination has occurred and that there is a chance for the occurrence of enteric pathogenic bacteria as well. There are several criteria that are essential for the selection of an indicator bacteria like enterococci. These are represented in the following list, as quoted by Maier, Pepper and Gerba, 2009:

1. The organism should be useful in all types of water
2. The organism should be present when enteric pathogens are present
3. The organism should have a longer survival time longer than that of the hardiest enteric pathogen
4. The organism should not grow in water
5. The testing method should be easy to perform
6. The density of the organism should relate to the degree of fecal pollution
7. The organism should be present in the intestinal tracts of warm-blooded animals

In 1986, the U.S. Environmental Protection Agency recommended enterococci for use as an indicator of fecal contamination in water systems in the United States (USEPA, 1986; Halliday and Gast, 2011). Enterococci was noted to have a direct relationship to swimming-related illness in freshwater (USEPA, 1984) and in ocean water (Moore et al., 2008). Finding enterococci in freshwater or marine water directly correlates the risk of gastrointestinal illness associated with swimming with the enterococci density (USEPA, 2006). Each state is required to develop their own monitoring and regulation plans for indicator bacteria and create plans for Total Maximum Daily Loads (TMDLs) to ensure water quality.

The EPA Water Quality Standard for recreational water samples is <33 cfu/100 mL for a 30 day geometric mean and for marine water <35 cfu/100 mL (USEPA, 1986). The regulation also dictates that "no sample should exceed a one sided confidence limit calculated using the following as guidance" for freshwater and marine water, listed in Table 2, below. These means and single sample densities are based on an acceptable swimming associated gastroenteritis rate per 1,000 swimmers of 8 for freshwater and 19 for marine water.

Table 2: Criteria for Indicator for Enterococci Bacteriological Densities

	Freshwater (CFU/100 mL)	Marine Water (CFU/100 mL)
Geometric Mean[1]	33	35
Designated bathing beach[2]	61	104
Moderate use for bathing[2]	89	158
Light use for bathing[2]	108	276
Infrequent use for bathing[2]	151	500

[1] Steady-State geometric Mean Indicator Density
[2]Single Maximum Allowable Density

2.3.2 Human health and pathogenic species

There are over 32 distinct species of *Enterococcus* present to date. In both human and animal gastrointestinal tracts, there are two main pathogenic species of enterococcus that are cause for concern: *E. faecalis* and *E. faecium*. Both are gram-positive, commensal bacteria which are harmful because of their negative effects on the gastrointestinal and urinary tracts of humans and mammals. In some cases, *E. faecalis* and *E. faecium* can be considered opportunistic pathogens targeting those with weakened immune systems (Franz et al., 1999). Some cases can lead to severe illness or death. Many consider *E. faecalis* to be more prevalent than *E. faecium* (Amyes, 2007).

Both *E. faecium* and *E. faecalis* species are potentially resistant to antibiotics used in treatment. Both of these species are the cause of a majority of clinical enterococcal infections found in humans (Moellering, 1992; Mundy et al., 2000; Shepard and Gilmore, 2002). The ability of these two species to acquire mobile genetic elements has contributed to their emergence as a leading hospital pathogen (Palmer et al., 2010). Clinical isolates tested from 1995 to 1997 identified *E. faecalis* nearly 80% of the time and *E. faecium* in the remaining 20% (Huycke et al., 1998). Current studies indicate that there has been an increase in *E. faecium* in clinical isolates (Mundy et al., 2000). Enterococci were found to be the leading cause of surgical site infection in intensive care units in the 1990's (Richards et al., 2000). Furthermore, *E. faecium* and its transfer from swine to humans has been blamed as a leading cause of disease and death in a 1998 outbreak in China (Lu et al., 2002). At that time, thousands of pigs died from hemorrhagic shock caused by *E. faecium*, which caused severe illness in 40 humans, killing 12.

Enterococci also play an important role in food production and in medicine. There are a few select cheeses (artisanal cheeses from southern Europe) and fermented milk products that utilize enterococci in their production (Franz et al., 1999). Additionally, enterococci can be used as probiotics to improve the microbial balance in the gut or to treat gastroenteritis (1999).

Enterococci have been found in food supplies, which is cause for concern with regard for human health. Most of these instances are a result of food coming in contact with fecal matter. Due to the tolerance of enterococci to temperature, pH and salinity, enterococci may

survive food handling processes if proper precautions are not in place. The antibiotic resistance profiles of 185 Enterococcus isolates from fresh produce including celery, cilantro, mustard greens, spinach, collards, parsley, dill, cabbage and cantaloupe which were harvested in the southwestern United States were evaluated by Johnston and Jaykus (2004). From those isolates, 52% were positive for E. *faecium*, 21% were *E. faecalis*, and the remaining 27% were other *Enterococcus* species. The detection of antibiotic resistant Enterococcus in raw meat from antibiotic-treated animals is low (Garofalo et al., 2007). The National Research Council estimated that humans were at low risk from drug residues in food (1999).

2.4 Mechanisms of antibiotic resistance in enterococci

Antibiotic resistance is a growing public health concern in both the United States and around the world. The concern stems from the method of which antibiotics are used in both human medicine and agriculture. Enterococci have multiple pathogenic species, so it is imperative to evaluate the implications of antibiotic resistance in these bacteria (Portillo et al., 2000; Aarestrup et al., 2001). In order to effectively do so, it is important to understand the mechanisms of resistance that drive the resistance patterns. Evidence shows enterococci are becoming more resistant to antibiotics and have acquired resistance genes (Aarestrup and Carstensen, 1998; Portillo et al., 2000; Jackson et al., 2004a; Boerlin et al., 2005; Pei et al., 2006; Storteboom et al., 2007; Bockelmann et al., 2009; Chee-Sanford et al., 2009; Chen et al., 2010; West et al., 2010; Graham et al., 2011; Heuer et al., 2011).

The development of resistance to antibiotics is part of the evolutionary response of organisms in the presence of selective pressure. The origin of antibiotic resistance genes is thought to evolve in antibiotic producing bacteria and protect such bacteria from the antibiotics produced by other microorganisms naturally (Hawkey, 2000). Antibiotic resistance patterns seen today are driven largely by human use of antibiotics (Hawkey, 2000). There are two mechanisms of antibiotic resistance in enterococci including acquired bacterial resistance and intrinsic resistance.

Enterococci have a large number of inherent and acquired resistance traits which can easily be transferred to other enterococci (Gilmore, 2002). Antibiotics and ARGs in the environment are both from animal and human sources (Chee-Sanford et al., 2001; Kolpin et

al., 2002; Kümmerer and Henninger, 2003). When a population of microorganisms is exposed to antibiotics, there is an increased chance that genetic modification will occur, and resistance will spread. Exposure to antibiotics is a major driving factor in the selective pressure for the development of antibiotic resistance. Over time, the resistance genes will dominate in the microbial population and render the antibiotic useless. This process occurs naturally in the environment (Zhang et al., 2009) and in the gut of warm-blooded animals (Schjorring and Krogfelt, 2011).

2.4.1 Intrinsic resistance: natural selection and mutational resistance

Intrinsic resistance is a function of both natural selection and mutational resistance in an organism. In some cases, the organism might be resistant to that antibiotic because the change in the target site of a particular organism. Mutation is the change of a genomic sequence of the bacteria to one that is resistant to antibiotics (Martinez and Baquero, 2000; Martinez, 2008). The change in genetic structure is manifested by altering the ribosome structure by the degrading enzymes. It has been shown that antibiotic resistance occurs naturally, even without the exposure to the antibiotics that the microorganism is resistant to (Jindal et al., 2006; Storteboom et al., 2007; Allen et al., 2010). In the natural environment, the concentration of antibiotic resistance genes and resistant bacteria should be at a minimum without selection pressure, or the external forces promoting the spread. Enterobacterial strains collected from 1917-1952 were evaluated during ‘pre-antibiotic’ conditions (Hughes and Datta, 1983) and found low abundance of resistance genes, which was confirmed in a study evaluating long-term trends in enteric bacteria (Houndt and Ochman, 2000). Mutation resistance involves the spontaneous change in bacterial genetic material. The development of antibiotic resistance involves multiple unique genes because of the differences in target, modes of access, or the pathways of protection against antibiotics in the bacterial cell (Martinez and Baquero, 2000). Resistance modes are shown in Figure 2.

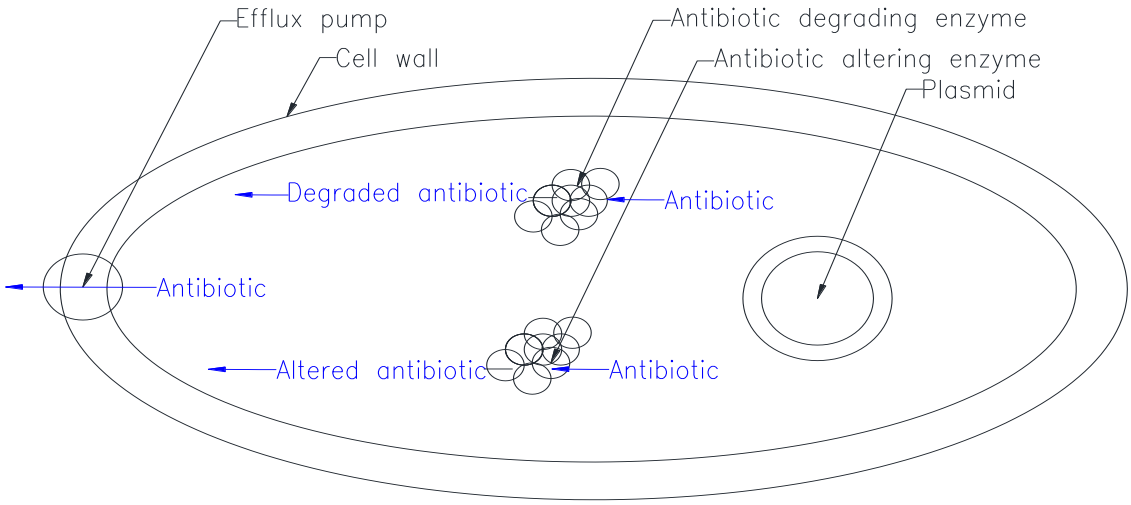


Figure 2: Mechanisms of antibiotic resistance modeled after Allen et al. 2010

The target gene can be made inaccessible to the antibiotic if there is a lack of an adequate transport system within the microorganism. Without an adequate transporter of the antibiotic, the antibiotic fails to reach its target. The administered drug can also be inactivated or altered by enzymes within the cell, typically by the modification of the target site. Target modification alters the binding site of the antibiotic (Tenover, 2006). In macrolides, the 23S rRNA is altered, a modification that can confer to cross-resistance to macrolides (Fluit et al., 2001). Additionally, gram-negative bacteria have a reduced permeability or uptake due to a unique cell wall that can establish a low permeability layer which prevents the antibiotic from entering. Multidrug efflux pumps are proteins that are responsible for the extrusion of antibiotics outside of the cell. By pumping the antibiotic out of the cell, the intracellular concentration of the antibiotic remains relatively low (Hawkey, 2000; Fluit et al., 2001). Enzymes degrade the antibiotic so that it is unable to reach the target site, and thus becomes ineffective (Wilke et al., 2005). The permeability of the cell can also be altered such that the antibiotic is unable to enter the cell. The plasmid can survive in the presence of antibiotics, which when transported between cells and pass along this altered genetic element. Gram positive bacteria produce enzymes within the cell that can be altered and inactivate the macrolide antibiotics (Hawkey, 2000) .

2.4.2 Acquired resistance and horizontal gene transfer

Acquired resistance is the modification of existing genetic material or the acquisition of new genetic material from another source. Horizontal gene transfer is the process where resistance gene DNA is transferred between individual bacteria of the same or different species Horizontal gene transfer and is a significant cause for the propagation of antibiotic resistance in animals and humans (de la Cruz and Davies, 2000). Resistance genes primarily transfer between bacteria of the same species (Morley et al., 2005), although horizontal gene transfer can also occur between different species.

There are three mechanisms of horizontal gene transfer, including: 1) transduction; 2), transformation; or 3) conjugation (Hawkey, 2000; Ochman et al., 2000). Transduction is the process by which bacteriophages transfer DNA between two similar bacteria. Transformation is the process by which DNA is taken up from the external environment from bacteria as a result of cell lysis or death. Conjugation is the process by which plasmids, transposons or other mobile genetic elements are transferred between two bacteria from cell contact. Conjugation is thought to be the most prevalent mechanism of horizontal gene transfer in humans and animals (Mazel and Davies, 1999; de la Cruz and Davies, 2000).

The most common mechanism for acquired resistance in enterococci to antibiotics such as tylosin and erythromycin is due to a reduced antibiotic binding to the 23S ribosomal RNA of the 50S ribosomal subunit (Jensen et al., 1999). In enterococci, this process is most commonly mediated by the *erm*B gene (Portillo et al., 2000) which signifies its importance in antibiotic resistance studies in this bacteria. Additionally, the *mef*A gene has been found to encode the efflux pump and impairs the function to ‘pump out’ the antibiotic (Tait-Kamradt et al., 1997). The genetic material surrounding the efflux system can be carried on plasmids, which can contribute to the intrinsic resistance within the cell.

Another issue in resistance acquired by horizontal gene transfer is that of co-selection. Co-selection is due to the genetic linkage between resistance genes of a similar structure (Andersson and Hughes, 2010). Co-selection may also occur when a drug confers resistance to other drugs with a similar structure (Andersson and Hughes, 2010) such as the macrolides tylosin and erythromycin. This type of horizontal gene transfer is problematic in human medicine and in agriculture when different variants of the same drug are used

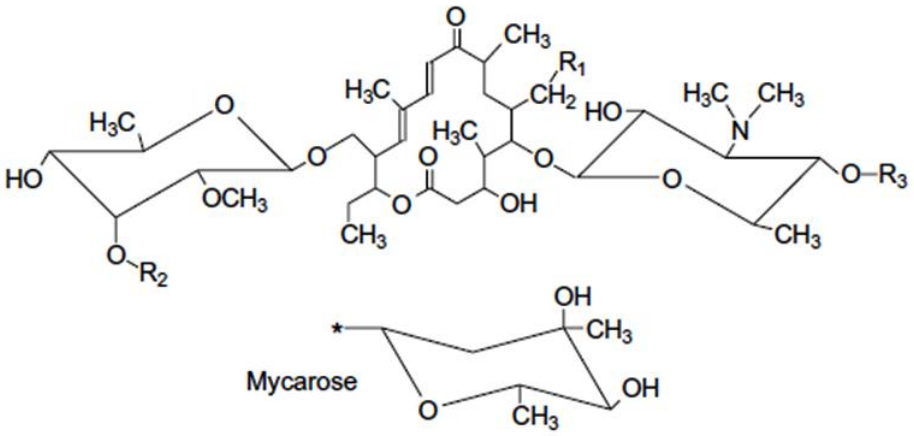
(Andersson and Hughes, 2010). Horizontal gene transfer of antibiotic resistant genes through enterococci has been reported (Palmer et al., 2010). Therefore, the frequency of resistance in one environment, such as in agriculture, could lead to increased susceptibility in human medicine.

2.5 Tylosin and tylosin resistance

2.5.1 Tylosin

Tylosin belongs to the MLS (macrolide-lincosamide-streptogramin) superfamily of antibiotics which are antibiotics that are structurally distinct, yet functionally related (Chen et al., 2007). Tylosin is strictly used on food animals and not intended for human use (Mellon et al., 2001).Tylosin is produced by *Streptomyces fradiae in* a fermentation process and is active mostly against gram-positive bacteria including *Enterococcus ssp.* (Sarmah et al., 2006). It is used in veterinary medicine therapeutically to treat disease and as a feed additive to promote growth in swine, cows and poultry. When used subtherapeutically, tylosin improved feed efficiency and increased the rate at which weight was gained in all stages of production (Mellon et al., 2001). Tylosin consists of a 16-membered lactone ring, amino sugar and two neutral sugars. Tylosin is composed of a mixture of four macrolides including Tylosin A, Tylosin B (desmycosin), Tylosin C (macrocin), and Tylosin D (relomycin) as shown in Figure 3.

Tylosin A is the main component making up over 80% of the compound (Horie et al., 1998; Loke et al., 2002). Tylosin is stable in neutral pH conditions (Sarmah et al., 2006), has high solubility (Sarmah et al., 2006), biodegradable (Hu and Coats, 2007; Hu and Coats, 2009), and is highly sorbed to manure and soils (Loke et al., 2002; Clay et al., 2005; Kolz et al., 2005a; Kolz et al., 2005b; Strock et al., 2005; Sassman et al., 2007; Hu and Coats, 2009). Due to its high water-solubility and positive ionic charge, tylosin binds to the negatively charged soil and manure particles and does not form complexes with metal ions (Loke et al., 2002). Kolz (2005b) summarizes some reported parameters in soil and swine manure including pH, organic content, partition coefficient, organic carbon sorption coefficient value, and specific values noted in literature.



	Tylosin A	Tylosin B	Tylosin C	Tylosin D
R_1	-CHO	-CHO	-CHO	$-CH_2OH$
R_2	$-CH_3$	$-CH_3$	-H	$-CH_3$
R_3	-Mycarose	-H	-Mycarose	-Mycarose

Figure 3: Chemical Structure of Tylosin modeled after Kolz et al. 2005a.

The half-life of tylosin varies depending on the type of media. Results of half-life tests indicate that tylosin should not persist in the environment. In swine manure, a half-life was reported of 7.6 days in the lab (Teeter and Meyerhoff, 2003) or less than 2 days under methanogenic conditions (Loke et al., 2000). Batch-sorption studies on soil-manure slurries found a range of half-life of 3.3-8.1 days (Ingerslev and Halling-Sorensen, 2001). At the field scale, half-lives of 49 and 67 days was found with no significant difference existing between the two (Halling-Sorensen et al., 2005). Assuming first-order reaction kinetics, a range was reported of 9.5-40 days in an anaerobic environment (Ingerslev et al., 2001). Tylosin applied to soil with manure had a reported half-life of 4.5 days, while without manure 6.1 days (Carlson and Mabury, 2006). Based on the results of the aforementioned studies, tylosin should not persist in the environment.

2.5.2 Tylosin resistance

Recommendations were made in 1969 by the Swann Committee to limit the concurrent use of the same antibiotics for both subtherapeutic treatment and treatment of disease (Aarestrup and Carstensen, 1998). The theory behind this development was to limit

the development of resistance of certain antibiotics because being used at low levels would inhibit the selection for resistance. Since then, however, many studies have shown that this was not the case.

Erm genes are among the most commonly acquired by many different strains of bacteria (Chen et al., 2007) and more than 40 classes of *erm* genes identified (Chen et al., 2007; Roberts, 2011). The *erm* gene codes for the RNA methylase enzyme that methylates a single adenine in 23S rRNA, which is a component of 50S rRNA. These genes code for rRNA methylase that changes the binding site for erythromycin and tylosin. It has been reported that *erm* genes are the most common resistance genes in enterococci (Roberts, 2003; Roberts, 2004).

In 113 erythromycin-resistant isolates of enterococci from human and animal origin, 88% were found to have the *ermB* gene (Jensen et al., 1999). *ermB* was also prevalent in 75 erythromycin-resistant *E. faecium* isolates with 93% testing positive for the gene (Schmitz et al., 2000). Tylosin resistance has been reported in animal production as *erm* genes have been found in environmental samples (Aarestrup and Carstensen, 1998; Whitehead and Cotta, 2001; Jost et al., 2003; Jost et al., 2004; Chee-Sanford et al., 2009; Chen et al., 2010; Koike et al., 2010).

The administration of tylosin at subtherapeutic levels has significantly increased resistance in commensal bacteria such as enterococci (Jackson et al., 2004b). Jackson et al. (2004b) reported 59% of isolates from swine waste collected from a farm administrating tylosin at subtherapeutic levels to be resistant to erythromycin, while 28% were resistant on a farm using tylosin for treatment only, and 2% on a tylosin-free farm. Of the total of 1,187 isolates from all three farms, 95% were positive for *erm*B. This confirms the theory that subtherapeutic tylosin use leads to increased resistance in swine manure and that resistance less frequently occurs in the absence of antibiotics. The use of tylosin in the feedstock of swine did not change the number of resistant fecal bacteria or *erm* gene copy number during a feeding trial (Kalmokoff et al., 2011). Kalmokoff (2011) also reported that a majority of isolates screened for *erm* genes were positive for *erm*B.

It is important to briefly discuss erythromycin and erythromycin resistance in environmental samples when tylosin resistance is present. Erythromycin is also a part of the

MLS antibiotic group, and used in humans. Since both tylosin and erythromycin are both macrolides and are effective in gram-positive organisms, it is highly likely that if enterococci were resistant to tylosin, it would also likely be resistant to erythromycin. This is cause for concern in terms of human health because erythromycin is the most commonly prescribed macrolide antibiotic in humans (Stephenson et al., 1997). Erythromycin has been used in many microbiological studies dealing with macrolide antibiotics (Ōmura, 2002). Erythromycin is commonly used in humans when the patient is allergic to penicillin (Madigan et al., 2000).

Recent studies have focused on erythromycin resistance in agriculture. In a study comparing the effects of antimicrobial use in swine on an organic farm where the swine received no antimicrobials found concentrations of tylosin less than the detection limit of 5 ng/g of wet manure (Zhou et al., 2010). Zhou (2010) showed that farms with a history of antibiotic use had MLS resistant fractions (MLS-Resistant/total bacteria) that were not different from organic farms without antibiotic use. Batch tests that were also conducted in the study showed that the prevalence of MLS increases in the short-term with increased manure, especially in the conventional farm manures, or with added antibiotics (Zhou et al., 2010). In a study of up and down gradient concentrations of enterococci and enterococci resistance, 18% of the enterococci were found to be resistant to erythromycin (Sapkota et al., 2007). However, in groundwater samples up and down-gradient from the swine facility, erythromycin resistant enterococci was found in 67% of the samples compared with 20% on the downstream side (Sapkota et al., 2007).

2.5.3 Transport of tylosin from manure into soil and water

The transport characteristics of antibiotics have been minimally studied in the past. Due largely to improvements in genotypic methods of analysis and molecule-mass-based methods, research is now possible at low concentrations of the antibiotic. It is hypothesized that antibiotics in manure will pass through the soil column, while losing a portion of that concentration to the sorbtive properties of the soil. Then, those remaining antibiotics will be released through the outlet of the subsurface drainage system.

Teeter and Meyerhoff (2003) reported a mean concentration of tylosin of 62.8 μg/g (ppm) in fresh swine manure samples and 4.1 μg/g in 30 day old samples. Typical antibiotic

concentrations in manure have been reported from 1-10 mg/kg with an upper limit of greater than 200 mg/kg (Kumar et al., 2005b). Manure application at agronomic rates of 168 kg N/ha can yield an application rate of 202 g/ha (Kumar et al., 2005b). Zhou compared the effects of antimicrobial use in swine with an organic farm in which the swine received no antimicrobials. Concentrations of tylosin less than the detection limit of 2 ng/g of dry weight were reported in manure (Zhou et al., 2010).

The effects of macropores, tile drainage, and tillage on pesticide transport have been widely reported. Studies by Kanwar (1999), Bakhsh (2005), Malone (2007), and Lawlor (2011), have found a strong correlation to nitrate transport, precipitation patterns and tile drain flow. Antibiotics, however, are just beginning to be studied in such a manner. Studies by Kay et al. (2004; 2005c; 2005b; 2005a) and Blackwell et al. (2007; 2009) indicated that low concentrations of antibiotics were found in drainage water and tylosin was not found at all. Tylosin and other antibiotics were measured in leachate and runoff from 2003-2005 (Dolliver and Gupta, 2008a). Tylosin concentrations ranged from 0.4-4.9 mg/kg in swine manure. Additionally, tylosin was detected in 8% of leachate water samples, with the highest concentration of 1.2 µg/L. Tylosin in runoff was quantified at a maximum concentration of 6 µg/L, which constituted less than 0.05% of the concentration found in the manure.

The fate of tylosin in the environment is likely controlled by two main processes: sorption and biodegradation. Sorption will have an impact on the ability of the antibiotic to spread from the soil or manure matrix, for example, and limit the availability in tile water. Kolz et al. (2005b) demonstrated rapid loss of tylosin in swine manure under both aerobic and anaerobic conditions and attributed this loss primarily to the sorption of tylosin to the manure slurry. Rabølle and Spliid (2000) reported a correlation between soil clay content and the sorption of tylosin. Additionally, 60-80% of tylosin was reported to have leached to a depth of 5 cm and 25 cm for sandy loam soil and sand soil respectively (Rabølle and Spliid, 2000). Similarly, Clay et al. (2005) found that desorption of tylosin from silty clay soils was < 0.2% which also illustrated the high sorptive properties of the antibiotic. The sorption of tylosin was also correlated to surface area, clay content and cation-exchange capacity in soils (Sassman et al., 2007).

Tylosin was not detected in leachates or soil after it was amended with manure slurry from swine operations feeding at rates of 100 g per ton of feed (Kay et al., 2005c; Kay et al., 2005b). Similarly, tylosin was shown to have little risk of accumulation in soil or groundwater when applied to soils in a slurry (Blackwell et al., 2007; Blackwell et al., 2009). Furthermore, Kay et al. (2004) also did not find tylosin in tile-drained clay soil. The measured concentrations of antibiotics in soil are often significantly less, if found at all, than in manure samples (Halling-Sorensen et al., 2005; Martinez-Carballo et al., 2007; Zhou et al., 2010). However, the effect of the soil and the binding of the antibiotics to the soil matrix is likely facilitating a gross underestimation of the actual concentrations in soil (Allaire et al., 2006; Hu and Coats, 2009; Heuer et al., 2011). This is due to the limitations of the extraction procedure to unbind the antibiotic compound from the soil. Concentrations of tylosin A in swine manure amended soil in Denmark found maximum concentrations in soil ranging from 25-50 μg/kg (Halling-Sorensen et al., 2005).

With reports of tylosin being found in elevated concentrations in the soil, studies have looked for tylosin in plants and in soil organisms. Plants such as corn, green onion and cabbage did not uptake tylosin from the soil through their roots, even though some took up other antibiotics such as chlortetracycline (Kumar et al., 2005a). Tylosin was found to have no effect at expected environmental concentrations in earthworms, springtails, and enchytraeids (Baguer et al., 2000). While the recent studies are few, general indications suggest that human and animal health is not negatively affected by tylosin uptake through plants and other animals

The runoff potential of tylosin was measured in clay loam, silty clay loam and silt loam soils by Hoese (2009). 8-12% of the applied tylosin was recovered in runoff (Hoese et al., 2009). The findings indicate that surface application of manure containing antibiotics such as tylosin facilitate the spread of antibiotics into the watershed if a precipitation event immediately follows manure application. Since manure slurry may not be fully incorporated into the soil immediately following application as recommended, Kay (2005a) performed assessed transport through crop stubble in the fall after application. After 24 hours, rainfall was simulated over the test plot. While Kay (2005a) reported finding oxytetracycline and sulphachloropyridazine, he did not find any tylosin in runoff in the clay loam soil, even

though all three antibiotics were present in the applied manure. Hu and Coats (2009) also agree that soil erosion and preferential flow processes are likely to be the mechanisms of tylosin transport. Another rainfall simulation showed that antibiotic transport in a field amended with seven of the most common antibiotics exhibited different runoff patterns for each antibiotic (Kim et al., 2010). The occurrence of antibiotics in surface water may have a negative effect on aquatic life and impact the growth of many organisms. Therefore, it is important to limit the input of antibiotics into any water supply to ensure high quality drinking water for years to come.

2.6 Antibiotic Resistance in Manure, Soil, and Water

One of the greatest issues today in the use of antibiotics is not in the treatment of humans or animals for disease, but rather the use of antibiotics at a subtherapeutic level to promote growth and prevent disease (Jackson et al., 2004b; Dolliver and Gupta, 2008b; Heuer et al., 2011). Antibiotics and metabolites are frequently in urine and feces from pigs (Mackie et al., 2006) and in other agricultural animals. Transport is crucial to understand the pathways that ARG's take to get from the source (manure) and end up in water. Presently, there is little information on how a reduction in subtherapeutic antibiotic use in agriculture will alter the occurrence of antibiotic resistance on a farm level (Heuer et al., 2011). The pathway resistant bacteria must take from the manure to the tile water is not direct. It must pass through the soil matrix before discharging though the tile drainage line.

2.6.1 Antibiotic resistance in manure

The use of tylosin at sub therapeutic concentrations will select and increase resistance to macrolides in enterococci living in the intestinal tract of pigs (Aarestrup and Carstensen, 1998). Resistant bacteria were found to occur naturally in organic manure in swine waste at five farms using culture-based and molecular methods with approximately 5% being resistant to tylosin, more than 25% resistant to tetracycline's and less than 5% resistant to both (Jindal et al., 2006). Furthermore, approximately 50% of the organic manure samples contained bacteria resistant to MLS (Jindal et al., 2006).

Antibiotic resistance in swine production is often related to the antibiotic used. Tetracycline resistant genes in fecal samples have been extensively studied (Chee-Sanford et

al., 2001; Morsczeck et al., 2004; Yu et al., 2005) from operations using tetracycline. *Erm*A, *erm*B, *erm*C, *erm*F, *erm*G, *erm*T, *erm*Q, and *erm*X genes have been found in elevated concentrations about expected background levels in swine waste lagoons (Chen et al., 2007; Koike et al., 2010) from operations using tylosin. Chen et al. (2007) developed real-time PCR assays to evaluate the persistence of six *erm* genes. In swine manure, he found 3.63 x 10^9 copies/gram of manure of *erm* genes. In all, just over 70% of those were *erm*B, 25% *erm*T, and 2% *erm*F. The study was able to weakly correlate the occurrence of *erm* genes to *tet* genes previously studied byYu et al. (2005) from the same samples. Koike et al. (2010) also reported that *erm* and *tet* genes were "mildly correlated" in swine waste. Koike et al. (2010) investigated the diversity, distribution and abundance of MLS methylases in groundwater wells and waste lagoons at swine farms with a history of tetracycline and tylosin use. These studies showed the importance of assessing *erm* gene resistance in swine manure.

Treatment and storage of the manure, in some cases, has a significant impact on the prevalence of antibiotic resistance reported. Composting of swine manure was found to decrease the prevalence and total quantity of some *erm* genes (Chen et al., 2007). Anaerobic digestion and storage in lagoons or pits were found to not have a significant impact on the persistence of *erm* genes in manure (Chen et al., 2010). Chen et al. (2010) concluded that antibiotic resistance genes are structurally different than chemical pollutants that can be removed with conventional treatment since the genes are located on mobile genetic elements, and can be transferred horizontally.

Tylosin-resistant bacteria have been reported to be persistent even following treatment in an aerobic thermophilic sequencing batch reactor (Chenier and Juteau, 2009). However, they found that while antimicrobial resistance persisted through treatment, the gene diversity was narrower following treatment. *Erm*B was the second most frequently detected gene. Seven *erm*B detects were reported before treatment, three following treatment. The total antibiotic resistant bacterial population also decreased following treatment, but only to the order to 10^2-10^8 copies/mL of treated swine manure. Aerobic thermophilic treatment does not prevent the propagation of antibiotic resistant bacteria or antibiotic resistant genes in the environment.

Pakpour et al. (2012) evaluated the resistance to tylosin in swine feces 2.5 years after antibiotic administration ceased at the research farm. Neither the sow nor piglets were administered antibiotics in their lifetimes, even though antibiotics had been used at facility in the past. *Erm*B was found in the suckling and weaning phase but not in the finishing stage, showing the persistence of this gene and other *tet* genes in the absence of antibiotics. While the withdrawal of tylosin in feed reduced the occurrence of tylosin-resistant genes, complete elimination of resistant genes is not likely in the short-term. Graham (2009a; 2009b) has also extensively studied the occurrence antibiotic resistant bacteria in farm workers, poultry products and the environment surrounding confined poultry operations in Delaware, Maryland and Virginia. The results of both studies indicate that typical storage practice of poultry manure does not eliminate antibiotic resistant bacteria (Graham et al., 2009a) and that flies may be another potential contributor to the spread of this resistant bacteria (Graham et al., 2009b). While poultry litter is not the focus of this report, an important connection can be made between farming practices in terms of storage and transport of antibiotic resistant bacteria.

2.6.2 Antibiotic resistance in soil

Antibiotic resistance can be found in the environment naturally, often at low levels. Since the soil matrix carries high populations of microorganisms, antibiotics can be found in the soil. Because tylosin is a natural fermentation product of *Streptomyces fradiae* (Clewell, 2008), it is possible that tylosin is present naturally in the soil. Only a few recent studies have quantified antibiotic resistance in the soil.

A significant difference in antibiotic resistant bacteria levels in agricultural soil was found when swine farms using subtherapeutic and therapeutic antimicrobials were compared with those that only used antimicrobials at therapeutic levels (Onan and LaPara, 2003). They reported that the proportion of tylosin-resistant bacteria to total bacteria was 7.2-16.5% higher at the subtherapeutic users vs. the therapeutic users (Onan and LaPara, 2003). Onan and LaPara (2003) also found that between 5.8-6.7% of the soil bacteria on plots with a manure history were antibiotic resistant. Ghosh and LaPara reported similar results (2007). Ten sampling locations were evaluated for chlortetracycline resistant bacteria, including three non-agricultural soil sites, three dairy farm (no antibiotics) and four finishing swine

facilities (Ghosh and LaPara, 2007). They found no significant difference in terms of the antibiotic resistant populations in the soils for all but one site, which allowed manure to accumulate outside of the pen and saw persistently higher background level at that site. Their results indicate that the proliferation of antibiotic resistance is not inherently related to antibiotic use and application (Ghosh and LaPara, 2007). Munir and Xagoraraki reported background levels of *tet* genes in soil before manure was applied and found levels elevated above that background measurement after application (2011). Andrews et al. (2004) reported total and tetracycline resistant enterococci concentrations of 2.5 x 10^5 cfu/g soil after swine manure application. Under simulated rainfall Hoang (2010) reported total and tylosin-resistant enterococci in manured soil averaged 9.8 × 10^3 cfu/g soil and 7.5 × 10^3 cfu/g soil, respectively

2.6.3 Antibiotic resistance in water

Precipitation facilitates the movement of pathogens, pathogen indicators, and through the soil and into the groundwater or surface water systems (Auckenthaler et al., 2002). One of the most prevalent and most concerning source of antibiotic resistance is in potable water supplies. While chlorination and other disinfection methods should provide adequate protection to consumers, this is often not the case (Huang et al., 2011). Kolpin et al. (2002) reported that 50% of surface waters in the United States tested positive for some sort of antibiotic, which Dolliver and Gupta (2008a) attributed to the combination of both agricultural and human sources. The treated drinking water must come from a source; therefore it is important to look at the occurrence of antibiotic resistance in source water such as surface water and groundwater. Preliminary findings of research by Costanzo et al. (2005) indicate that antibiotics in the aquatic system have the potential to threaten ecosystem function and pose threats to human health. Studies in California, have found a substantial amount of pharmaceuticals in groundwater which may provide an additional source for resistance (Fram and Belitz, 2011). Artificial groundwater recharge systems studied in Belgium, Spain, and Italy (Bockelmann et al., 2009) were found to harbor antibiotic resistance genes and bacteria which is consequently used as a source water.

In the last thirty years, there has been a major influx in the research of antibiotic resistant bacteria and antibiotic resistant genes in water, water supplies and in wastewater

effluents. For many researchers, the primary concern is based on the possibility that either human contact or ingestion of water contaminated with antibiotic resistant bacteria will decrease the body's ability to respond to antibiotics, and potentially leading to severe sickness or death. Two studies conducted in the early 1980's were some of the first to address the issue of antibiotic resistant bacteria in drinking water (Armstrong et al., 1981; Armstrong et al., 1982). The first evaluated water from six separate communities, finding bacteria in over one-third of the water samples were resistant to multiple antibiotics and indicated the selection of the treatment process to the occurrence of the resistant bacteria (Armstrong et al., 1981). The second found that selective parameters exist in water treatment facilities which may lead to an increased portion of antibiotic resistant bacteria in drinking water supplies (Armstrong et al., 1982).

Other studies have looked at the transport of antibiotic resistant genes within the distribution system. The prevalence and persistence of antibiotic resistant genes from source water to tap water was researched by the University of Michigan (Xi et al., 2009). With the use of qPCR, an increase in resistant genes in the distribution system from the water treatment plant to tap water in a home was noted (Xi et al., 2009). In 2004, a study evaluated the effects of low chlorination of drinking water and multi-drug resistance (Shrivastava et al., 2004). A laboratory scale test discovered that the antibiotic resistant strains found after chlorination were resistant to many antibiotics and only at high dosages of chlorination were the strains completely destroyed (Shrivastava et al., 2004) While the distribution is typically modeled as a closed system, the implications of this to environmental research are quite large. If in fact antibiotic resistant bacteria is in environmental water samples, special treatment may be required make sure that it does not end up in drinking water. As noted, the lack of effective wastewater and water treatment for the removal of antimicrobials, and the selective pressures that come with those microbials, is a risk to human health.

Groundwater has long been one of the more pristine sources of drinking water. However, pharmaceutical compounds are being found in California in approximately 3% of water samples (Fram and Belitz, 2011). "Old" and "new" groundwater samples were compared to determine if the groundwater was from the facilitated recharge by assessing tritium levels (Fram and Belitz, 2011). Samples of water after 1952, termed "new" likely

have higher tritium concentrations than "old" water due to testing of atomic bombs. The detection of antibiotics in the ground water suggests that the artificial recharge and the use of pharmaceuticals in humans may cause an increase in antibiotic resistance in water supplies. In Europe, like in California, groundwater recharge systems are used to store treated water or to replenish low water tables (Bockelmann et al., 2009). However, the reintroduction of water has led to an increase in antibiotic resistance genes in three systems in Belgium, Spain and Italy (Bockelmann et al., 2009). The most effective way to treat the antibiotic resistant bacteria was through ultrafiltration and reverse osmosis (Bockelmann et al., 2009). While in most cases groundwater sources are more pristine than surface waters, certain treatment is more effective than others to prevent contamination.

Groundwater samples taken from within the vicinity of swine production facilities were found to contain tetracycline (*tet*) resistance genes. Identical sequences were also found in a nearby lagoon signifying the transport from the lagoon to the groundwater (Chee-Sanford et al., 2001). Similarly, 87% of 250 coliform bacteria were found to be resistant to at least one antibiotic in untreated, rural groundwater supplies in West Virginia (McKeon et al., 1995).

A rainfall simulation by Hoang (2010) used PCR and bacterial methods to quantify tylosin resistance in liquid swine manure, treated soil and tile drainage water. Samples were collected after manure application and rainfall simulation, and again the following spring. Between 68% and 100% of enterococci were tylosin-resistant in manure samples. Total and tylosin-resistant enterococci in soil samples averaged 8.8×10^6 cfu/g of soil and 8.9×10^6 cfu/g of soil, respectively. In tile drainage water, total enterococci ranged from 1.3×10^1 cfu/100 mL-5.0×10^3 cfu/100 mL while tylosin-resistant enterococci ranged from 1.3×10^1 cfu/100 mL-1.2×10^3 cfu/100 mL. *Erm*B and *erm*F were detected in 69% and 78% of 200 isolates selected from all three matrices. Presently, there is insufficient information on antibiotic and resistant gene transport to tile waters under natural conditions, which this study aims to address.

2.7 Detection and quantification methods

In order to accurately detect and quantify enterococci and tylosin in environmental samples, various microbiological methods are required. There are two main methods used in

the bacterial analysis: phenotypic and genotypic. Phenotypic methods are the more conventional approach-they rely on culturing bacteria on a selective medium that permits growth. An example of a phenotypic method is standard membrane filtration. Genotypic methods compare the genetic makeup of an organism with reference to a specific genes or traits.

Common methods include polymerase chain reaction (PCR) and real-time quantitative polymerase chain reaction (qPCR). Unfortunately, there is no method which is the best in all applications. Rather, bacterial and resistant-bacterial identifications are categorized as either phenotypic methods or genotypic methods (Cowan and Talaro, 2008). The phenotypic and genotypic methods can be used in conjunction with one another to provide a more thorough understanding, as shown by Noble (2010). Noble (2010) attempted to correlate qPCR results with standard phenotypic methods for recreational water quality monitoring and found that qPCR underestimated the quantity of fecal indicator bacteria *E. coli* and *enterococcus spp*. Tylosin and other antibiotics can be quantified by mass spectrometry. Significant developments in the laboratory procedures have played a key role in improving understanding of antibiotic resistant bacteria and genes.

2.7.1 Phenotypic methods

Membrane filtration is the most common method for the detection and enumeration of bacteria in water and is the focus of this review. Membrane filtration is an Environmental Protection Agency (EPA) certified method for the detection and enumeration of bacteria in water (USEPA, 2006) and is described in Method 1600: Enterococci in Water by Membrane Filtration Using membrane-Enterococcus Indoxyl-β-D-Glucoside Agar (mEI) (USEPA, 2006). It is a single-step method which modifies EPA Method 1106.1. This method provides a direct count of the bacteria in terms of a colony forming units (CFU) by culturing the growth of the bacteria on a selective medium. The membrane filtration method has been used by many on environmental samples in recent research (Sapkota et al., 2007; Watkinson et al., 2007; Moore et al., 2008; Pathak and Gopal, 2008; Sharma et al., 2009; Hoang, 2010; Holzel et al., 2010b; Luczkiewicz et al., 2010; Hoa et al., 2011; Walczak and Xu, 2011).

The number of Colony Forming Units for enterococci is found by filtering a water sample, or series of ten-fold dilutions, through a 0.47μm filter and placing the filter, which

retains the bacteria, on an agar media and incubating for 48 hours. For enterococci, the mEnterococcus, mE, (Difco, Detroit, MI) selective agar is one option. After incubation, the plate is removed from the incubator and the total CFU are counted. The number of CFUs is only the count of culturable bacterial cells, or the number of cells that are still viable. The rest of the cells present, which do not appear on the media, are termed 'viable but nonculturable,' or VBNC (Oliver, 2005). The results of water samples are expressed as the number of CFU/100 mL whereas soil or manure samples are in the form of CFU/gram media dry weight.

The count of the number of bacteria resistant to a particular antibiotic can be determined by adding to an agar mix a quantity of the desired antibiotic. The breakpoint concentration for tylosin is 35 mg/L (FDA, 2009) Portillo et al. (2000) reported a range of breakpoint concentrations ranging from 0.125-128 µg/ml (mg/L) and the concentrations in the present study are less than the lower bound of this range. When plated on this media, only those bacterial colonies that are resistant to tylosin will grow. When this number is compared with the total CFU, as measured in the agar without antibiotics, the percent resistant can be found. This method is limited by high concentrations of bacteria, typically greater than 400, of the turbidity of the sample. These limitations can be overcome by serial dilutions.

2.7.2 Genotypic methods

Two of the common methods are the use of polymerase chain reaction (PCR) and quantitative polymerase chain reaction (qPCR). PCR is based on thermal cycling with the sample DNA, forward and reverse primers for the target of interest and an enzyme to synthesize the new DNA strand. Even more recently, qPCR has been developed which enables the detection and quantification of one or more specific sequences in a DNA sample. Based on similar principals as PCR, the key feature is that the amplified DNA is detected by inserting a florescent dye and the quantity measured as the reaction progresses in real-time. Both methods determine the occurrence of the resistance genes in environmental samples.

There are a few known limitations of the PCR based genotypic methods. First and foremost, PCR analysis on environmental samples is subject to limitations due to the nature of the sample including inhibition due to humic substances and metals (Maier et al., 2009). Other concerns include non-specific amplification, failure of primers to amplify the genes

coding for some function. One of the most pressing issues, however, is that the nucleic acids detected in the reaction cannot be distinguished between live or dead cells. The use of both phenotypic methods and genotypic methods then allow the verification of the vitality of the cell of interest assuming culturability the organisms of interest. Both PCR and qPCR were used in recent studies of resistance genes (Chee-Sanford et al., 2001; Yu et al., 2005) and provide a unique and through evaluation.

2.7.2.1 PCR

The Polymerase Chain Reaction was discovered by Kerry Mullis in 1985. Today, it is common practice in microbiological laboratories. PCR is a "simple enzymatic reaction used to generate copies of a target DNA sequence through a series of temperature cycles" (Maier et al., 2009). Each PCR cycle, doubles (theoretically) the amount of target DNA in each well. However, due to limitations in the amplification process caused by inhibitors, reagent limitations or high DNA concentrations, the practical maximum amplification is only a 1 x 10^6 increase (Maier et al., 2009). PCR assays were used on environmental samples to detect and evaluate macrolide resistance (Jackson et al., 2004a; Jackson et al., 2004b; Chen et al., 2007; Patterson et al., 2007).

A typical PCR cycle has three main steps. First, the double stranded DNA is denatured into two single strands of target DNA at a temperature of 94°C (Maier et al., 2009). Two primers, the forward and reverse, are added to the reaction mixture. The region of DNA that will be amplified is called the PCR product. The second step is the primer annealing, which occurs at a temperature between 50 and 70°C (Maier et al., 2009). In this step, the polymerase will bind to the target sequence which begins DNA synthesis. The third step is the extension which synthesized a complimentary strand to the original DNA. Extension is optimized at a temperature of around 72°C (Maier et al., 2009). This process is repeated for 25 to 30 cycles. This procedure requires the optimization of temperature, time and concentrations or the primer pairs to maintain the desired level of sensitivity. The molecular weight of the DNA produced by this procedure can be determined an agarose gel electrophoresis and compared to standards also run on the same gel.

PCR has been used to confirm the presence of resistant genes (Sutcliffe et al., 1996; Chen et al., 2007; Bockelmann et al., 2009). While PCR may yield a detectable fragment of

the gene of interest, due diligence is required to confirm the results. For example, the primer concentration needs to be checked as well as the verification of annealing temperatures of the primers. The inclusion of positive and negative controls is required to check for purity or contamination of the DNA template and ensure that false positives are eliminated.

2.7.2.2 qPCR

Real-Time Quantitative Polymerase Chain Reaction (qPCR) was first reported in 1992 and was described as a method where the reaction tube could remain undisturbed during the reaction (Higuchi et al., 1992). This method is used to quantify target DNA as the initial concentration can be estimated due to the changes in PCR product concentration with the amplification cycles (Zhang et al., 2009). The reaction is based on the presence of fluorescent molecules that monitor production of PCR product,, typically a fluorescent dye such as SYBR Green(Morsczeck et al., 2004). These fluorescent molecules then mix and bind to double stranded DNA of the amplicons after the annealing step in each PCR cycle. This process outputs a plot of amplification (fluorescence) rather than expressing the results on a gel like in traditional PCR. The fluorescence signal for a sample increases above a background level with positive PCR reaction. The point in time at which the signal crosses the threshold value it called the Ct value (Eurogentec, 2009). This C_t can be plotted against the known concentration of standard, and used to calculate the concentration of the target gene of interest.

qPCR is more accurate than conventional PCR because the quantification is based on the exponential phase of the amplification whereas PCR only is an endpoint measurement (Maier et al., 2009). qPCR includes a graphical software interface, which allows for immediate data analysis and processing using hard numbers rather than photographs or observations. While this interface allows for real-time analysis, it also leaves the operator to use scientific judgment, which has a direct impact on the results.

A melting curve is typically run during a qPCR run to measure the florescence in a sample to confirm the specificity of the reaction (Maier et al., 2009). Melting curves verify that nonspecific amplification did not occur in the samples. The fluorescence is measured as the temperature increases. The first derivative of the plot of the fluorescent signal vs. temperature is made, which shows the temperature at which the denaturing step was

occurring. If this temperature is the same as the desired amplicon temperature, the desired qPCR reaction was achieved. Melting curves can be added into the analysis protocol by heating the block from 65°C to 95°C, reading the fluorescence every 2 °C, and holding on each temperature for 1 second.

Tests of inhibition play an important role in the validation of the qPCR process. Inhibition is most likely found in complex biological samples, particularly environmental samples. Commercial purification kits are available that address some of the natural inhibition in these samples. Inhibition within the matrix can be evaluated by the use of an external spiking control.

Along with inhibition analysis, the reproducibility of results is necessary. Reproducibility consists of the evaluation of both intra-plate similarity and inter-plate similarity. This can be accomplished by running a standard in triplicate on one plate and comparing that with the standards from another run. The standard deviation should be small between the replications to ensure high reproducibility. Additionally, DNA sequencing can be used with the PCR products to verify that the targeted gene was quantified (Thompson et al., 2007). By providing the sequencer with the forward and reverse primers, the forward and reverse sequence can be produced. These can then be inverted and combined to create the initial gene that was sequenced using specializes software such as BLAST, Basic Local Alignment Search Tool (Altschul et al., 1990). This validates the results if a sequence matches the forward and reverse primers.

2.7.3 Extraction and Analysis by Mass Spectrometry

The use of mass spectrometry to determine the concentration of an antibiotic in water is a standard approach in research today. The method for extraction is a major part of obtaining accurate results using mass spectrometry (either GC-MS or LC-MS). It has been reported that one of the best performing methods is to use the Oasis HLB (hydrophilic-lipophilic balanced) cartridges (Le-Minh et al., 2010) for the Solid-Phase Extraction (SPE). High Performance Liquid Chromatography (HPLC) coupled with mass spectrometry is one of the most widely used methods to detect antibiotics in environmental matrices. However, this method is often limited by the matrix of the environmental sample (Kim and Carlson, 2005). Each sample matrix requires different sample preparation steps, particularly those

with high suspended solids such as soils and stream water. The limits of the process can be tested by conducting necessary recovery tests when using the SPE methods by spiking a known amount of the antibiotic into the sample and measuring the recovery to ensure that the mass spectrometer results are representative. Recoveries as low as 35% were reported (Kay et al., 2004) and as high as over 150% (Kim and Carlson, 2005).

Several recent studies have used molecule mass analysis to determine the concentrations of antibiotics in samples. Josamycin, kitasamycin, mirosamicin, spiramycin, and tylosin, all macrolides, were recovered from samples of meat using HPLC-UV analysis at once (Horie et al., 1998). The stability of tylosin A under both methanogenic and aerobic conditions was studied using HPLC (Loke et al., 2000). Sixteen compounds, including tylosin, were investigated in Missouri lakes, rivers and groundwater using liquid chromatography coupled with tandem mass spectrometry highlighting the capabilities of this technology and its applicability to environmental studies (Wang et al., 2011).

Yang and Carlson (2004) conducted an extensive study on macrolide antibiotics in wastewater and natural environments. They reported recoveries of tylosin of 94.3% and erythromycin of 93.6% in environmental water samples and 86.1% and 84.8% in samples from wastewater treatment facilities using Oasis HLB cartridges. They were able to achieve detection limits of 0.05 and 0.07 μg/L for tylosin and erythromycin, respectively and showed that (SPE) combined with high-performance liquid chromatography-ion trap tandem mass spectrometry (LC-MS-MS) is viable for environmental samples.

2.8 Summary

Antibiotics are used to promote growth in the swine industry, and introduce high levels of tylosin into the environment. This in turn has likely led to an increase in tylosin resistance in the natural environment. The study of antibiotic-resistant bacteria and antibiotic resistance genes plays an important role in agriculture and in human health. This literature review has provided an overview of these broad topics and how they relate to the environmental concern at hand.

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CHAPTER 3: TYLOSIN-RESISTANT ENTEROCOCCI, ERM GENES, AND TYLOSIN IN DRAINED FIELDS RECEIVING SWINE MANURE

A paper to be submitted

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Abstract

The use of tylosin at subtherapeutic levels by the swine industry provides selective pressure for the development of antibiotic resistance in gastrointestinal bacteria. The land application of swine manure to drained agricultural fields might introduce elevated levels of total and tylosin-resistant enterococci, *erm* genes and tylosin. The goal of this study was to develop an understanding of the occurrence and transport of total and tylosin-resistant enterococci, *erm* genes and tylosin in tile-drained chisel plow and no-till agricultural fields that have received multi-year application of liquid swine manure through injection over two growing seasons.

Resistance to tylosin in manure, soil and water samples was investigated at the field scale level using phenotypic based (membrane filtration) and genotypic based (qPCR) methods and compared with samples from control plots treated with urea and ammonium nitrate (UAN). Tylosin was quantified using LC-MS/MS. Plots in a corn-soybean rotation were identified for sampling from 2010-2012. Soil samples were collected from each manure plot, from both the direct area of injection and from the area between the manure bands and from control plots. Each one-acre plot is drained separately and tile water samples were collected directly from the discharge tile line weekly while the tiles were flowing. The results of this study suggest that tylosin usage has increased the short-term occurrence of total and tylosin-resistant enterococci, *erm* genes, and tylosin in soils, but has had minimal effect on tile drainage water quality under dryer than average conditions.

3.1 Introduction

Antibiotic resistance is becoming a greater concern on a global scale, even leading some members of the EU to ban the use of agricultural antibiotics for growth promotion (Aarestrup et al., 2001). Both agricultural and human use of antibiotics are potentially contributing to a population of antibiotic resistant organisms that might spread resistance to pathogenic bacteria. The practice of land application of swine manure provides large-scale introduction of antibiotics into the environment. Once released, the antibiotics are transported via soil particles and potentially into groundwater (Campagnolo et al., 2002; Chee-Sanford et al., 2009).

Tylosin is a macrolide antibiotic frequently used by the swine industry for growth promotion and therapeutic purposes. Tylosin is not completely metabolized in the pigs, and metabolites are excreted in manure (Teeter and Meyerhoff, 2003), which is typically applied to agricultural fields as fertilizer (Chee-Sanford et al., 2009). The presence of tylosin in waste pits and in manure amended soil can lead to selective pressures on antibiotic resistant bacteria (ARB). These selective pressures confer resistance on antibiotic resistant genes (ARG). Antibiotic resistant genes are most commonly are carried on mobile genetic elements, which disseminate between microorganisms (Mazel and Davies, 1999; de la Cruz and Davies, 2000; Roberts, 2004). However, ARG are also mobilized by the processes of transduction and transformation (Ochman et al., 2000). Recent studies have suggested that swine manure can contribute significantly to the antibiotic resistance in the environment (Chee-Sanford et al., 2009; Chen et al., 2010; Koike et al., 2010).

Pathogens and pathogen indicators persist in the environment and provide a medium in which antibiotic resistance can reside. Enterococci are one such bacterium found in the intestinal tract of warm-blooded animals and in humans. There are two main pathogenic strains: *Enterococcus faecalis* and *Enterococcus faecium* (Franz et al., 1999; Shepard and Gilmore, 2002). Enterococci are gram positive bacteria which are targeted by macrolides like tylosin. Enterococci play an important role as indicators of pathogens, for their use in regulatory standards (USEPA, 1986), and in past studies on bacteria transport (Soupir et al., 2006; Sapkota et al., 2007). Pappas et al. (2008) found higher concentrations of enterococci

in tile water than *E. coli* or fecal coliform in a central Iowa study and Hoang (2010) reported high incidences in tile water.

Tylosin is structurally related to erythromycin, which is the most commonly prescribed macrolide-lincosamide-streptogramin (MLS) antibiotic in humans (Stephenson et al., 1997; Portillo et al., 2000). Both tylosin and erythromycin inhibit protein synthesis by binding to the 23S ribosomal RNA of the 50S subunit (Roberts, 2004). *Erm* genes are responsible for tylosin-resistance in bacteria and the *erm* class of genes is among the most commonly acquired genes conferring resistance to MLS antibiotics (Chen et al., 2007). In enterococci, MLS resistance is most commonly mediated by the *erm*B gene (Portillo et al., 2000). Tylosin use in animal production has been shown to lead to increased levels of erythromycin resistance in enterococci isolates (Jackson et al., 2004b). Various e*rm* genes have been found in swine waste lagoons including e*rm*A, *erm*B, *erm*C, *erm*F, *erm*G, *erm*T, *erm*Q, and *erm*X (Chen et al., 2007; Koike et al., 2010). Additionally, a wide variety of resistance genes are found naturally in soils, even in the absence of manure application (Schmitt et al., 2006; Allen et al., 2010). Tylosin has previously been detected in tile flow (Dolliver and Gupta, 2008a). However, no study has looked at the comprehensive release of enterococci, tylosin-resistant enterococci, *erm* genes, and tylosin from tile-drained fields receiving swine-manure application.

Land application of swine manure introduces excess enterococci, tylosin-resistant enterococci, *erm* genes, and tylosin into the environment. Between 25-35% of cropland in Iowa is artificially drained (Zucker and Brown, 1998). However, these highly developed drainage systems may facilitate pollutant transport to downstream water bodies. Additionally, high concentrations of confined swine operations have resulted in the common use of swine manure in corn production. Antibiotics and bacteria move through the matrix, but can move faster in no-till fields versus chisel-plow plots via transport through macropores (Cullum, 2009). The quality of tile drainage water in highly drained areas, such as Iowa, is an important concern. Studies by Kanwar et al. (1999), Bakhsh et al. (2005), Malone et al. (2007), and Lawlor et al. (2011), have found a strong correlation between nitrate transport, precipitation patterns and tile drain flow. Therefore, it is possible that antibiotics and antibiotic-resistant bacteria will also be related to precipitation and hydrology.

Concentrations of pathogens reaching tile drainage during high flows have been reported (Dean and Foran, 1992; Joy et al., 1998; Hunter et al., 2000).

Presently, there is insufficient information on antibiotic and resistance gene transport to tile waters under natural conditions. Previously, Hoang (2010) used PCR and membrane filtration to quantify tylosin resistance in *Enterococcus* spp. from liquid swine manure, treated soil and tile drainage water under an artificial rainfall simulation. Between 68% and 100% of enterococci were tylosin-resistant in manure while 100% were tylosin-resistant in soils immediately after application. In tile water, total and tylosin-resistant enterococci concentrations were reported up to 5×10^3 cfu/100 mL and 1×10^3 cfu/100 mL respectively. *Erm*B and *erm*F were detected in 69% and 78% of 200 resistant isolates selected from all three matrices indicating that these genes are likely to be found in quantifiable levels. This study aims to extend the initial analysis by Hoang (2010) by performing a similar analysis under natural conditions over two study years and includes quantitative PCR and analysis of tylosin.

The goal of this research project is to further understand the occurrence and movement of total and tylosin-resistant enterococci, *erm* genes and tylosin in tile-drained agricultural fields that have received multi-year application of liquid swine manure through injection. The objectives of this study were to: 1) in liquid swine manure, soil and tile drainage water: a) investigate the occurrence and transport of total and tylosin-resistant enterococci; b) quantify the occurrence of *erm*B, *erm*F and *erm*T; c) quantify tylosin; 2) compare the effects of tillage on total and tylosin-resistant enterococci, *erm* genes and tylosin concentrations; 3) compare the effects of season on total and tylosin-resistant enterococci, *erm* genes and tylosin concentrations. These objectives were met by evaluating plots of both tillage types receiving multi-year application of liquid swine manure and comparing them to non-manured control plots. The results of this study will improve understanding of the effects of liquid swine manure application from facilities using tylosin for growth promotion on water quality in the upper Midwest.

3.2 Materials and Methods

3.2.1 Study Site and Sample Collection

Two sets of four plots were identified for sampling at Iowa State University's Northeast Research and Demonstration Farm near Nashua, IA, USA (43.0° N, 92.5° W) from 2010-2012. The soils are moderately well to poorly drained Floyd loam, Kenyon silty-clay loam and Readlyn loam which overlie loamy glacial till, as described previously by Fathelrahman et al. (2011). Soil slopes vary from 1 to 3%. These agricultural plots are instrumented with a subsurface water quality monitoring system operational since 1988. Each one-acre plot is drained separately with 10 cm diameter subsurface drain lines installed in the center of the plot at a depth of 1.2 m below ground surface and a drain spacing of 28.5 m (Kanwar et al., 1999). Cross flow between plots is avoided by border drains. Central drainage lines from each plot are connected to individual sumps equipped with an effluent pump and Neptune T-10, 1" diameter flow meter. Flow meters record subsurface drainage flow as a function of pumped volume and are recorded weekly while the tile lines are flowing.

The plots for this study were selected based on manure application rate, tillage practice, and crop rotation. The plots are in the corn-soybean rotation; therefore, a total of 8 plots were selected to obtain 2 years of data. In the first year of the study, only 4 plots were sampled (hereafter referred to as plot system A, or PSA). In the second year of the study, 4 additional plots were added (hereafter referred to as plot system B, or PSB) along with PSA. A summary of the plots selected for sampling are presented in Table 3. The selected plots encompass two tillage practices, chisel plow (CP) and no-till (NT), and manure was applied to one plot of each tillage type while the second plot of each type received urea and ammonium nitrate (UAN) and served as a control for assessing background levels. All corn plots receive swine manure or UAN fertilizer as a nitrogen source prior to each crop season. The control plots have no history of manure since 1978, while the manured plots have been in various manure rotations since 1993. Specific plot locations at the project site are presented by Kanwar et al. (1999).

Manure was injected 10 to 15 cm below the soil surface with a shank forming a band of treated soil, as described by Al-Kaisi and Kwaw-Mensah (2007), on October 28 in both 2010 and 2011 only in the year and plots specified in Table 3. The manure was applied to

field plots at rates to provide 168 kg N ha^{-1} which was roughly 42,000 L ha^{-1} (PSA) and 31,000 L ha^{-1} (PSB). The manure was from a commercial finishing facility currently feeding tylosin at sub-therapeutic levels of 40 gram/ton for growth promotion for 16 out of 20 weeks of each animal rotation, or 2.5 turns per year (personal communication, facility manager). UAN was knifed into the control plots in late April both years. The chisel plow fields were tilled (20 cm depth) within two weeks of manure application in November following harvest and field cultivated (10 cm depth) prior to planting the next May (Al-Kaisi and Kwaw-Mensah, 2007). Manure samples were collected directly from the manure applicator.

Table 3: Northeast Research Farm plots selected for sample collection

Plot	Tillage	Nitrogen Management
23†	CP	2010 Fall inject swine manure at 168 kg N ha^{-1}
24†	CP	Spring preplant spoke inject UAN at 168 kg N ha^{-1}
25†	NT	2010 Fall inject swine manure at 168 kg N ha^{-1}
34†	NT	Spring preplant spoke inject UAN at 168 kg N ha^{-1} with Cover Crop
29‡	CP	Spring preplant spoke inject UAN at 168 kg N ha^{-1}
30‡	CP	2011 Fall inject swine manure at 168 kg N ha^{-1}
19‡	NT	Spring preplant spoke inject UAN at 168 kg N $ha^{-1}$with Cover Crop
20‡	NT	2011 Fall inject swine manure at 168 kg N ha^{-1}

† Plots are part of PSA, and have data for 2 full years (2010-2012)
‡ Plots are part of PSB, and have data for 1 full year (2011-2012)

Soil samples were collected following manure application in both the fall of 2010 and 2011. Six composite soil samples were collected from each manure plot, three from the direct area of injection (manure band) and three from the area between the manure bands (inter-band). Each sample was a composite of 3 cores to 15 cm depth. Three composite samples were also collected from the control (no-manure) plots. Sampling equipment was cleaned with 75% ethanol between sampling in the manure injection band, inter-band and non-manured soils. Samples were collected in gallon plastic bags and placed on ice in a cooler and transported back to Iowa State University. Samples were mixed using surface sterilized spatulas. A subsample was removed for analysis of total enterococci and tylosin-resistant enterococci and processed within 24 hours. Another subsample was removed for moisture analysis and the remaining sample was frozen for DNA and tylosin extraction. A second set of soil samples were collected in mid-April using the same samples and analysis protocol as

in the initial sampling. The manure bands were flagged in the fall to allow accurate repeat sampling. Mean soil moisture content from all samples was 17%, 16%, 19%, and 24% for the fall 2010, spring 2011, fall 2011 and spring 2012 respectively.

Tile water samples were collected directly from the discharge tile line in the sump (see Kanwar et al. (1999)) for each plot. Samples were collected weekly during the spring and early summer during each year until flow ceased. Samples were also collected following major rainfall events during this period. A total water volume of 2,500 mL was collected: 250 mL for analysis of tylosin, 250 mL for DNA extraction, and 2,000 mL for analysis of total and tylosin-resistant enterococci. The 250 mL samples for tylosin were collected in brown glass bottles and the samples for DNA extraction and enterococci analysis were collected in plastic bottles. Samples were transported to the Water Quality Research Lab in Ames on ice and analyzed within 24 hours (enterococci) or 48 hours (tylosin).

3.2.2 Enterococci and Enterococci Resistance to Tylosin

Manure, soil, and tile water samples were assayed for enterococci and enterococci resistant to tylosin by the membrane filtration technique (APHA, 1998) using a 0.45 micron filter (Millipore, Billerica, MA) within 24 hours. Soil and manure samples were diluted (1 g/ 9 mL) with distilled water prior to filtration. Concentrations of total and tylosin-resistant enterococci were determined by enumeration on mEnterococcus (mE) agar (Difco, Detroit, MI) without antibiotics and mE agar infused with tylosin tartrate at 35 mg/L (Kaukas et al., 1988; FDA, 2009; CLSI, 2010). Agar was infused with 1 mL of a stock solution made from tylosin tartrate (Sigma-Aldrich, St. Louis, MO) by dissolving 410 mg in 10 mL of 50% methanol as recommended in previous studies (Kaukas et al., 1988; Benning and Mathers, 1999). The 410 mg accounts for 85% purity of tylosin tartrate. After filtration, the membrane was placed on each respective media and incubated at 35 ± 0.5°C for 48 hours. All samples were analyzed in triplicate. Colonies enumerated on mE media accounted for the total enterococci population and colonies enumerated on tylosin infused media accounted for tylosin-resistant enterococci. Results for manure or soil were expressed on a dry weight basis in terms of colony forming units (cfu)/g dry weight and results for water were expressed as cfu/100 mL.

3.2.3 PCR assays for detection of *Enterococcus faecalis* and *Enterococcus faecium*

Qualitative PCR assays were performed on isolates selected from the filtration process to determine if isolates were human pathogenic strains, *Enterococcus faecalis* or *Enterococcus faecium* (Franz et al., 1999; Shepard and Gilmore, 2002). Isolates (218 total) were selected from the mE agar plates to encompass tile water samples (132), soil samples (36) and manure (50) from both control and manured plots. Isolates were streaked on mE media to verify culture viability before screening. Controls consisted of strains of *E. faecalis* (ATCC 19433, 360 bp) and *E. faecium* (ATCC 19434, 215 bp) and PCR grade water was the negative control (Jackson et al., 2004a). The PCR templates were prepared by suspending a single bacterial colony in 100 µl sterile water and boiling the cells at 95°C for 15 minutes. PCR master mixes containing primers for *E. faecalis* and *E. faecium* were prepared. For this assay, the 20 µl reactions were performed in 96-well plates containing 0.5 µL boiled cells, 10 µL 2x Taq PCR Master Mix (Qiagen, Valencia, CA), 0.2 µL each primer, and 8.7 µL RNase-free water. Amplification was conducted in a BioRad iCycler Thermocycler (BioRad, Hercules, CA) with initial denaturation at 94°C for 3 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 30 seconds and extension at 72°C for 3 minutes, and a final extension at 72°C for 7 minutes (Jackson et al., 2004a). PCR product (8 µL) was analyzed by gel electrophoresis on 1% TAE agarose with 1 kb Plus DNA ladder as a standard (Invitrogen, Carlsbad, CA) followed by ethidium bromide staining.

3.2.4 DNA Extraction

Quantitative PCR assays were performed to quantify three *erm* genes: *erm*B, *erm*F and *erm*T. Tile water extractions (250 mL) were performed using the MoBio Power Water DNA kit from samples for all plots within 48 hours of collection. Samples were only collected from tile lines in the first year after manure application. Soil extractions (10 g, wet weight) were performed using the MoBio UltraClean Soil DNA kit. Due to the complexity of the manure matrix, the repeated bead beating plus column extraction method (RBBC) as described by Yu and Morrison (2004) on 250 µL manure slurry was combined with Qiagen QIAamp DNA Stool protocol. This method uses bead beating in the presence of a lysis buffer with sodium dodecyl sulfate (SDS), salt and EDTA. Extracted DNA was frozen until qPCR

analysis. The concentration of DNA after extraction and purification was determined with an Eppendorf biophotometer (Hauppauge, New York).

3.2.5 Quantitative Real-Time Polymerase Chain Reaction

Primers developed for *erm* genes and validated in previous studies (Chen et al., 2007; Koike et al., 2010) were used in this study (Table 2). Quantitative real-time PCR was performed on triplicate DNA extracts in independent runs for *erm*B, *erm*F and *erm*T. Each qPCR reaction was carried out a MJ Research Opticon2 qPCR instrument with total reaction volume of 25 µL containing 2.5 µL DNA, 12.5 µL Qiagen SYBR Green Master Mix, and 5 µL of each primer (forward and reverse). The qPCR conditions for all genes consisted of an initial denaturation of 95°C for 15 minutes; followed by 40 cycles of 30 seconds of denaturation at 95°C, one minute of annealing at the temperature specified in Table 4 and one minute of extension at 70°C. This is followed by a final extension at 70°C for 10 minutes. A melt curve was run following each plate for primer specificity. The reported temperatures for *erm*B, *erm*F, and *erm*T were optimized for this study to 58.4°C, 54.3°C, and 51.0°C, respectively. The abundance of each gene in each sample was calculated by multiplying the number of copies per well by the total volume of DNA per well (2.5 µL) and total volume of DNA extracted derived from 1 g dry weight (manure or soil adjusted to a dry weight basis after extraction) or 100 mL (water). DNA standards were prepared from *E. coli* strains carrying plasmids with *erm* gene fragment inserts (Table 4). *Erm*B and *erm*T were constructed from *Enterococcus* isolates Man T1-C and Soil T3-R, respectively, previously characterized by (Hoang, 2010). PCR products from these isolates were purified and cloned into pCR-4TOPO using the TOPO TA cloning kit (Invitrogen Corp., Carlsbad, CA). A reference *E coli* stain with a plasmid carrying *Erm*F was provided by M. C Roberts lab (University of Washington). Both negative controls and blanks were run with each assay. Negative controls for PCR consisted of *Pseudomonas stutzeri* genomic DNA (ATCC 14405) and PCR grade water.

The effect of inhibitory substances co-extracted with the samples were characterized by spiking soil and manure samples with known amounts of standard DNA and comparing actual and theoretical recoveries for each *erm* gene. Amplified DNA from SYBR Green assays were subjected to melting curve analysis and gel electrophoresis to assure primer

specificity. Samples of DNA were also selected from soil and water matrices for PCR product sequencing. DNA extracted from soil (both band and inter-band samples) and tile water from manured plots were amplified with both forward and reverse primers (without SYBR green to prevent interference with the sequencing process) and the reaction products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). The purified product subsamples were then submitted to the DNA Facility of the Iowa State University Office of Biotechnology for sequencing.

Table 4: qPCR primer sequences, targets, annealing temperatures, and amplicon lengths used in this study

Primer	Gene targeted	Primer Sequence (5'→3')	Amplicon Size (bp)	Primer annealing temp. (°C)	Reference
Erm B-FW *Erm* B-RV	*Erm* (B)	GGTTGCTCTTGCACACTCAAG CAGTTGACGATATTCTCGATTG	191	58.4	Koike et al. 2010
Erm F-189f *Erm* F-497r	*Erm* (F)	CGACACAGCTTTGGTTGAAC GGACCTACCTCATAGACAAG	309	54.3	Chen et al. 2007
Erm T-52f *Erm* T-420r	*Erm* (T)	CATATAAATGAAATTTTGAG ACGATTTGTATTTAGCAACC	369	51.0	Chen et al. 2007

3.2.6 Tylosin Extraction and Analysis

Analytical methods were developed and validated for tylosin A (hereafter referred to as tylosin) extraction and analysis and tested by spiking non-manured Clarion soil from the Boyd Farm research plots near Ames, IA with tylosin. Briefly, soils (15 g) were extracted twice with a solution of 85% acetonitrile and 15% of 0.1 M ammonium acetate. The manure samples (30 g) were extracted twice with two solutions: 85% acetonitrile + 15% ammonium acetate and 95% acetonitrile + 5% isopropyl alcohol. The acetonitrile in the combined extracts was evaporated and the remaining aqueous extract was passed through an Oasis HLB solid phase extraction (SPE) column (Waters Corporation, Milford, MA). The tylosin was eluted with 2 mL of methanol and evaporated to approximately 0.5 mL. This final extract was brought to 2 mL volume with 10 mM ammonium acetate, filtered and analyzed on an Agilent 1100 LC/MSD mass spectrometer. Quantification of tylosin A ((m/z) 916.4 [M+1]) was performed using multiple reaction monitoring (MRM) with isolation of the parent mass and internal standard (simetone) calibration. Positive identification of tylosin

was performed with a second method using MRM with isolation of the parent ion (916.4) followed by fragmentation. If the primary fragment 772.4 was present along with ions 598.2 and 754, the presence of tylosin A was confirmed. Tylosin recovery from 4 replicate soil samples averaged 88%.

Tylosin was extracted from the tile water samples by filtering 250 mL through an Oasis HLB solid phase extraction (SPE) column cartridge. Method validation studies were performed with water from the South Fork of the Iowa River, which is heavily fed by tile drainage. The laboratory study found that 250 mL stream water samples could be passed through the SPE column without clogging, thus avoiding pretreatment of the sample to remove suspended material. Tylosin recovery from distilled water compared to stream water was not different, showing that SPE columns did not concentrate organic materials that affect recovery or chromatography. Recovery of tylosin (mean of 3 replicates) from distilled water and stream water averaged 71%. This analysis was conducted in part to develop limits of detection (2 ng/mL) and quantification (6.8 ng/mL) in the extracts from the first study year where concentrations of tylosin as low as 2 ng/mL (ppb) were detected. In the second year, optimizing the procedure allowed for tylosin A to be detected at 0.3 ng/mL and quantified at 0.8 ng/mL.

3.2.6 Statistical Tests and Analysis

Statistical analysis was performed using R, version 2.14.1 (R Development Core Team, 2011). Data were first log-transformed to meet assumptions of normality and equality of variances. Non-detects were taken as ½ of the limit of detection (Croghan and Egeghy, 2003) for the *erm* gene and tylosin data. Pearson's correlation coefficient was determined for the concentrations of enterococci and *erm* genes in tile water over time after manure application and tile flow rate. Effects were considered significant at $r \geq 0.9$. Analysis of variance was performed using the effects of tillage (chisel plow or no-till), treatment (manure band, inter-band, or no-manure), season (fall or spring), and year (2010 or 2011) on soil and water data. Interaction effects were examined between tillage and treatment, and between season and treatment. Akaike's Information Criterion was used to select the best-fitting covariance structure for a model that initially included tillage, treatment, season, year, and interactions of tillage:treatment and season:treatment. Mean separation was conducted from

pairwise differences of least squares means. Effects were considered significant at $p \leq 0.1$. Data are reported as back-transformed means.

3.3 Results and Discussion

Swine manure, soil, and tile water samples were collected over two growing seasons to quantify the total and tylosin-resistant enterococci, *erm* genes, and tylosin. Additionally, precipitation and tile flow data were also collected for a frame of reference for these results. In general, the tile flow decreased throughout the growing season due to an increase in evapotranspiration and plant moisture demands, with periodic increases following precipitation events. The two years included in this study were dryer than normal, which might provide an explanation for some of the deviations from the hypothesized results. The 10-year rainfall average during the first 6 months of the year is 37.4 cm; 2011 experienced 30.8 cm and 2012 experienced 21.2 cm (Iowa State University Department of Agronomy, 2012). The tile flow was less than the 10-year average of total tile flow for two plots in 2011, and an average of 40% less in all 4 plots for 2012, as shown in Table 5. This indicates that quantity of bacteria (and potentially *erm* genes and tylosin) in tile drainage water might be less than that expected during a year under normal flow. It has been demonstrated that rainfall duration and intensity directly affect protozoan transport through the soil (Ramirez et al., 2009), therefore, the results of this study potentially underestimate the concentration of total and tylosin-resistant enterococci, erm genes, and tylosin in tile water during an average flow year. Under normal conditions, it is likely that there would have been more transport through the tile lines and/or macropore flow in the no-till plots over chisel plow plots (Cullum, 2009; Ramirez et al., 2009).

Table 5: Tile Flow Data† for January-June, averaged from 2003-2012. Data are an average of plots in PSA and PSB.

Plot	Total Tile Flow (m^3)		
	2011	2012	10-year average
Chisel Plow with Manure	408.5	213.5	337.7
Chisel Plow with No Manure	119.4	63.8	161.2
No-Till with Manure	381.1	220.3	286.0
No-Till with No Manure	141.6	109.6	192.1

†Data Source: Personal Communication with Carl Pederson, Iowa State University (2003-2012)

3.3.1 Enterococci and enterococci resistant to tylosin

Manure

Total enterococci concentrations were highest in liquid swine manure, second highest in soil in the manure application band, and lowest in tile water. Average concentrations of enterococci in manure were 56,571 cfu/g and 8,635 cfu/g for year 1 (PSA, 2010) and year 2 (PSB, 2011), respectively. Of those, 39,653 cfu/g (70%) and 7,216 cfu/g (81%) were resistant to tylosin in PSA and PSB respectively. The concentration of enterococci and tylosin-resistant enterococci were significantly lower ($p<0.1$) in the second year, so it is therefore likely to see lower concentrations of enterococci in manure amended soils in the second year. In 2011, the analysis was done three days after application, while in 2010 sampling and analysis took place the day after application, therefore, bacterial die off may account for some of the differences between the years. These results are similar to a study by Onan and LaPara (2003) where 69% of the bacteria from manure samples were resistant to tylosin. Holzel (2010a) reported enterococci concentrations of up to 13,800 cfu/g in swine manure. Hoang (2010) found between 68% and 100% of enterococci were tylosin-resistant in manure samples collected from the same swine facility used in the present study.

Soil

In soil, enterococci concentrations were the greatest in the manure injection band and the lowest in the no-manure (control) plots. Mean concentrations were calculated for season (fall and spring) and treatment location (manure bands, inter-band or no-manure) because these parameters were found to be statistically significant. Tillage had no statistical effect on enterococci populations. Table 6 provides the seasonal and treatment means for enterococci and tylosin-resistant enterococci in soils for the first year after manure application for PSA and PSB while Table 7 shows the means over two years. For both tables, the ANOVA showed that enterococci populations in soil after manure application in the 2010-2011 period (PSA) is significantly greater than 2011-2012 period (PSB), which is expected due to the difference between the concentrations of enterococci in the applied manure. Additionally, these tables show that, particularly in the manure band, that there is a significant decrease in concentrations from the fall after manure application to the following spring.

Table 6: Mean† enterococci (ENT, cfu/g) and tylosin-resistant enterococci (TYL, cfu/g) concentrations in soil in the first year after manure application for plot PSA and PSB. Standard deviations are presented in parenthesis.

Application	Sampling	Treatment					
		Manure Band	Manure Inter-band	No-Manure	Manure Band	Manure Inter-band	No-Manure
		ENT			TYL		
2010‡	Fall 2010	826 **a** (±43)	78 **a** (±13)	24 **a** (±27)	45 **a** (±46)	0 **a**	0 **a**
	Spring 2011	246 **b** (±252)	36 **a** (±13)	34 **a** (±42)	73 **a** (±82)	0 **a**	0 **a**
	Annual Mean	536 **x** (±410)	57 **y** (±29)	29 **y** (±7)	59 **x** (±19)	0 **y**	0 **y**
2011§	Fall 2011	346 **a** (±164)	202 **a** (±208)	15 **a** (±23)	416 **a** (±188)	7 **a** (±14)	0 **a**
	Spring 2012	6 **b** (±5)	78 **b** (±126)	13 **a** (±1)	1 **a** (±3)	0 **b**	1 **b** (±3)
	Annual Mean	176 **x** (±240)	140 **xy** (±87)	14 **y** (±1)	209 **x** (±293)	1 **y** (±5)	1 **y** (±1)
Treatment Means¶,		356 **x** (±254)	99 **yz** (±58)	22 **z** (±10)	134 **x** (±105)	1 **y** (±10)	1 **z** (±1)

† Treatment means are averaged across tillage. Means in columns within study years followed by the same letter (a, b, c) or rows within analysis type (x, y, z) are not significantly different ($P \leq 0.1$).
‡ PSA plots, as shown in Table 3.
§ PSB plots, as shown in Table 3.
¶ Mean over both 2010 and 2011.

The mean concentrations of enterococci in the manure bands were significantly greater than populations in the no-manure controls at $p<0.1$ (Table 6, where statistical significance denoted with x,y,z across rows). However, the enterococci population in manure bands was nearly ten times greater than populations in the inter-band soil during the 2010-2011 sampling period, but differences were not observed during the 2011-2012 sampling period. The treatment means, which are the mean concentration across both years, are presented at the bottom of Table 6. The treatment means for the first year after manure application show that the enterococci concentration in the manure band is significantly greater than in the manure inter-band. However, the mean inter-band enterococci concentration is not significantly greater than the no-manure control.

The concentration of enterococci in soil over two years following manure application in 2010 to PSA, shows that the concentration decreased in the manure band and reached concentrations equivalent to the no-manure and inter-band samples after two years (Table 7).

In the first year, enterococci concentrations in the band are statistically greater than the no-manure and the no band samples. However, the enterococci populations in the no-manure and inter-band samples were not statistically different. In year 2, the means relative to treatment (data not shown) were not statistically different. Enterococci in manure band samples from 2011 were not statistically different from the no-manure or inter-band samples from 2010, indicating that the manured plots return to the 'background' levels measured in the manure-free plots.

Table 7: Mean† enterococci (ENT, cfu/g) and tylosin-resistant enterococci (TYL, cfu/g) concentrations in soil over 2-year monitoring for PSA. Standard deviations are presented in parenthesis.

Application	Sampling	Treatment					
		Manure Band	Manure Inter-band	No-Manure	Manure Band	Manure Inter-band	No-Manure
		ENT			TYL		
2010‡	Fall 2010	826 **a** (±43)	78 **a** (±13)	24 **a** (±27)	45 **a** (±46)	0 **a**	0 **a**
	Spring 2011	246 **b** (±252)	36 **a** (±13)	34 **a** (±42)	73 **a** (±82)	0 **a**	0 **a**
	Fall 2011	52 **c** (±111)	9 **ab** (±17)	29 **a** (±28)	0 **b**	1 **b** (±2)	0 **a**
	Spring 2012	NS	5 **b** (±11)	11**a** (±14)	NS	0 **a**	0 **a**
	Mean	375 **x** (±403)	32 **y** (±34)	25**z** (±10)	59 **x** (±20)	1 **y**	0 **z**

† Treatment means are averaged across tillage. Means in columns followed by the same letter (a, b, c) or rows within analysis type (x, y, z) are not significantly different ($P \leq 0.1$).
‡ System A plots, as shown in Table 3.
NS-No Sample, this collection period is not included in the calculation of the overall mean

Manure application has a significant effect on the concentration of enterococci and tylosin-resistant enterococci in soil; it increases enterococci levels above the background level of the controls, and in band samples taken one year later. Tylosin-resistant enterococci concentrations from soil are also shown in Table 6 and Table 7. Resistant enterococci were most frequently detected in the manure band soils, and rarely detected in the inter-band or controls. On average, 36%, 2%, and 1% of the enterococci from the manure bands, inter-bands and controls respectively were resistant to tylosin in all soil samples. These results compare to a study by Onan and LaPara (2003) where 5.8-6.7% of the soil bacteria on plots

with a manure history were antibiotic resistant. Andrews et al. (2004) reported total and tetracycline resistant enterococci concentrations of 2.5 x 10^5 cfu/g soil after swine manure application. Prior to simulated rainfall, Hoang (2010) reported total and tylosin-resistant enterococci in manured soil averaged 9.8 × 10^3 cfu/g soil and 7.5 × 10^3 cfu/g soil, respectively. The enterococci concentrations immediately after manure application in this study were slightly less, and 2 orders of magnitude less for tylosin-resistant enterococci. Since most of the tylosin-resistant enterococci were detected in the manure injection band, it suggests that manure is the driving factor in the occurrence of resistant bacteria, while a small fraction of resistant bacteria are present naturally in all soils.

Tile Water

Enterococci concentrations in tile water were highly variable relative to time after manure application (Figure 4) and drainage flow rate (data not shown). Enterococci concentrations were expected to be highest at the start of tile flow and decrease over the growing season. However, there was no correlation ($r<0.5$) between enterococci concentrations relative to drainage flow or time after manure application; the data was therefore analyzed by tillage, treatment, and year subsets for analysis.

There was no statistical difference on the concentration of enterococci in tile water due to manure application or study year (2010-11 compared to 2011-12 as shown in Figure 4). The second year of monitoring from PSA (data not shown) also showed that there was no statistical difference due to tillage or manure treatment. The geometric mean for enterococci in recreational water is 33 cfu/100 mL (USEPA, 1986). In the water samples collected in 2011 from PSA, a total of 18 samples exceeded this water quality standard. Seven of the exceedences occurred early in the tile-flow season, most likely due to the first "flushing" of the system since the previous fall when manure was applied. Of the exceedences, nearly 40% were detected in samples collected from the control plots. In PSB, 8 samples exceeded this standard, with 6 from plots with manure history, and 2 from controls. Pappas et al. (2008) measured the concentration of enterococci, *E. coli* and fecal coliform in drainage water in central Iowa over 3 years under both manure and manure free conditions. Pappas et al. (2008) reported mean, flow weighted concentrations of enterococci in tile water under both

manure-free and for manure applied at 168 kg N ha^{-1} conditions and found enterococci concentrations of 52 cfu/100 mL from plot types.

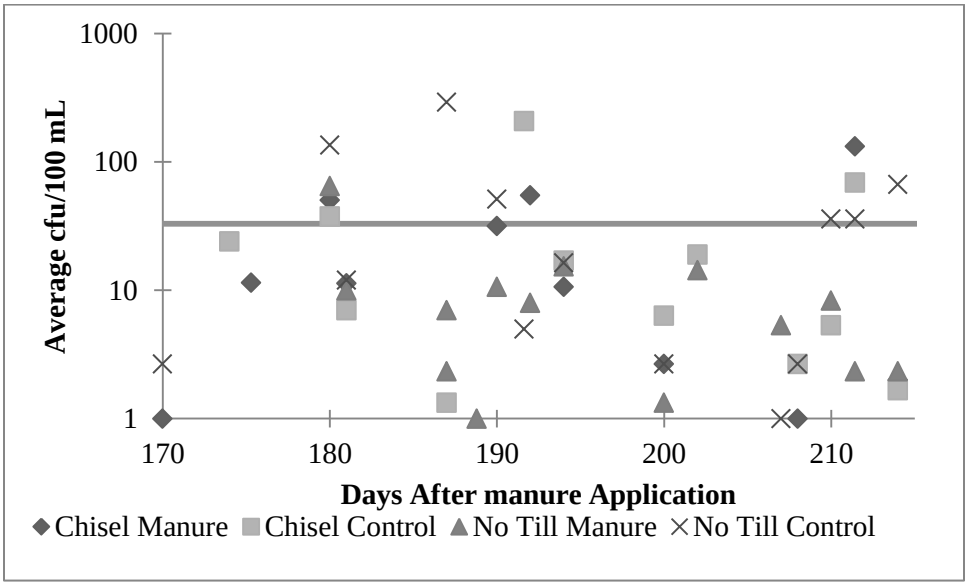

Figure 4: Enterococci in individual tile water samples in 1st year after manure application for both PSA and PSB. The recreational mean (33 cfu/100 mL) is shown for reference.

Tylosin-resistant enterococci concentrations in the tile water were rarely detected, and when present the maximum concentration was 1 cfu/100 mL (data not shown). In PSA, tylosin-resistant enterococci were found in 16% and 2% of the total of 86 tile water samples collected the summers of 2011 and 2012 respectively. System B plots from the summer of 2012 had only 5% of 46 samples with enterococci resistant to tylosin. Sapkota et al. (2007) and Pappas et al. (2008) reported an increase in down gradient concentrations of MLS-resistant enterococci in groundwater near a swine facility, which may be similar to tile drainage in the present study. However, that study also found in lesser quantities of MLS-resistant enterococci in up gradient samples as well. This indicates that low, "background" levels of enterococci are present in the environment, and should be expected in unamended areas, similar to the controls in the present study.

The relative abundance of total and tylosin-resistant enterococci were low compared to those described by Hoang (2010). That study reported total enterococci ranging from 1.3×10^1 to 5.0×10^3 cfu/100 mL while tylosin-resistant enterococci ranged from 1.3×10^1 to 1.2

$\times 10^3$ cfu/100 mL. The greatest concentration of tylosin-resistant enterococci in the present study was only 1 cfu/100 mL. The main difference between the present study, and the study by Hoang (2010) is that Hoang collected tile water samples generated by an artificial rainfall event immediately following manure application, whereas in the present study, tile drains remained dry for over five months after application. It is apparent that a significant decrease in bacterial concentration occurs over time, in the present studies as previously found by Cook et al. (1997) and Warnemuende and Kanwar (2000).

The samples collected in this study were either weekly grab samples, or samples collected soon after precipitation events. The samples following a rainfall event are likely from the falling limb of the hydrograph which may differ from the peak concentration during the storm event, as shown by previous research on surface runoff (Soupir et al., 2006), or different from a flow-weighted mean. That study evaluated the relationship between *E. coli* and enterococci concentrations and the runoff hydrograph. Bacteria concentrations frequently mimicked the runoff hydrograph: the bacteria concentrations peaked at the same time as the flow, and then rapidly fell off (Soupir et al., 2006). Specific work on tile line hydrographs show that the flow falls off rapidly after reaching the peak (Cullum, 2009), and this study likely misses the peak enterococci concentration as well. The concentrations of pathogens also reaching tile drainage discharge during high flows have been reported (Dean and Foran, 1992; Joy et al., 1998; Hunter et al., 2000). The grab samples in the present study may not represent an average concentration at the tile line discharge.

Detection of *Enterococcus faecalis* and *Enterococcus faecium*

There were 218 isolates selected from tile water, soil and manure samples. Of those, 11 (5%) were *E. faecalis*. No isolates were identified as *E. faecium*. Nearly all (10 of the 11) were colonies initially plated on mE media without tylosin. All but one of the isolates was from tile water, with 6 from a manured plot and 4 from no-manure control. The remaining was from a manure band soil sample. All 11 isolates of *E. faecalis* were confirmed to be resistant to tylosin following PCR. While *E. faecalis* was detected, the relative occurrence is very low and similar to recent work at the same research farm that found 11% (23/204) of enterococci isolates were *E. faecalis* (Hoang, 2010 unpublished data). Holzel (2010b) reported that 63% and 39% of swine manure samples were positive for *E. faecalis* and *E.*

faecium, respectively, with 47.6% and 13.4% resistant to tylosin, which was different from this study.

3.3.2 Occurrence and quantification of *erm* genes in environmental samples

Quantitative PCR analysis was conducted on DNA extracted from manure, soil, and water samples for *erm*B, *erm*F and *erm*T. This study is one of the first to look specifically for *erm* genes in soil and tile water. Average detection limits are reported in Table 8 and vary based on the performance of the standards for each individual qPCR run. *Erm*B and *erm*F were found in all three matrices, while *erm*T was not detected.

Table 8: Detection Limits for *erm* Genes

Year	Soil (copies/gram)			Water (copies/100 mL)			Manure (copies/gram)	
	*erm*B	*erm*F	*erm*T	*erm*B	*erm*F	*erm*T	*erm*B	*erm*F
2010	2.1×10^5	5.0×10^5	6.1×10^6	2.7×10^1	7.2×10^5	4.6×10^5	2.0×10^6	1.0×10^7
2011	6.3×10^3	7.0×10^3	3.7×10^6	4.8×10^2	2.3×10^3	4.4×10^6	5.0×10^4	2.0×10^7
Mean	1.1×10^5	2.5×10^5	4.9×10^6	2.5×10^2	3.6×10^5	2.4×10^6	1.0×10^6	1.5×10^7

A test of matrix inhibition was conducted by spiking selected manure, soil and water samples with an aliquot of standard (plasmid DNA) and determining the percent recovery for each matrix. Mean recoveries for *erm*B, *erm*F and *erm*T in manure, soil and water ranged from 73%-251%. There appeared to be no inhibition of PCR due to the sample matrix, as recovery of *erm* genes was greater than 100%. This variability in recovery can be explained by the inherent nature of using qPCR on environmental samples (Osborn and Smith, 2009) and the large standard deviation between the replications.

The *erm*B and *erm*F PCR products were purified and sequenced at the ISU DNA Facility. The forward and reverse sequences were aligned and consensus sequences were developed using Vector NTI software. The ermB sequence size was 182-185 bp. The *erm*F fragment was 310 bp. According to Koike et al (2010) and Chen et al (2007), the primers we used produced an *erm*B amplicon of 191 bp and an *erm*F amplicon of 309 bp, respectively. All of the products derived from PCR of standards and samples for *erm*B were successfully sequenced. However, only the the PCR products from standards and the water samples (matrix) produced consensus sequences for *erm*F. Matches to the consensus sequences were

identified using Mega BLAST searches of the NCBI nucleotide database. The Mega BLAST search produced matches to ermB and ermF and indicated that gram positive gut bacteria included various species of *Enterococcus*, *Streptococcus*, *Clostridium*, *Bacteriodes* and *Capnocytophaga* are the likely host of the *erm* genes found in this study. No npon-specific matches were obtained.

Manure

In manure, the mean *erm*B concentrations were 8 x 10^8 copies/g in 2010 and 6 x 10^{12} copies/g in 2011 respectively. The mean *erm*F concentrations were 4 x 10^7 copies/g and 3 x 10^{12} copies/g in the first and second study years, respectively. *Erm*T was not detected in manure in either year. Once again, since the 2011 analysis was performed three days after application, rather than one day after application in 2010, the actual concentrations reported in the second year might be underrepresented due to bacteria host die off. Chen et al. (2007) reported concentrations of 1 x 10^9 copies/g for *erm*B, *erm*F, and *erm*T respectively in liquid swine manure. Also, *erm*B and *erm*F were found at concentrations of 1 x 10^9 by Koike et al. (2010). The differences between the results from the present study and those by Chen et al. (2007) or by Koike et al. (2010) may be due to the spatial or ecological differences in the manure or manure storage.

Soil

In soil, the mean *erm*B concentrations were the greatest in the manure injection band followed by the inter-band and no-manure soil, as shown in Table 9 in the first year after manure application. Statistical analysis found that the effects of tillage were not significant. The *erm*B level in the manure band sampled in 2011 was less than in the band sampled in 2010. In 2011, the sampling and analysis was done three days after application, while in 2010 sampling took place the day after application. The mean concentrations of *erm*B for both PSA and PSB plots relative to treatment were statistically different ($p<0.1$), as shown in Table 9. Manure application has a significant treatment effect, and increases the levels of *erm*B in the manure injection zone relative to concentrations found outside of the injection zone, or in control plots.

The abundance of *erm*B declined in the manure band in the two years after manure application in fall of 2010 (PSA) and reached concentrations equivalent to concentrations in the inter-band and no-manure control soils by one year after manure application (Figure 5). There was no statistical difference in *erm*B concentrations from the manure injection band sampled in spring of 2011 and the concentrations in the inter-band samples from both years. Similarly, there was no significant difference between the mean *erm*B concentrations in soil from the manure injection band in 2011 (4×10^8 copies/g) and the concentrations in control plots in 2010 or 2011 (3×10^5 copies/g). The no-manure control soils are representative of the background level of *erm*B in the soil. *Erm*B in the manure injection band returns to this background level after a full year.

Table 9: Mean† *erm*B (copies/g dry weight) concentrations in soil in the first year after manure application. Standard deviations are presented in parenthesis.

Application	Sampling	Treatment		
		Manure Band	Manure Inter-band	No-Manure
2010‡	Fall 2010	1×10^9 **a** ($\pm 2 \times 10^8$)	2×10^6 **a** ($\pm 3 \times 10^5$)	3×10^5 **a** ($\pm 2 \times 10^4$)
	Spring 2011	9×10^7 **b** ($\pm 1 \times 10^7$)	3×10^6 **a** ($\pm 8 \times 10^5$)	3×10^5 **a** ($\pm 4 \times 10^4$)
	Annual Mean ‡	5×10^8 **x** ($\pm 5 \times 10^8$)	2×10^6 **y** ($\pm 6 \times 10^5$)	3×10^5 **y** ($\pm 3 \times 10^4$)
2011§	Fall 2011	5×10^8 **a** ($\pm 2 \times 10^5$)	1×10^6 **a** ($\pm 1 \times 10^6$)	$<6.3 \times 10^3$ **a**
	Spring 2012	2×10^6 **b** ($\pm 1 \times 10^6$)	1×10^5 **a** ($\pm 1 \times 10^5$)	2×10^4 **b** ($\pm 0 \times 10^0$)
	Annual Mean §	2×10^8 **x** ($\pm 2 \times 10^8$)	6×10^5 **x** ($\pm 4 \times 10^5$)	2×10^4 **x** ($\pm 0 \times 10^0$)
Treatment Means¶		4×10^8 **x** ($\pm 4 \times 10^8$)	1×10^6 **y** ($\pm 1 \times 10^6$)	3×10^5 **z** ($\pm 1 \times 10^5$)

† Means are averaged across tillage. Means in columns followed by the same letter (a, b, c, d) or rows (x, y, z) are not significantly different ($P \leq 0.1$).
‡ System A plots, as shown in Table 3.
§ System B plots, as shown in Table 3.
¶ Mean over both 2010 and 2011.

The mean concentrations of *erm*B across the two-year study period for the manure band, inter-band and controls was 4×10^8 copies/g, 1×10^6 copies/g, and 2×10^5 copies/g respectively. While there is no significant difference ($p<0.1$) between the means of the *erm*B concentrations in the control and inter-band samples, the mean *erm*B concentration in the manure band, which includes the sample taken right after manure application, is still

significantly higher than the controls or inter-band samples. This suggests that the long-term manure application history causes an increase in *erm*B in the soil, particularly within the year of manure application, and then the level decreases to levels observed in the controls after a complete year. Since the plots in this study are in a corn-soybean rotation, the long-term mean for plots under biennial manure application will likely remain higher than controls. However, a continuous corn rotation might maintain high levels of *erm*B and not experience the biennial decrease reported in the present study.

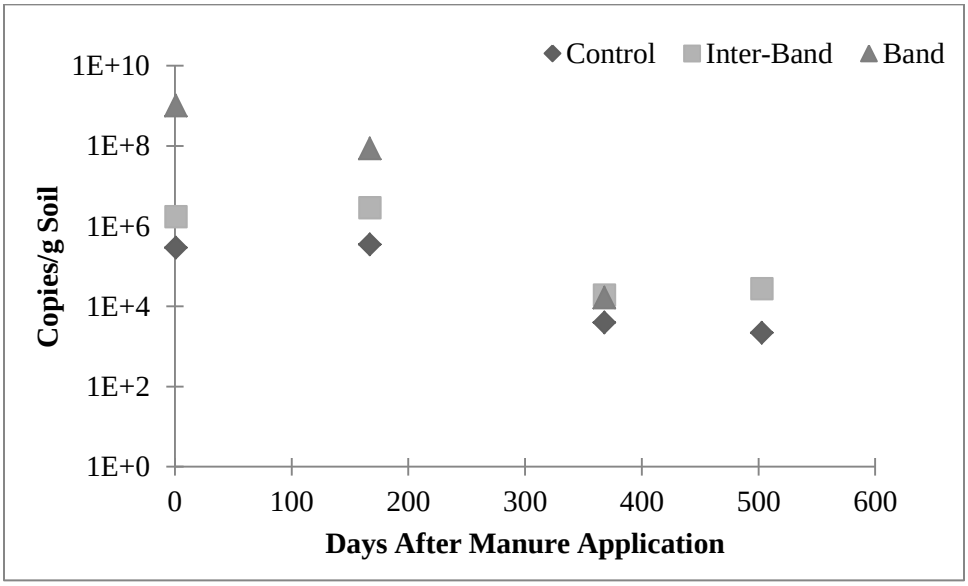

Figure 5: Persistence of *erm*B in soil over 2 years (PSA) from plots receiving manure in 2010. No manure band samples were collected in spring of 2012 as the bands were no longer visible.

Mean *erm*F concentrations in soil were the greatest in the manure injection band, with a reduced concentration found in the inter-bands, and the lowest concentration in the non-manured soils (Table 10). Similar to *erm*B, statistical analysis found that the effects of tillage were not significant. There was no significant difference in *erm*F concentrations between the no-manured plots (both 2010 and 2011) and inter-bands (both 2010 and 2011) at $p<0.1$ (Table 10 columns). Again, this indicates that manure application has a significant treatment effect, and increases the levels of *erm*F in the manure injection zone to concentrations greater than those found outside of the injection zone, or in no-manure plots.

The abundance of *erm*F declined in the manure band over two years after manure application in PSA in 2010 and reached concentrations equivalent to concentrations in the inter-band and to the no-manure control soils by one year after manure application, as shown in Figure 6. The mean concentrations of *erm*F across the two-year study period for the manure band, inter-band and controls was 1 x 10^{12} copies/g, 3 x 10^{6} copies/g, and 4 x 10^{3} copies/g respectively. There is no statistical difference in *erm*F concentrations between the inter-bands for 2010 and 2011. Additionally, there is no statistical difference between the inter-bands and controls across the two years. The concentrations in the manure band in 2010 were significantly greater than the concentrations in the band in 2011. The relative abundance of *erm*F in all soils is converging on a low level, and in the spring of 2012, concentrations were below detection in all treatments. Manure application causes an increase in *erm*F in the soil, particularly within the year of manure application.

Table 10: Mean† *erm*F (copies/g dry weight) concentrations in soil in the first year after manure application. Standard deviations are presented in parenthesis.

Application	Sampling	Treatment		
		Manure Band	Manure Inter-band	No-Manure
2010‡	Fall 2010	4 x 10^{12} **a** (±2 x 10^{12})	2 x 10^{4} **a** (±6 x 10^{3})	3 x 10^{3} **a** (±3 x 10^{1})
	Spring 2011	2 x 10^{9} **b** (±1 x 10^{9})	1 x 10^{7} **b** (±9 x 10^{6})	1 x 10^{4} **a** (±2 x 10^{3})
	Annual Mean ‡	2 x 10^{12} **x** (±2 x 10^{12})	7 x 10^{6} **y** (±1 x 10^{7})	8 x 10^{3} **z** (±6 x 10^{3})
2011§	Fall 2011	5 x 10^{8} **a** (±3 x 10^{8})	5 x 10^{6} **a** (±58 x 10^{6})	< 7.0 x 10^{3} **a**
	Spring 2012	4 x 10^{6} **a** (±3 x 10^{6})	1 x 10^{5} **a** (±1 x 10^{5})	< 7.0 x 10^{3} **a**
	Annual Mean §	2 x 10^{8} **x** (±3 x 10^{8})	3 x 10^{6} **y** (±5 x 10^{6})	< 7.0 x 10^{3} **z**
Treatment Means¶		9 x 10^{11} **x** (±1 x 10^{12})	7 x 10^{6} **y** (±7 x 10^{6})	6 x 10^{3} **z** (±6 x 10^{3})

† Means are averaged across tillage. Means in columns followed by the same letter (a, b) or rows (x, y, z) are not significantly different ($P \leq 0.1$).
‡ System A plots, as shown in Table 3.
§ System B plots, as shown in Table 3.
¶ Mean over both 2010 and 2011.

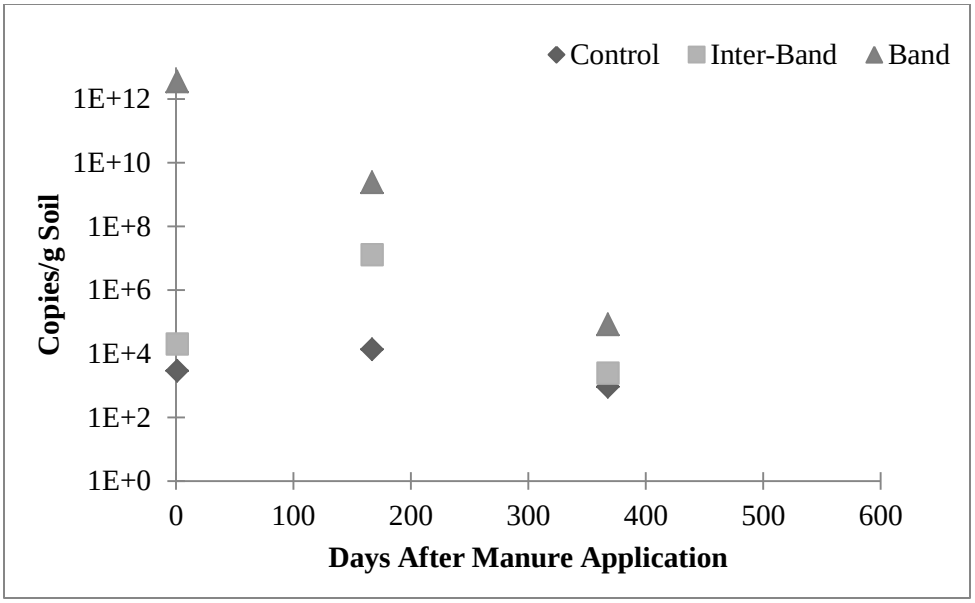

Figure 6: Persistence of *erm*F in soil over 2 years (PSA) from plots receiving manure in 2010. No manure band samples were taken in spring of 2012 as the bands were no longer visible and the control and inter-band samples were below detection.

Tile Water

The relative abundance of both *erm*B (Figure 7) and *erm*F (Figure 8) in tile water was low. *Erm*B was detected in 93% of tile water samples in the first year (2010), and 60% in the second year (2011) with a two year mean concentration of 9.0 x 10^3 copies/100 mL. *Erm*F was detected in 35% of tile water samples in the first year, and 27% in the second year with a two year mean concentration of 2.4 x 10^5 copies/100 mL. There was no correlation ($r<0.5$) between *erm*B or *erm*F concentrations relative to flow or time after manure application. There was also no significant statistical difference between the tillage or treatment for each year. Furthermore, *erm* gene concentrations were significantly less in 2012 when compared to 2011, likely due to the difference in tile flow (an average of 40% less between 2011 and 2012). Even though four different plots were used in each study year, the tillage and treatment practices were the same in both years. Both Figure 7 and Figure 8 show that *erm*B and *erm*F were found in drainage water from the control plots as well as drainage water from plots receiving manure. This was expected since the concentration of *erm* genes in soil also showed a level of background concentrations. Figure 7 and Figure 8 also show that time after manure application did not have an effect on the concentration of *erm* genes present in the

water. As mentioned previously, the data from the *erm* gene analysis potentially underestimates concentrations in tile water during an average flow year, where it is likely that there would have been more transport through the tile lines and/or macropore flow in the no-till over chisel plow plots.

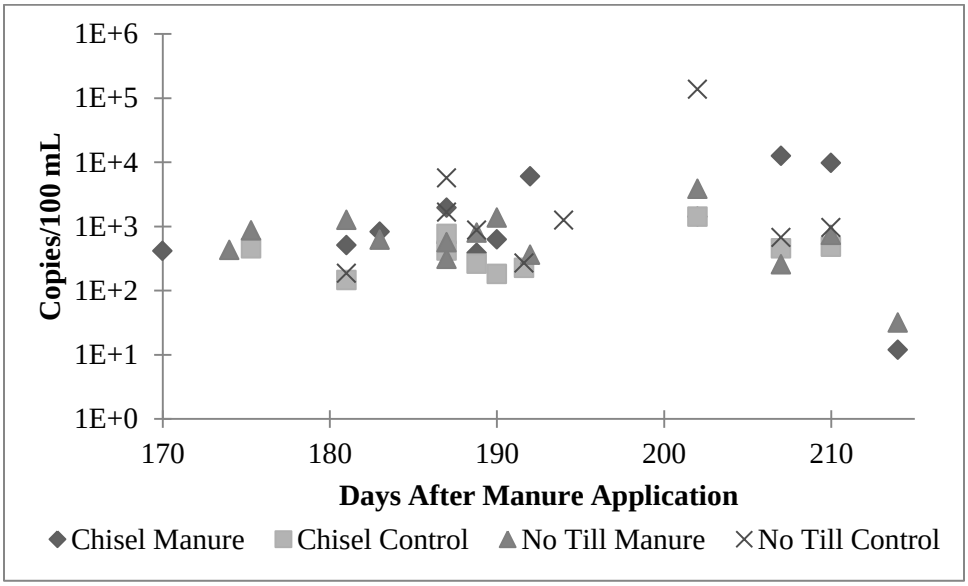

Figure 7: ermB in tile water in 1st year after manure application for PSA and PSB. The x-axis is time after manure application for both plot systems.

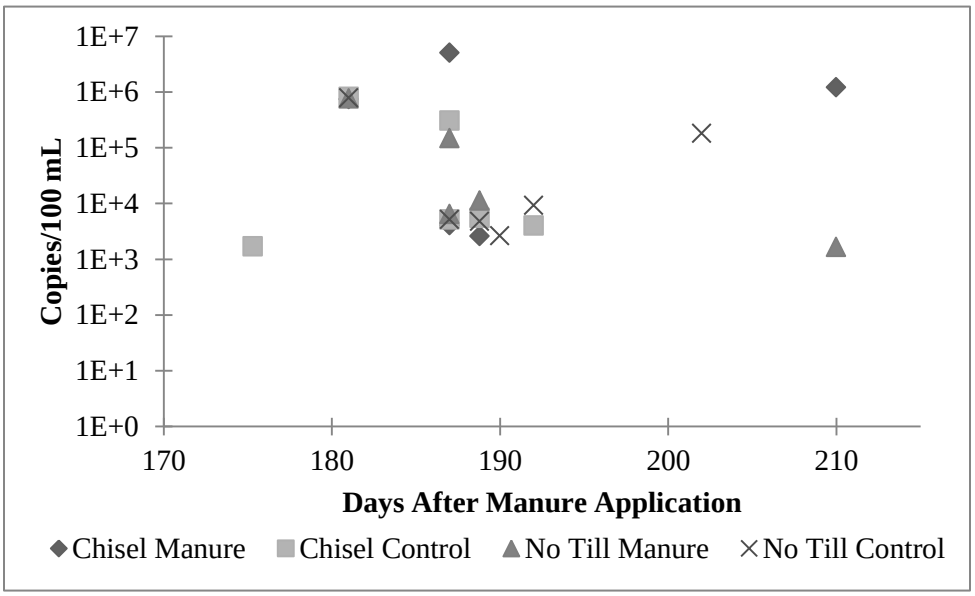

Figure 8: *erm*F in tile water in 1st year after manure application for PSA and PSB. The x-axis is time after manure application for both plot systems.

To date, no other published study has quantified the occurrence of *erm* genes in tile water. However, groundwater samples have tested positive for *erm*B and *erm*F, among other genes in recent studies (Koike et al., 2007; Bockelmann et al., 2009). Bockelmann (2009) detected *erm*B in groundwater at concentrations ranging from 4 x 10^3-1.35 x 10^5 copies/ 100 mL while Koike et al. (2010) detected both *erm*B and *erm*F in shallow groundwater wells near swine lagoons. *Erm*F was always less than the quantification limit of 36 copies/100 mL, whereas nine samples of *erm*B were within the detection range of 40-4 x 10^8 copies/100 mL (Koike et al., 2010).

3.3.3 Quantification of Tylosin

Manure

Tylosin A was detected in manure applied to plots in both 2010 and 2011 (PSA and PSB). Manure samples had an average tylosin concentration of 17±1.5 ng/g (ppb) in PSA and 128±19 ng/g in PSB. The difference in concentration between the two years might be attributed to the animal rotation at the swine facility as there are approximately 2.5 turns/year with tylosin being administered 16 out of 20 weeks per turn; manure applied on PSA might be from the beginning of a new dosage cycle, which would have lower amounts of tylosin in the excreted manure. Dolliver and Gupta (2008a) quantified tylosin at levels ranging from 0.4-4.9 µg/g in swine manure while Kolz et al. (2005a) reported concentrations of tylosin B and D ranging from 50-1700 ppb and 15-270 ppb, respectively in swine lagoons. The concentrations found in the present study are significantly less. This might be due to the administration practice (no indication of the tylosin administration rate to hogs was presented in either previously mentioned study). The administration rate of 40 g/ton is within the typical range, and therefore these concentrations are potentially indicative of the concentrations from a finishing facility. Teeter and Meyerhoff (2003) reported a mean concentration of tylosin of 62.8 µg/g (ppm) in spiked swine manure samples and 4.1 µg/g in 30 day old samples. Kolz et al. (2005b) also demonstrated rapid loss of tylosin in swine manure, which also might explain some of the differences in results reported in the present study.

Soil

In soil, tylosin concentrations were affected by the treatment and year. Mean concentrations (including the non-detects) for the manure band, inter-band and controls for 2010 were 1.33 ng/g (ppb), 0.22 ng/g, and 0.09 ng/g respectively. There is no statistical difference in concentrations between the inter-band and band, and no difference between the inter-band and control. For 2011, the mean concentrations were 0.97 ng/g, 0.34 ng/g, and 0.37 ng/g respectively, with no statistical difference between the three means. The mean concentrations of tylosin in soils across the two-year study of the PSA for the manure band, inter-band and controls were 1.17 ng/g, 0.79 ng/g, and 0.57 ng/g respectively, and there was no statistical difference between the three means over the two years. The measured concentrations of antibiotics in soil are often significantly less, if found at all, than in manure samples (Halling-Sorensen et al., 2005; Martinez-Carballo et al., 2007; Zhou et al., 2010). Concentrations of tylosin A in swine manure amended soil in Denmark ranged from 25-50 x 10^3 µg/g (Halling-Sorensen et al., 2005).

Tile water

The occurrence of tylosin in tile water in all samples tested was less than 1 ng/mL (ppb), as shown in Table 11. In 2010, tylosin was detected frequently, although at low levels. However, in 2011 tylosin was only detected once, which is likely due to the differences in precipitation patterns, as previously discussed. The breakpoint for tylosin resistance is 35 mg/L (FDA, 2009) so it is unlikely that the levels of <1 ng/mL will introduce additional inhibitory effects in the water. However, the concentrations necessary to select for resistance are not nearly so well-known and are potentially well below breakpoints for sensitive isolates. Each genus responds differently and therefore would have a different range of MIC and breakpoints. Portillo et al. (2000) reported a range of MICs from 0.125-128 µg/ml (mg/L) and the concentrations in the present study are less than the lower bound of this range. The limit of detection in 2010 ranged from 0.016 ng/mL for the first 7 samples to 0.0096 ng/mL for the last 8. For 2011, that detection limit was further refined to 0.0024 ng/mL by improvement of the signal to noise ratio, which made it easier to interpret the analyte (tylosin A) response. Concentrations of tylosin up to 1.2 µg/L (ng/mL) have been detected in tile flow (Dolliver and Gupta, 2008a). Kay (2004), however, was unable to detect

tylosin in tile-drained clay soil at a quantification limit of 0.35 µg/L(ng/mL). Concentrations of tylosin from samples collected in this study were between these two extremes, possibly because of the weekly and post-event collection schedule.

Table 11: Mean concentrations (ng/mL) of tylosin in tile water in the first year after application for PSA and PSB.

		Chisel w/ Manure	Chisel Control	No Till w/ Manure	No Till Control
PSA	Mean of detects†	0.20	0.24	0.03	0.04
	Mean of all data‡	0.15	0.21	0.01	0.02
PSB	Mean of detects†	-	-	-	0.004
	Mean of all data‡	-	-	-	0.0004

† Samples are a mean of only the samples above the detection limit
‡ Samples are a mean of all samples, using ½ of the detection limit for those falling below the detection limit

3.4 Discussion

It was expected to find higher concentrations of enterococci, tylosin-resistant enterococci, *erm* genes, and tylosin in the manured plots, and in drainage water from the manured plots. It was also expected to find higher concentrations of these parameters in tile water samples collected from the no-till plots due to advanced macropore development. No-till fields typically have increased macropore connectivity due to more worm holes and root channels than a tilled field; this enhances movement of water and bacteria through soil (Shipitalo et al., 2000). The capacity of pathogen transport to subsurface waters is greater though no-till plots than conventional tillage (Gagliardi and Karns, 2000) indicating that a balance must be found between soil loss and pathogen transport. However, neither a relationship due to treatment or tillage was observed in this study, which is likely attributed to the drier conditions that the research farm experienced during the study period. These dry conditions likely increased bacterial die off in the manure and in the soil after application. This reduces the host bacteria for the *erm* genes, and possible reduces selective pressure of the tylosin in both the manure and soil. Under more normal conditions, these processes would still exist, but possibly at different rates.

The results of the total and tylosin-resistant enterococci can be compared to previous studies on the occurrence of ARB in the manure, soil and tile water. Resistant bacteria

occurred naturally in organic manure (Jindal et al., 2006) and in soil (Onan and LaPara, 2003). In the same study, Onan and LaPara (2003) found that an increased proportion of tylosin-resistant bacteria were detected in fields amended with cattle, swine and chicken manure (25% resistant) associated with sub therapeutic use of antibiotics when compared with fields where only organic manure was applied (2% resistant). Andrews et al. (2004) reported a five order of magnitude decrease in fecal enterococci concentration after five weeks in inoculated soil. Of the water samples from the non-manured control plots in the present study, less than 8% of the samples had detectable resistant enterococci. Low levels of tylosin-resistant bacteria in the control plot soils are not completely unexpected considering that tylosin is a natural product of *Streptomyces fradiae* in soil (Sarmah et al., 2006) and ARBs have been previously detected in environments not obviously impacted by animal agriculture (Allen et al., 2010). The use of tylosin at subtherapeutic concentrations selects and increases resistance to macrolides in enterococci living in the intestinal tract of pigs (Aarestrup and Carstensen, 1998), but the results of this study indicate that a very small fraction of these resistant enterococci are present in tile drainage water samples, potentially due to dry conditions.

Since higher concentrations of enterococci, tylosin-resistant enterococci, *erm*B, and *erm*F were found in manure band soils, it was expected to find higher concentrations in the tile water from manured plots. However, this was not the case. The manure band was estimated to be 4 cm in width. For the 0.4 ha plot, the total "treated" soil is less than 1% of the surface area of the plot. Therefore, a significant area of the plot essentially received no manure and likely explains why the concentrations of enterococci, tylosin-resistant enterococci, *erm*B and *erm*F in tile water from manured and control plots were not distinctly different. However, this small area had a relative *erm* gene abundance of 2-3 log units greater than the inter-bands or controls. As previously mentioned, the greatest effect on the *erm* gene concentrations in water was not a function of treatment, but the year, 2011 or 2012. Therefore, the results of this two year study (under dry conditions) show that manure application does not increase gene abundance in tile water.

It was expected to see natural fluctuations of *erm* gene concentrations in both soil and tile water due to seasonal and environmental effects in the soil. Such factors might include

ambient air temperature, or precipitation. The occurrence of *erm* genes in tile water from both manured and manure-free plots, suggests that the organisms found in the manure in which the *erm* genes reside are not well adapted to the soil environment. However, the search of the NCBI nucleotide database indicated that enterococcus was one of the bacterial hosts of the *erm* genes following PCR product sequencing. The field samples showed no difference in gene abundance between the manured and manure-free soils and did not confirm the hypothesis of a significant treatment effect. This also might explain why such a significant decrease in *erm* gene concentration was observed over time in the manure band soils. Koike (2010) attributed the reduction in *erm* gene prevalence in groundwater from manure storage pits to the limited dispersal of *erm*-hosting bacteria, which is likely the case in tile drainage water as well.

The results of the *erm* gene analysis is consistent with a study by Hoang (2010) who used conventional PCR on samples from the same research farm. Hoang (2010) detected *erm*B, *erm*F and *erm*T in 69%, 78% and 9.5% of 200 isolates from manure, soil and water samples. However, *erm*B was the most prevalent macrolide resistant gene in this study, followed by *erm*F. The prevalence of *erm*B in animal manures was described previously (Chen et al., 2007; Graham et al., 2009a). *Erm*F was found less frequently than *erm*B, which is similar to the study by Chen (2007) where *erm*F was only slightly lower than *erm*B.

In previous studies, tylosin was not detected in leachates or soil after it was amended with manure slurry from swine operations feeding at rates of 100 g per ton of feed (Kay et al., 2005c; Kay et al., 2005b). Similarly, tylosin was shown to have little risk of accumulation in soil or groundwater when applied to soils in slurry (Blackwell et al., 2007; Blackwell et al., 2009). Furthermore, Kay (2004) did not find tylosin in tile-drained clay soil. Some (Allaire et al., 2006; Hu and Coats, 2009; Heuer et al., 2011) have suggested that the binding of the antibiotics to the soil is likely facilitating a gross underestimation of the actual concentrations in soil due to limitations of the extraction procedure to unbind the antibiotic compound from the soil matrix. However, since recoveries in soil in the present study were near 90%, it is unlikely that this was the case. Tylosin concentrations were less than the detection limit of 2 ng/g soil in silt loam fields under either organic or swine manure application (Zhou et al., 2010).

It is likely that tylosin was not effectively transported to the tile lines because of the high sorption and low desorption characteristics of tylosin on soil (Clay et al., 2005). Tylosin was found to be immobile in clay loam soils in a study by ter Laak (2006), similar to the findings of the present study. Previous studies have reported average half-lives of tylosin as 2-8 days in swine manure and soil–manure mixtures; and 10-40 days in surface water simulation systems (Loke et al., 2000; Ingerslev and Halling-Sorensen, 2001; Ingerslev et al., 2001; Teeter and Meyerhoff, 2003). However, the half-life might increase substantially if the tylosin was tightly sorbed to soils (Clay et al., 2005). This may account for some of the high persistence of tylosin throughout the growing season in the tile water seen in the first year after manure application.

There are many factors that might also impact the leaching of total and tylosin-resistant enterococci, *erm* genes, and tylosin from waste-amended soils including waste characteristics or manure storage. The manure was stored in a waste pit for up to nine months before it was applied, leaving a potentially large window to facilitate conditions in which the bacteria and tylosin would dissipate in the system, similar to previous findings (Teeter and Meyerhoff, 2003; Kolz et al., 2005a). Based on the findings of Teeter and Meyerhoff (2003) as described previously, the time the manure spent in the lagoon presumably has a direct relationship to the final concentration applied on the field.

While the underlying goal of this study was to evaluate the movement of total and tylosin-resistant enterococci, *erm* genes and tylosin under drained conditions, the larger goal is to determine the effects of long-term manure application on the environment. In other words, has tylosin use by the swine industry led to an increase of tylosin and tylosin resistance in agricultural soil greater than natural background levels? According to Figure 5 and Figure 6, it appears that *erm*B and *erm*F persist in manure amended soils in concentrations greater than controls for at least 1 year. There was no significant difference in concentrations between the control and the inter-band samples for *erm*B, but there was for *erm*F over two years. In the soil samples within 1 year of manure application, an increased level of *erm* genes was found in the manure band relative to the background levels in the controls. Over time, the manure band concentrations decreased to equivalent levels in the controls. This is likely due to a reduction in *erm*-hosting bacteria in the soil following manure

application, even though the manure had high concentrations of both *erm* genes. The same trend was seen in the decline of total enterococci populations over time potentially due to die off and other environmental factors. Zhou et al. (2010) reported that land application of swine manure did not increase MLS resistance in soils over time in manured plots over controls (both no manure application or no antimicrobial use), which was not entirely the case in the present study.

The results of this study suggest that the use of tylosin in swine production is causing an increase in *erm* genes at the field level greater than background concentrations in soils receiving manure. This increase has a high level of seasonal variability, as previously shown in Table 9, Table 10, Figure 5, and Figure 6. Tylosin concentrations are very low in the soil and water, and do not likely impact the selective pressures on *erm* genes in either matrix. *Erm* gene concentrations in tile water are not different between treatments. While the use of tylosin over time has potentially increased abundance of tylosin resistance genes in soils, the results of this study indicate that this increase is not transported into drainage water under dry conditions. However, according to recommendations by Schilling and Helmers (2008), the effects of tile drainage on watershed hydrology are important in water quality monitoring. Perhaps, the further consideration of hydrologic parameters would provide more information to predict bacterial and *erm* gene transport patterns on a field scale.

3.5 Conclusions

The results of this study suggest that tylosin usage has increased the short-term occurrence of total and tylosin-resistant enterococci, *erm* genes, and tylosin in soils, but has had minimal effect on tile drainage water quality under dryer than average conditions. Average concentrations of enterococci in manure were 56,571 cfu/g and 8,635 cfu/g for year 1 and year 2, respectively and nearly 75% of the enterococci were resistant to tylosin. In soil, mean enterococci concentrations ranged from 22 cfu/g to 356 cfu/g and tylosin-resistant concentrations ranged frm 1-134 cfu/g. Concentrations of enterococci in tile water were low, and rarely exceeded the geometric mean for recreational waters while tylosin-resistant enterococci was rarely detected. No effect of tillage was found in either year.

Erm genes were detected in concentrations comparable to recent studies in the manure, and in elevated concentrations in the soil manure injection zone relative to inter-

band or no-manure soil. Drainage water from manured soils and non-manured soils, as well as no till and chisel plow fields had similar concentrations of *erm* genes. In the manure, *erm*B concentrations averaged 7.96 x 10^8 copies/g and 6.44 x 10^{12} copies/g in the first and second study years, respectively. The mean *erm*F concentrations were 6.94 x 10^7 copies/g and 4.67 x 10^{12} copies/g in the first and second study years. No *erm*T was detected in manure, soil or water. The mean concentrations of *erm*B across the two-year study period for the manure band, inter-band and controls was 4 x 10^8 copies/g, 1 x 10^6 copies/g, and 2 x 10^5 copies/g respectively. The mean concentrations of *erm*F across the two-year study period for the manure band, inter-band and controls was 1 x 10^{12} copies/g, 3 x 10^6 copies/g, and 4 x 10^3 copies/g respectively. The relative abundance of *erm*B and *erm*F in tile water was low; e*rm*B was detected in approximately 75% of tile water samples and *erm*F was detected in 30% of tile water samples. Two year mean concentrations for *erm*B and *erm*F in tile water were 9.0 x 10^3 copies/100 mL and 2.4 x 10^5 copies/100 mL, respectively.

In manure, the two-year mean concentration of tylosin was 73 ng/g (ppb). The mean concentrations of tylosin in soils over two-years for the manure band, inter-band and controls were 1.17 ng/g, 0.79 ng/g, and 0.57 ng/g respectively, with no difference between the three means. In tile water, mean tylosin concentrations in all samples tested was less than 1 ng/mL (ppb) and no difference was found between both tillage practices.

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CHAPTER 4: CONCLUSIONS

4.1 General Discussion and Conclusions

Field and laboratory experiments were conducted to detect and quantify the transport of total and tylosin-resistant enterococci, *erm* genes and tylosin from liquid swine manure, through no-till or chisel plow soils, and into the tile drainage system. Specifically, the goal of this research project was to further understand the occurrence and movement of total and tylosin-resistant enterococci, *erm* genes and tylosin in tile-drained agricultural fields that have received multi-year application of liquid swine manure through injection by:

1. In liquid swine manure, soil and tile drainage water
   a. Quantify the occurrence of total and tylosin-resistant enterococci
   b. Quantify the occurrence of *erm*B, *erm*F and *erm*T
   c. Quantify tylosin
2. Compare occurrence of tylosin, total and tylosin-resistant enterococci and resistance genes between no-till and chisel plow fields relative to controls.
3. Compare the quantity of total and tylosin-resistant enterococci, *erm*B, *erm*F, *erm*T, and tylosin in liquid swine manure, soil and tile drainage water over two years

The results of this study suggest that tylosin usage has increased the short-term occurrence of total and tylosin-resistant enterococci, *erm* genes, and tylosin in soils, but has had minimal effect on tile drainage water quality under dryer than average conditions. Average concentrations of enterococci in manure were 56,571 cfu/g and 8,635 cfu/g for year 1 and year 2, respectively and nearly 75% of the enterococci were resistant to tylosin. In soil, mean enterococci concentrations ranged from 22 cfu/g to 356 cfu/g and tylosin-resistant concentrations ranged frm 1-134 cfu/g. Concentrations of enterococci in tile water were low, and rarely exceeded the geometric mean for recreational waters while tylosin-resistant enterococci was rarely detected. No effect of tillage was found in either year.

Erm genes were detected in concentrations comparable to recent studies in the manure, and in elevated concentrations in the soil manure injection zone relative to inter-band or no-manure soil. Drainage water from manured soils and non-manured soils, as well

as no till and chisel plow fields had similar concentrations of *erm* genes. In the manure, *erm*B concentrations averaged 7.96 x 10^8 copies/g and 6.44 x 10^{12} copies/g in the first and second study years, respectively. The mean *erm*F concentrations were 6.94 x 10^7 copies/g and 4.67 x 10^{12} copies/g in the first and second study years. No *erm*T was detected in manure, soil or water. The mean concentrations of *erm*B across the two-year study period for the manure band, inter-band and controls was 4 x 10^8 copies/g, 1 x 10^6 copies/g, and 2 x 10^5 copies/g respectively. The mean concentrations of *erm*F across the two-year study period for the manure band, inter-band and controls was 1 x 10^{12} copies/g, 3 x 10^6 copies/g, and 4 x 10^3 copies/g respectively. The relative abundance of *erm*B and *erm*F in tile water was low; e*rm*B was detected in approximately 75% of tile water samples and *erm*F was detected in 30% of tile water samples. Two year mean concentrations for *erm*B and *erm*F in tile water were 9.0 x 10^3 copies/100 mL and 2.4 x 10^5 copies/100 mL, respectively.

In manure, the two-year mean concentration of tylosin was 73 ng/g (ppb). The mean concentrations of tylosin in soils over two-years for the manure band, inter-band and controls were 1.17 ng/g, 0.79 ng/g, and 0.57 ng/g respectively, with no difference between the three means. In tile water, mean tylosin concentrations in all samples tested was less than 1 ng/mL (ppb) and no difference was found between both tillage practices.

4.2 Implications and Recommendations for Future Research

The results of this study suggest that tylosin usage has increased the short-term occurrence of tylosin-resistant enterococci, *erm* genes and tylosin in soils, but has had minimal effect on tile drainage water quality. However, this study only assessed tile waters on approximately a weekly basis. It is likely that these samples underestimate the total and tylosin-resistant enterococci, *erm* genes and tylosin during high tile flow after a precipitation event. Both study years were dryer than average, so it is also likely that higher concentrations of total and tylosin-resistant enterococci, *erm* genes and tylosin would be found during a wet year. Specifically, the following research points should potentially be considered in the future:

- Determine total and tylosin-resistant enterococci, *erm* genes and tylosin concentrations on a flow-weighted basis following precipitation events
- Assess total and tylosin-resistant enterococci, *erm* genes and tylosin concentrations from full-scale field (possibly quarter-section) samples to validate the results of the present study

APPENDIX A: SAMPLING DATES AND PROCESSING

Table 12: Sample Collection and Processing Dates

Collection Date	Sample Type	Number of Samples	Sample Processed Within/on		
			Bacterial Analysis[a]	DNA Extraction[b]	Tylosin Extraction
October 28, 2010	Manure	3	24 hours	7/12-28/2011[c]	7/12-28/2011[c]
October 29, 2010	Soil	18	24 hours	7/12-28/2011[c]	7/12-28/2011[c]
March 23, 2011	Water	4	24 hours	24 hours	72 hours[d]
April 13, 2011	Soil	18	24 hours	7/12-28/2011[c]	7/12-28/2011[c]
April 26, 2011	Water	4	24 hours	24 hours	72 hours[d]
April 27, 2011	Water	4	24 hours	24 hours	72 hours[d]
May 3, 2011	Water	4	24 hours	24 hours	72 hours[d]
May 18, 2011	Water	4	24 hours	24 hours	72 hours[d]
May 23, 2011	Water	4	24 hours	24 hours	72 hours[d]
May 26, 2011	Water	4	24 hours	24 hours	72 hours[d]
June 1, 2011	Water	4	24 hours	24 hours	72 hours[d]
June 8, 2011	Water	4	24 hours	24 hours	72 hours[d]
June 10, 2011	Water	4	24 hours	24 hours	72 hours[d]
June 15, 2011	Water	4	24 hours	24 hours	72 hours[d]
June 22, 2011	Water	4	24 hours	24 hours	72 hours[d]
June 29, 2011	Water	4	24 hours	24 hours	72 hours[d]
July 6, 2011	Water	3	24 hours	24 hours	72 hours[d]
October 28, 2011	Manure	2	72 hours	6/7/2012	2/13-3/31/12
October 31, 2011	Soil	36	24 hours	2/13-3/30/12	2/13-3/31/12
March 14, 2012	Soil	30	24 hours	2/13-3/30/12	2/13-3/31/12
April 15, 2012	Water	5	24 hours	24 hours	72 hours[d]
April 19, 2012	Water	6[e]	24 hours	24 hours	72 hours[d]
April 20, 2012	Water	5	24 hours	24 hours	72 hours[d]
April 28, 2012	Water	4	24 hours	24 hours	72 hours[d]
May 2, 2012	Water	7	24 hours	24 hours	72 hours[d]
May 3, 2012	Water	7[e]	24 hours	24 hours	72 hours[d]
May 5, 2012	Water	8	24 hours	24 hours	72 hours[d]
May 7, 2012	Water	8[e]	24 hours	24 hours	72 hours[d]
May 9, 2012	Water	8	24 hours	24 hours	72 hours[d]
May 15, 2012	Water	8	24 hours	24 hours	72 hours[d]
May 23, 2012	Water	8	24 hours	24 hours	72 hours[d]
May 26, 2012	Water	8[e]	24 hours	24 hours	72 hours[d]
May 29, 2012	Water	8	24 hours	24 hours	72 hours[d]
June 6, 2012	Water	7	24 hours	24 hours	72 hours[d]
June 11, 2012	Water	5	24 hours	24 hours	72 hours[d]

[a] Total and tylosin-resistant enterococci plates were incubated for 48 hours and counted
[b] DNA was frozen and analyzed by qPCR September 6-11, 2011
[c] Tylosin manure/soil samples were frozen until TYL and DNA extraction July 12-28, 2011
[d] Tylosin water samples were extracted within 72 hours of sample collection
[e] ISCO sample was used in place of grab sample for that time

APPENDIX B: FIELD SITE DESCRIPTION

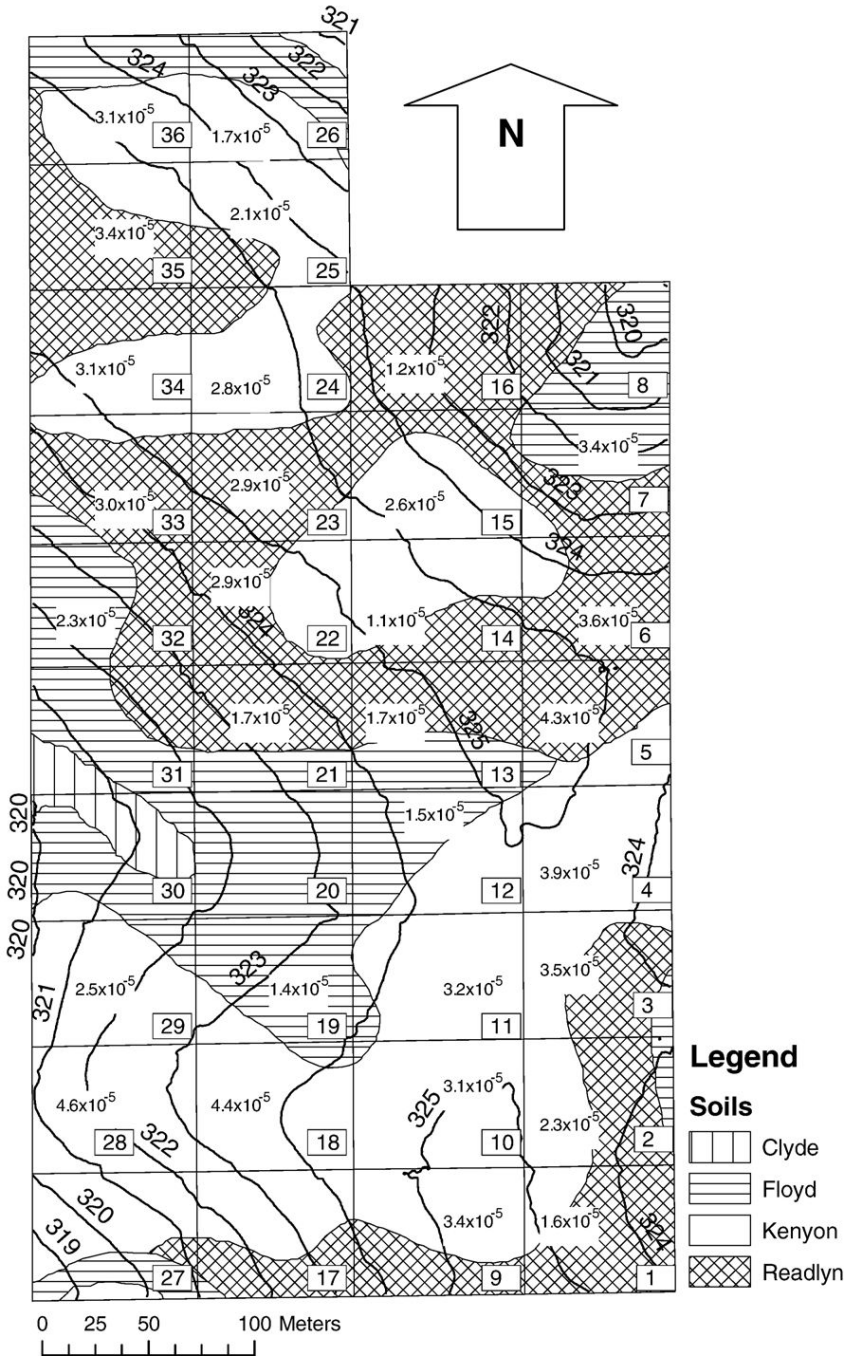


Figure 9: Schematic of the 36 plots at the ISU Northeast Research Farm.
The plots used in this study were 23, 24, 25, 34, 19, 20, 29, and 30. Lines are digitized elevation and scientific numbers in each plot are calibrated lateral hydraulic gradients. Source: L. Ma et al (2007)

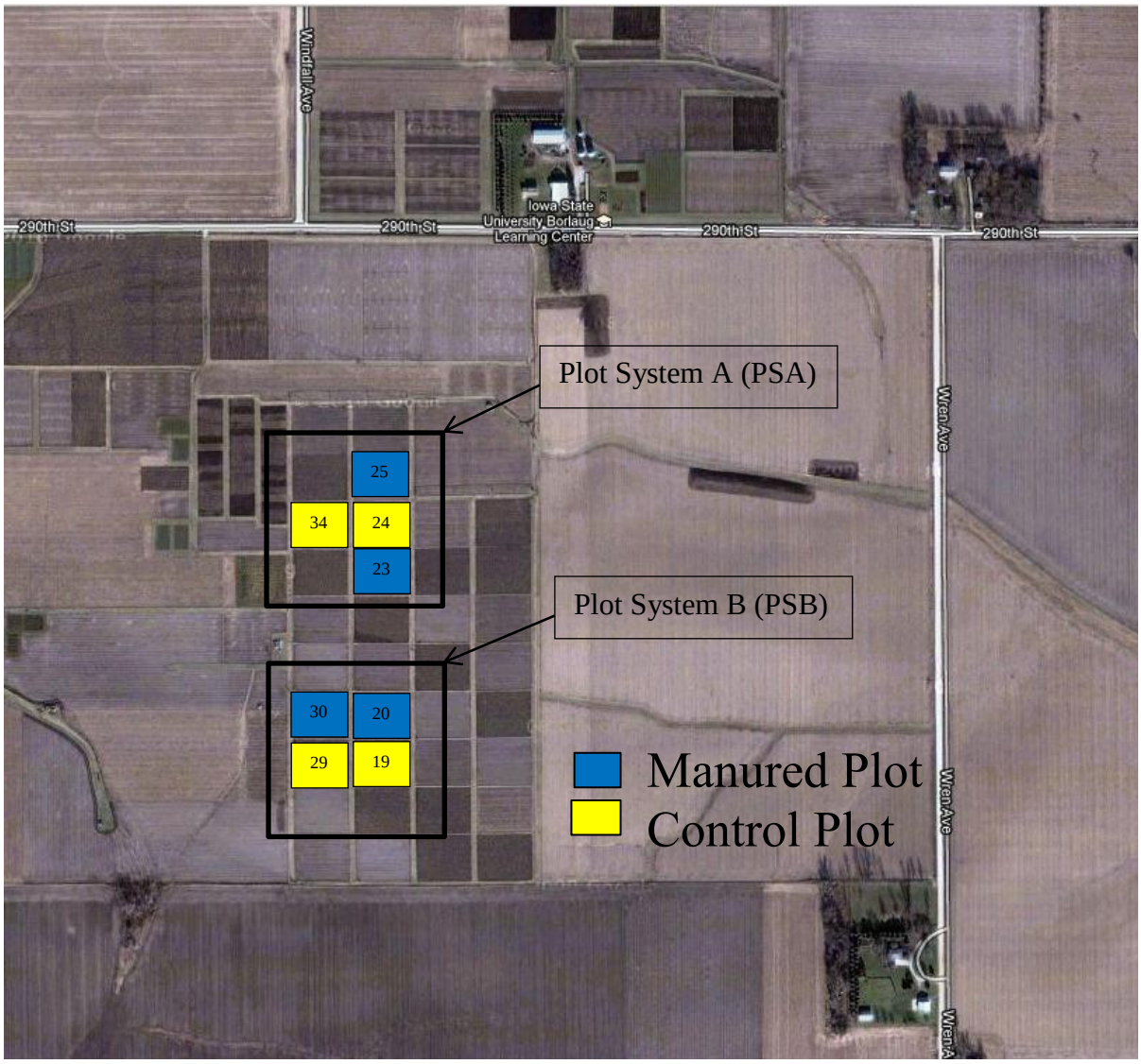


Figure 10: Aerial image of the 36 plots at the ISU Northeast Research Farm with the study plots outlined. The plots used in this study were 23, 24, 25, 34, 19, 20, 29, and 30.

Table 13: List of plots in the study

	Plot	Tillage	Type
2010 manure application plots (PSA)	23	Chisel Plow	Manure
	24	Chisel Plow	Control
	25	No Till	Manure
	34	No Till	Control
2011 manure application plots (PSB)	19	No Till	Control
	20	No Till	Manure
	29	Chisel Plow	Control
	30	Chisel Plow	Manure

APPENDIX C: RAW DATA

Soil Sample Collection Notes and Labels

October 29, 2010
Manure applied: 10/28/2010

Plot	Rep	Position	Treatment	Lab Code	%Moisture	ww/dw
24	A		Control	1	15.79	1.19
24	B		Control	2	16.66	1.20
24	C		Control	3	15.33	1.18
34	A		Control	4	14.89	1.17
34	B		Control	5	15.09	1.18
34	C		Control	6	15.53	1.18
23	A	NO-BAND	Manure	7	15.97	1.19
23	B	NO-BAND	Manure	8	14.90	1.18
23	C	NO-BAND	Manure	9	15.54	1.18
25	A	NO-BAND	Manure	10	15.43	1.18
25	B	NO-BAND	Manure	11	15.84	1.19
25	C	NO-BAND	Manure	12	15.50	1.18
23	A	BAND	Manure	13	18.85	1.23
23	B	BAND	Manure	14	21.12	1.27
23	C	BAND	Manure	15	19.42	1.24
25	A	BAND	Manure	16	18.67	1.23
25	B	BAND	Manure	17	18.13	1.22
25	C	BAND	Manure	18	18.50	1.23
				MI	94.73	18.99
				MII	94.01	16.70
				MIII	94.09	16.93

April 13, 2011
Manure applied: 10/27/2010

Plot	Rep	Position	Treatment	Lab Code	%Moisture	ww/dw
24	A		Control	19	15.76	1.19
24	B		Control	20	14.96	1.18
24	C		Control	21	15.27	1.18
34	A		Control	22	14.72	1.17
34	B		Control	23	14.23	1.17
34	C		Control	24	15.72	1.19
23	A	NO-BAND	Manure	25	15.45	1.18
23	B	NO-BAND	Manure	26	15.06	1.18
23	C	NO-BAND	Manure	27	15.90	1.19
25	A	NO-BAND	Manure	28	17.37	1.21
25	B	NO-BAND	Manure	29	17.11	1.21
25	C	NO-BAND	Manure	30	15.93	1.19
23	A	BAND	Manure	31	15.21	1.18
23	B	BAND	Manure	32	15.67	1.19
23	C	BAND	Manure	33	17.11	1.21
25	A	BAND	Manure	34	17.08	1.21
25	B	BAND	Manure	35	16.42	1.20
25	C	BAND	Manure	36	15.98	1.19

October 31, 2011
Manure applied: 10/27/2010

Plot	Rep	Position	Treatment	Lab Code	%Moisture	ww/dw
24	A		Control	37	18.10	1.18
24	B		Control	38	18.12	1.18
24	C		Control	39	17.38	1.17
34	A		Control	40	16.34	1.16
34	B		Control	41	18.28	1.18
34	C		Control	42	17.76	1.18
23	A	NO-BAND	Manure	43	16.98	1.17
23	B	NO-BAND	Manure	44	18.57	1.19
23	C	NO-BAND	Manure	45	17.90	1.18
25	A	NO-BAND	Manure	46	18.55	1.19
25	B	NO-BAND	Manure	47	17.62	1.18
25	C	NO-BAND	Manure	48	19.51	1.20
23	A	BAND	Manure	49	17.49	1.17
23	B	BAND	Manure	50	17.68	1.18
23	C	BAND	Manure	51	17.87	1.18
25	A	BAND	Manure	52	18.89	1.19
25	B	BAND	Manure	53	18.27	1.18
25	C	BAND	Manure	54	17.90	1.18

March 14, 2012
Manure applied: 10/27/2010

Plot	Rep	Position	Treatment	Lab Code	%Moisture	ww/dw
24	A		Control	55	21.87	1.22
24	B		Control	56	23.09	1.23
24	C		Control	57	22.47	1.22
34	A		Control	58	22.56	1.23
34	B		Control	59	22.21	1.22
34	C		Control	60	22.49	1.22
23	A	NO-BAND	Manure	61	22.88	1.23
23	B	NO-BAND	Manure	62	22.55	1.23
23	C	NO-BAND	Manure	63	22.09	1.22
25	A	NO-BAND	Manure	64	23.04	1.23
25	B	NO-BAND	Manure	65	22.12	1.22
25	C	NO-BAND	Manure	66	23.41	1.23

October 31, 2011
Manure applied: 10/28/2011

Plot	Rep	Position	Treatment	Lab Code	%Moisture	ww/dw
19	A		Control	101	16.14	1.16
19	B		Control	102	18.86	1.19
19	C		Control	103	18.53	1.19
29	A		Control	104	15.70	1.16
29	B		Control	105	17.55	1.18
29	C		Control	106	19.36	1.19
20	A	NO-BAND	Manure	107	17.12	1.17
20	B	NO-BAND	Manure	108	19.23	1.19
20	C	NO-BAND	Manure	109	19.13	1.19
30	A	NO-BAND	Manure	11	17.76	1.18
30	B	NO-BAND	Manure	111	18.50	1.18
30	C	NO-BAND	Manure	112	24.09	1.24
20	A	BAND	Manure	113	21.81	1.22
20	B	BAND	Manure	114	21.71	1.22
20	C	BAND	Manure	115	28.76	1.29
30	A	BAND	Manure	116	23.23	1.23
30	B	BAND	Manure	117	23.84	1.2384
30	C	BAND	Manure	118	27.96	1.27955
				MIV	95.15	20.6259
				MV	95.15	20.60

March 14, 2012
Manure applied: 10/28/2011

Plot	Rep	Position	Treatment	Lab Code	%Moisture	ww/dw
19	A		Control	119	24.87	1.25
19	B		Control	120	23.09	1.23
19	C		Control	121	22.19	1.22
29	A		Control	122	24.48	1.24
29	B		Control	123	22.92	1.23
29	C		Control	124	22.62	1.23
20	A	NO-BAND	Manure	125	22.97	1.23
20	B	NO-BAND	Manure	126	26.78	1.27
20	C	NO-BAND	Manure	127	22.02	1.22
30	A	NO-BAND	Manure	128	27.72	1.28
30	B	NO-BAND	Manure	129	17.91	1.18
30	C	NO-BAND	Manure	130	22.70	1.23
20	A	BAND	Manure	131	27.22	1.27
20	B	BAND	Manure	132	24.60	1.25
20	C	BAND	Manure	133	30.84	1.31
30	A	BAND	Manure	134	25.46	1.25
30	B	BAND	Manure	135	24.74	1.25
30	C	BAND	Manure	136	24.52	1.25

Manure ENT

Manure Sample Data

October 29, 2010													
		Count (Ent)				C.F.U / gram ww	C.F.U / gram dw	Count (Tyl-Ent)				C.F.U / gram ww	C.F.U / gram dw
Sample	Dilution	1	2	3	Average			1	2	3	Average		
MI	10^-3	57	53	40	50	5000	94954	38	48	37	41	4100	77862
MII	10^-3	15	12	10	12	1233	20592	4	5	3	4	400	6678
MIII	10^-3	25	35	36	32	3200	54166	19	25	17	20	2033	34418
					average		56571				average		39653

October 29, 2011													
		Count (Ent)				C.F.U / gram ww	C.F.U / gram dw	Count (Tyl-Ent)				C.F.U / gram ww	C.F.U / gram dw
Sample	Dilution	1	2	3	Average			1	2	3	Average		
MIV	10^-2	49	35	76	53	533	11001	61	64	60	62	617	12719
MV	10^-2	51	22	27	33	333	6867	46	39	47	44	440	9064
					average		8934				average		10892

Soil ENT

Fall 2010 Soil-Plots 23,24,25,34

Plate Code	ENT Bacteria Count (ENT=AA, ENT+TYL=TA)				C.F.U / gram ww	C.F.U / gram dw
	1	2	3	Average		
1 AA	0	0	1	0.33	3	4
2 AA	0	0	1	0.33	3	4
3 AA	3	0	3	2.00	20	24
4 AA	0	4	3	2.33	23	27
5 AA	0	2	0	0.67	7	8
6 AA	5	5	9	6.33	63	75
7 AA	6	8	7	7.00	70	83
8 AA	7	7	6	6.67	67	78
9 AA	9	7	8	8.00	80	95
10 AA	2	10	5	5.67	57	67
11 AA	0	6	9	5.00	50	59
12 AA	3	11	8	7.33	73	87
13 AA	78	55	69	67.33	673	830
14 AA	62	76	60	66.00	660	837
15 AA	70	65	64	66.33	663	823
16 AA	62	68	77	69.00	690	848
17 AA	53	76	54	61.00	610	745
18 AA	68	66	79	71.00	710	871
1 TA	0	0	0	0.00	0	0
2 TA	0	0	0	0.00	0	0
3 TA	0	0	0	0.00	0	0
4 TA	0	0	0	0.00	0	0
5 TA	0	0	0	0.00	0	0
6 TA	0	0	0	0.00	0	0
7 TA	0	0	0	0.00	0	0
8 TA	0	0	0	0.00	0	0
9 TA	0	0	0	0.00	0	0
10 TA	0	0	0	0.00	0	0
11 TA	0	0	0	0.00	0	0
12 TA	0	0	0	0.00	0	0
13 TA	0	0	0	0.00	0	0
14 TA	5	3	1	3.00	30	38
15 TA	0	6	9	5.00	50	62
16 TA	8	8	1	5.67	57	70
17 TA	4	0	6	3.33	33	41
18 TA	5	9	1	5.00	50	61

Spring 2011 Soil-Plots 23,24,25,34

Plate Code	ENT Bacteria Count (ENT=AA, ENT+TYL=TA)				C.F.U / gram ww	C.F.U / gram dw
	1	2	3	Average		
19 AA	4	2	24	10.00	100	119
20 AA	2	2	1	1.67	17	20
21 AA	0	6	2	2.67	27	31
22 AA	0	2	2	1.33	13	16
23 AA	0	0	2	0.67	7	8
24 AA	0	0	3	1.00	10	12
25 AA	5	1	0	2.00	20	24
26 AA	2	5	7	4.67	47	55
27 AA	5	4	2	3.67	37	44
28 AA	3	3	4	3.33	33	40
29 AA	3	1	4	2.67	27	32
30 AA	2	1	2	1.67	17	20
31 AA	8	3	5	5.33	53	63
32 AA	17	12	11	13.33	133	158
33 AA	78	54	49	60.33	603	728
34 AA	11	4	3	6.00	60	72
35 AA	37	9	31	25.67	257	307
36 AA	17	10	10	12.33	123	147
19 TA	0	0	0	0.00	0	0
20 TA	0	0	0	0.00	0	0
21 TA	0	0	0	0.00	0	0
22 TA	0	0	0	0.00	0	0
23 TA	0	0	0	0.00	0	0
24 TA	0	0	0	0.00	0	0
25 TA	0	0	0	0.00	0	0
26 TA	0	0	0	0.00	0	0
27 TA	0	0	0	0.00	0	0
28 TA	0	0	0	0.00	0	0
29 TA	0	0	0	0.00	0	0
30 TA	0	0	0	0.00	0	0
31 TA	2	1	0	1.00	10	12
32 TA	3	2	0	1.67	17	20
33 TA	13	16	15	14.67	147	177
34 TA	0	0	0	0.00	0	0
35 TA	7	31	6	14.67	147	175
36 TA	5	3	5	4.33	43	52

Fall 2011 Soil-Plots 23,24,25,34

Plate Code	ENT Bacteria Count (ENT=AA, ENT+TYL=TA)				C.F.U / gram ww	C.F.U / gram dw
	1	2	3	Average		
37 AA	0	0	0	0.00	0	0
38 AA	2	2	2	2.00	20	24
39 AA	3	9	3	5.00	50	59
40 AA	0	0	0	0.00	0	0
41 AA	6	5	6	5.67	57	67
42 AA	2	1	4	2.33	23	27
43 AA	1	0	0	0.33	3	4
44 AA	1	0	0	0.33	3	4
45 AA	4	4	3	3.67	37	43
46 AA	0	0	0	0.00	0	0
47 AA	0	0	0	0.00	0	0
48 AA	0	0	0	0.00	0	0
49 AA	20	29	22	23.67	237	278
50 AA	0	0	0	0.00	0	0
51 AA	1	0	0	0.33	3	4
52 AA	0	2	0	0.67	7	8
53 AA	2	0	0	0.67	7	8
54 AA	1	0	2	1.00	10	12
37 TA	0	0	0	0.00	0	0
38 TA	0	0	0	0.00	0	0
39 TA	0	0	0	0.00	0	0
40 TA	0	0	0	0.00	0	0
41 TA	0	0	0	0.00	0	0
42 TA	0	0	0	0.00	0	0
43 TA	0	0	0	0.00	0	0
44 TA	0	0	0	0.00	0	0
45 TA	0	0	0	0.00	0	0
46 TA	0	0	0	0.00	0	0
47 TA	0	0	0	0.00	0	0
48 TA	1	0	0	0.33	3	4
49 TA	0	0	0	0.00	0	0
50 TA	0	0	0	0.00	0	0
51 TA	0	0	0	0.00	0	0
52 TA	0	0	0	0.00	0	0
53 TA	0	0	0	0.00	0	0
54 TA	0	0	0	0.00	0	0

Spring 2012 Soil-Plots 23,24,25,34

Plate Code	ENT Bacteria Count (ENT=AA, ENT+TYL=TA)				C.F.U / gram ww	C.F.U / gram dw
	1	2	3	Average		
55 AA	0	0	0	0.00	0	0
56 AA	4	2	1	2.33	23	29
57 AA	0	2	5	2.33	23	29
58 AA	1	1	0	0.67	7	8
59 AA	0	0	0	0.00	0	0
60 AA	0	0	0	0.00	0	0
61 AA	3	3	1	2.33	23	29
62 AA	0	0	0	0.00	0	0
63 AA	0	0	0	0.00	0	0
64 AA	0	0	0	0.00	0	0
65 AA	0	0	0	0.00	0	0
66 AA	0	1	0	0.33	3	4
55 TA	0	0	0	0.00	0	0
56 TA	0	0	0	0.00	0	0
57 TA	0	0	0	0.00	0	0
58 TA	0	0	0	0.00	0	0
59 TA	0	0	0	0.00	0	0
60 TA	0	0	0	0.00	0	0
61 TA	0	0	0	0.00	0	0
62 TA	0	0	0	0.00	0	0
63 TA	0	0	0	0.00	0	0
64 TA	0	0	0	0.00	0	0
65 TA	0	0	0	0.00	0	0
66 TA	0	0	0	0.00	0	0

Fall 2011 Soil-Plots 19,20,29,30

Plate Code	ENT Bacteria Count (ENT=AA, ENT+TYL=TA)				C.F.U / gram ww	C.F.U / gram dw
	1	2	3	Average		
101 AA	1	3	0	1.33	13	15
102 AA	0	2	0	0.67	7	8
103 AA	0	0	0	0.00	0	0
104 AA	0	0	0	0.00	0	0
105 AA	1	0	1	0.67	7	8
106 AA	8	3	4	5.00	50	60
107 AA	3	7	7	5.67	57	66
108 AA	5	1	0	2.00	20	24
109 AA	32	40	40	37.33	373	445
110 AA	0	0	0	0.00	0	0
111 AA	0	0	0	0.00	0	0
112 AA	66	33	64	54.33	543	674
113 AA	37	34	31	34.00	340	414
114 AA	4	4	0	2.67	27	32
115 AA	3	38	36	25.67	257	330
116 AA	26	27	34	29.00	290	357
117 AA	42	46	29	39.00	390	483
118 AA	45	30	32	35.67	357	456
101 TA	0	0	0	0.00	0	0
102 TA	0	0	0	0.00	0	0
103 TA	0	0	0	0.00	0	0
104 TA	0	0	0	0.00	0	0
105 TA	0	0	0	0.00	0	0
106 TA	0	0	0	0.00	0	0
107 TA	0	3	6	3.00	30	35
108 TA	0	0	0	0.00	0	0
109 TA	0	0	0	0.00	0	0
110 TA	0	0	1	0.33	3	4
111 TA	0	0	0	0.00	0	0
112 TA	0	0	0	0.00	0	0
113 TA	41	43	37	40.33	403	491
114 TA	8	5	5	6.00	60	73
115 TA	33	51	47	43.67	437	562
116 TA	22	35	24	27.00	270	333
117 TA	38	37	38	37.67	377	466
118 TA	33	55	45	44.33	443	567

Spring 2012 Soil-Plots 19,20,29,30

Plate Code	ENT Bacteria Count (ENT=AA, ENT+TYL=TA)				C.F.U / gram ww	C.F.U / gram dw
	1	2	3	Average		
119 AA	4	2	2	2.67	27	33
120 AA	0	0	0	0.00	0	0
121 AA	0	0	0	0.00	0	0
122 AA	0	0	1	0.33	3	4
123 AA	1	1	1	1.00	10	12
124 AA	0	0	0	0.00	0	0
125 AA	0	0	1	0.33	3	4
126 AA	0	0	0	0.00	0	0
127 AA	0	0	0	0.00	0	0
128 AA	0	0	0	0.00	0	0
129 AA	21	34	20	25.00	250	295
130 AA	14	13	15	14.00	140	172
131 AA	0	1	1	0.67	7	8
132 AA	0	0	0	0.00	0	0
133 AA	0	0	0	0.00	0	0
134 AA	0	0	2	0.67	7	8
135 AA	0	1	1	0.67	7	8
136 AA	1	1	1	1.00	10	12
119 TA	0	0	0	0.00	0	0
120 TA	0	0	0	0.00	0	0
121 TA	0	0	0	0.00	0	0
122 TA	0	0	0	0.00	0	0
123 TA	0	0	0	0.00	0	0
124 TA	0	2	0	0.67	7	8
125 TA	0	0	0	0.00	0	0
126 TA	0	0	0	0.00	0	0
127 TA	0	0	0	0.00	0	0
128 TA	0	0	0	0.00	0	0
129 TA	0	0	0	0.00	0	0
130 TA	0	0	0	0.00	0	0
131 TA	0	0	0	0.00	0	0
132 TA	0	0	0	0.00	0	0
133 TA	0	0	0	0.00	0	0
134 TA	0	0	0	0.00	0	0
135 TA	0	0	0	0.00	0	0
136 TA	0	0	0	0.00	0	0

Water ENT

Plot	Date	ENT Bacteria Count				ENT+TYL Bacteria Count			
		1	2	3	Average	1	2	3	Average
23	4/26/11	87	13	51	50.33	0	0	0	0.00
23	4/27/11	19	9	6	11.33	0	0	0	0.00
23	5/3/11	0	0	0	0.00	1	0	0	0.33
23	5/18/11	0	0	0	0.00	0	0	0	0.00
23	5/23/11	0	0	0	0.00	0	0	0	0.00
23	5/26/11	0	0	0	0.00	0	0	0	0.00
23	6/1/11	0	0	0	0.00	0	0	0	0.00
23	6/8/11	205	198	197	100.00	0	0	0	0.00
23	6/10/11	408	422	396	204.33	0	0	0	0.00
23	6/15/11	3	13	15	10.33	0	0	0	0.00
23	6/22/11	3	1	7	3.67	0	0	0	0.00
23	6/29/11	9	3	4	5.33	0	0	0	0.00
24	4/26/11	50	57	6	37.67	0	0	0	0.00
24	4/27/11	4	14	3	7.00	0	0	0	0.00
24	5/3/11	2	1	1	1.33	0	0	0	0.00
24	5/18/11	5	67	42	19.00	0	0	0	0.00
24	5/23/11	2	0	0	0.33	0	0	0	0.00
24	5/26/11	3	4	9	5.33	0	0	0	0.00
24	6/1/11	0	3	2	1.67	0	0	0	0.00
24	6/8/11	0	0	0	0.00	0	0	0	0.00
24	6/10/11	5	4	2	1.83	0	0	0	0.00
24	6/15/11	15	7	11	11.00	0	0	0	0.00
24	6/22/11	5	3	4	4.00	0	0	0	0.00
24	6/29/11	3	4	7	4.67	0	0	0	0.00
24	7/6/11	6	3	6	5.00	0	0	0	0.00
25	4/26/11	46	115	34	65.00	0	0	0	0.00
25	4/27/11	9	10	11	10.00	0	0	0	0.00
25	5/3/11	2	0	5	2.33	0	0	0	0.00
25	5/18/11	23	41	22	14.33	1	2	3	1.00
25	5/23/11	6	10	16	5.33	0	0	0	0.00
25	5/26/11	7	8	10	8.33	0	0	0	0.00
25	6/1/11	2	1	6	3.00	0	0	0	0.00
25	6/8/11	0	0	0	0.00	0	0	0	0.00
25	6/10/11	0	5	3	1.33	0	0	0	0.00
25	6/15/11	198	246	228	224.00	0	0	0	0.00
25	6/22/11	6	7	5	6.00	1	0	0	0.33

Plot	Date	ENT Bacteria Count				ENT+TYL Bacteria Count			
		1	2	3	Average	1	2	3	Average
25	6/29/11	11	9	4	8.00	0	0	0	0.00
25	7/6/11	1	0	0	0.33	0	0	0	0.00
34	4/26/11	106	209	91	135.33	0	0	0	0.00
34	4/27/11	11	8	17	12.00	0	0	0	0.00
34	5/3/11	1	1	0	0.67	0	0	0	0.00
34	5/18/11	0	0	0	0.00	0	0	0	0.00
34	5/23/11	3	2	1	1.00	0	0	0	0.00
34	5/26/11	44	29	35	36.00	1	1	0	0.67
34	6/1/11	14	2	1	5.67	0	0	0	0.00
34	6/8/11	9	9	14	5.33	0	0	0	0.00
34	6/10/11	63	47	54	27.33	0	1	2	0.50
34	6/15/11	23	26	34	27.67	1	0	0	0.33
34	6/22/11	316	205	196	239.00	1	0	1	0.67
34	6/29/11	2	0	0	0.67	0	0	0	0.00
34	7/6/11	40	36	31	35.67	0	0	0	0.00
19	4/15/12	5	1	2	2.67	0	0	0	0.00
19	5/2/12	313	338	224	291.67	0	0	0	0
19	5/3/12	50	30	74	51.33				0
19	5/5/12				51.33	0	0	0	0
19	5/6/12				5.00				0
19	5/9/12	13	19	17	16.33	1	0	0	0.33
19	5/15/12	4	3	1	2.67	0	0	0	0
19	5/23/12	1	6	1	2.67	0	0	0	0
19	5/26/12				36.00				0
19	5/29/12	81	54	65	66.67	0	0	0	0
19	6/6/12	25	25	21	23.67	0	0	0	0
19	6/11/12	13	6	6	8.33	0	0	0	0
20	4/20/12				0.00				0
20	4/28/12	0	0	0	0.00				0
20	5/2/12	6	12	3	7.00	0	0	0	0
20	5/3/12				1.00				0
20	5/5/12	7	14	11	10.67	0	0	0	0
20	5/7/12				8.00				0
20	5/9/12	4	27	15	15.33	1	0	0	0.33
20	5/15/12	2	1	1	1.33	0	0	0	0
20	5/23/12	0	0	2	0.67	0	0	0	0
20	5/26/12				2.33				0
20	5/29/12	1	4	2	2.33	0	0	0	0

Plot	Date	ENT Bacteria Count				ENT+TYL Bacteria Count			
		1	2	3	Average	1	2	3	Average
20	6/6/12	1	1	1	1	0	0	0	0
29	4/19/12				24.00				0
29	4/20/12				0.00				0
29	5/3/12				0.00				0
29	5/5/12	0.00	0.00	0.00	0.00	0	0	0	0
29	5/6/12				208.00				0
29	5/9/12	17	16	18	17.00	0	0	0	0
29	5/15/12	5	7	7	6.33	0	0	0	0
29	5/23/12	2	0	6	2.67	0	0	0	0
29	5/26/12				69.00				0
29	5/29/12	4	1	0	1.67	0	0	0	0
29	6/6/12	4	1	0	1.67	0	0	0	0
30	4/15/12	0	0	3	1.00	0	0	0	0
30	4/19/12				0.00				0
30	4/20/12				11.50				0
30	4/28/12	0	1	0	0.33	0	0	0	0
30	5/2/12	0	0	0	0.00	0	0	0	0
30	5/3/12				0.00				0
30	5/5/12	26	36	33	31.67	0	0	0	0
30	5/7/12				55.00				0
30	5/9/12	8	9	15	10.67	0	0	0	0
30	5/15/12	1	4	3	2.67	0	0	0	0
30	5/23/12	3	0	0	1.00	0	0	0	0
30	5/26/12				132.00				0
30	5/29/12	1	3	2	2.00	0	0	0	0
30	6/6/12	1	0	0	0.33	0	0	0	0
30	6/11/12	2	9	2	4.333	0	0	0	0
23	4/19/12				1.00				0
23	4/20/12				45.50				0
23	5/2/12	5	3	1	3.00	0	0	0	0
23	5/3/12				0.00				0
23	5/5/12	9	10	12	10.33	0	0	0	0
23	5/6/12				42.00				0
23	5/9/12	1	0	0	0.33	0	0	0	0
23	5/15/12	1	1	0	0.67	0	0	0	0
23	5/23/12	0	0	0	0.00	0	0	0	0
23	5/26/12				84.00				0
23	5/29/12	3	6	13	7.33	0	0	0	0

Plot	Date	ENT Bacteria Count				ENT+TYL Bacteria Count			
		1	2	3	Average	1	2	3	Average
23	6/6/12	0	0	0	0	0	0	0	0
24	4/15/12	1	7	3	3.67	0	0	0	0
24	4/28/12	0	0	0	0.00	0	0	0	0
24	5/2/12	2	8	3	4.33	0	0	0	0
24	5/3/12				0.00				0
24	5/5/12	0	0	0	0.00	0	0	0	0
24	5/6/12				16.00				0
24	5/9/12	1	1	0	0.67	0	0	0	0
24	5/15/12	0	0	0	0.00	0	0	0	0
24	5/23/12	0	0	1	0.33	0	0	0	0
24	5/26/12				3.33				0
24	5/29/12	5	3	2	3.33	0	0	0	0
24	6/6/12	0	0	0	0	0	0	0	0
24	6/11/12	3	0	0	1	0	0	0	0
25	4/15/12	2	3	1	2.00	0	0	0	0
25	4/19/12				10.00				0
25	4/20/12				11.00				0
25	4/28/12	1	0	0	0.33	0	0	0	0
25	5/2/12	5	12	6	7.67	0	0	0	0
25	5/3/12				0.00				0
25	5/5/12	28	15	13	18.67	0	0	0	0
25	5/6/12				182.00				0
25	5/9/12	8	6	11	8.33	0	0	0	0
25	5/15/12	12	23	50	28.33	0	0	0	0
25	5/23/12	20	22	42	28.00	0	0	0	0
25	5/26/12				470.00				0.5
25	5/29/12	13	13	11	12.33	0	0	0	0
25	6/6/12	6	8	7	7	0	0	0	0
25	6/11/12	1	1	0	0.667	0	0	0	0
34	4/15/12	50	24	63	45.67	0	0	0	0
34	4/19/12				2.00				0
34	4/20/12				41.00				0
34	5/2/12	38	43	44	41.67	0	0	0	0
34	5/3/12				5.00				0
34	5/5/12	22	31	33	28.67	0	0	0	0
34	5/6/12				122.00				0
34	5/9/12	9	15	10	11.33	0	0	0	0
34	5/15/12	1	2	2	1.67	0	0	0	0

Plot	Date	ENT Bacteria Count				ENT+TYL Bacteria Count			
		1	2	3	Average	1	2	3	Average
34	5/23/12	4	0	0	1.33	0	0	0	0
34	5/26/12				1000.00				0
34	5/29/12	36	30	30	32.00	0	0	0	0
34	6/6/12	3	2	3	2.67	0	0	0	0
34	6/11/12	0	0	0	0	0	0	0	0

Tile Flow

Total tile flow for first 6 months of each year (January-June) measured in cubic meters.

Plot	2003	2004	2005	2006	2007	2008	2009	2010
19	164.1	79.1	20.6	154.8	375.9	509.1	227.8	164.4
20	379.5	124.5	235.7	217.0	567.0	539.6	442.1	446.1
23	115.0	144.0	69.9	167.9	279.9	438.6	58.0	225.9
24	159.3	187.6	72.7	169.3	236.2	361.1	152.3	181.5
25	184.9	123.2	109.6	156.0	275.9	458.7	52.2	205.0
29	122.7	90.4	19.6	76.9	253.1	490.3	209.8	75.7
30	428.4	277.3	282.7	339.9	604.2	931.8	623.4	523.7
34	177.5	205.2	101.6	162.5	236.9	420.6	165.8	173.5

Plot	2011	2012	10-year average
19	184.0	100.6	198.0
20	567.0	281.8	380.0
23	145.0	102.3	174.7
24	152.7	95.4	176.8
25	195.3	158.9	192.0
29	86.0	32.1	145.7
30	672.1	324.7	500.8
34	99.2	118.5	186.1

Precipitation

Total precipitation, in cm, for the first 6 months of each year (January-June) based on the ISU Mesonet weather station for Nashua, IA (Iowa State University Department of Agronomy, 2012).

2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	Average
37.9	53.6	45.5	26.3	33.3	57.0	35.8	32.6	30.8	21.2	37.4

ErmB

ErmB soil & manure data calculation/summary 2010-2011

Label	Average Copy	StDev	Copy/gram dry	Average Copy/gram dw	StDev
1A	266884	46963	317591		
2A	210063	0	252076		
3A	207451	0	244793	271487	32736
4	554315	0	648548		
5	244353	25883	288337		
6	0	0	0	312295	265310
7	1175859	117414	1399272		
8	1826847	171408	2155680		
9	783283	210513	924274	1493075	507076
10	1069064	80732	1261495		
11	1922929	180118	2288286		
12	2057444	235140	2427784	1992522	520041
13A	945171200	101577089	1162560576		
14A	1119288889	144119426	1421496889		
15A	751653689	140628027	932050574	1172036013	199927923
16	336946489	80575410	414444181		
17(10)	776832711	393888897	947735908		
18(10)	957826844	912766874	1178127019	846769036	319842254
19	330424	50338	392213		
20	362082	0	425809		
21	293791	94886	346673	388232	32429
22	262323	25560	307705		
23	220480	640	257080		
24	302842	48501	359473	308086	41803
25	2854834	413510	3377268		
26	1219534	367194	1435392		
27	1336500	263155	1589098	2133920	881417
28	1725385	142352	2087716		
29	3508935	1026087	4231776		
30	3869013	975874	4604126	3641206	1108951
31	26932907	19656580	31753897		
32	103469013	86774204	122714250		
33	113765333	27903177	137200992	97223046	46669930
34	13341326	900906	16089639		
35	98362311	8536733	117641324		
36	81201067	21763094	96629269	76786744	43768181
M1	9.17.E+08	1.53.E+08			
M11	8.79.E+08	1.54.E+08			
M111	5.91.E+08	6.45.E+07		7.96.E+08	145328061

ErmB water data calculation/summary 2011 and 2012

Date	Days After Manure Application	Chisel Manure	Chisel Control	No Till Manure	No Till Control
4/27/2011	181	511	145	1263	185
5/3/2011	187	1955	759	309	1665
5/18/2011	202	1410	1410	3837	136379
5/23/2011	207	12511	457	255	670
5/26/2011	210	9697	481	737	957
6/1/2011	216	7127	930	6149	9932
6/8/2011	223	344			1154
6/15/2011	230	1753	1241	1322	21094
6/22/2011	237	931	17180	1561	694
6/29/2011	244	3166	114	263	404
7/6/2011	251		39	59	51
4/15/2012	170	416.4			
4/19/2012	174			431.1	
4/20/2012	175.2917		452.1	866.3	
4/28/2012	183	822.7		620.0	
5/2/2012	187	549.7	417.9	566.8	5702.6
5/3/2012	188.7917	386.6	260.7	794.3	879.1
5/5/2012	190	626.8	181.3	1365.1	
5/6/2012	191.625		225.0		267.9
5/7/2012	192	6020.4		362.0	
5/9/2012	194				1243.1
5/15/2012	200				
5/23/2012	208				
5/26/2012	211.4375				
5/29/2012	214	11.9		31.7	
6/6/2012	222	665.9		3148.6	15.9
6/11/2012	227	350105.4			3116.5

ErmB soil data calculation/summary 2011-2012

Label	Average Copy	StDev	Copy/gram dry	Average Copy/gram dw	StDev
37	0.0	0.0	0.00		
38	0.0	0.0	0.00		
39	3609.5	0.0	4236.73	4237	
40	3196.9	0.0	3719.20		
41	0.0	0.0	0.00		
42	0.0	0.0	0.00	3719	
43	6687.6	735.7	7823.51		
44	19565.6	1025.3	23199.83		
45	11926.8	3486.4	14061.22	15028	6314
46	21970.1	1984.6	26046.09		
47	22136.7	3561.8	26036.50		
48	14621.9	3684.1	17474.84	23186	4038
49	10616.6	2528.3	12473.40		
50	15529.6	1919.5	18275.20		
51	3772.4	0.0	4446.54	11732	
52	13966.5	5676.5	16605.06		
53	18415.0	4060.4	21779.58		
54	21746.0	5568.3	25637.74	21341	3701
55	0.0	0.0	0.00		
56	2645.4	0.0	3256.24		
57	1570.5	461.3	1923.43	2590	
58	868.5	323.1	1064.43		
59	678.7	82.7	829.46		
60	2805.2	0.0	3436.08	1777	
61	0.0	0.0	0.00		
62	0.0	0.0	0.00		
63	11766.6	0.0	14366.19	14366	
64	22932.4	6571.7	28216.96		
65	49230.8	3155.6	60118.32		
66	28005.0	990.9	34561.14	40965	13789

ErmB soil & manure data calculation/summary 2010-2011

Label	Average Copy	StDev	Copy/gram dry	Average Copy/gram dw	StDev
101	0.0	0.0			
102	0.0	0.0			
103	0.0	0.0			
104	0.0	0.0			
105	0.0	0.0			
106	0.0	0.0			
107	3352181.9	374573.5	3922052.86		
108	24278.5	4978.5	28891.42		
109	0.0	0.0		1975472	
110	13851.3	0.0	16344.51		
111	26517.9	0.0	31291.10		
112	30153.2	18820.1	37389.98	28342	
113	231963309.2	46469686.5	282995237.26		
114	162216915.8	64168262.4	197904637.24		
115	255397552.2	53719573.5	329462842.36	270120906	54474469
116	37303280.4	4357084.4	45883034.85		
117	189178853.3	22938335.4	234581778.05		
118	1312898725.3	1150994289.5	1680510368.3	653658394	730169175
119	0.0	0.0			
120	0.0	0.0			
121	0.0	0.0			
122	14871.5	0.0	18440.67		
123	0.0	0.0			
124	0.0	0.0		18441	
125	16038.3	5533.3	19727.17		
126	39136.4	5280.5	49703.24		
127	5219.1	409.2	6367.31	25266	18120
128	8148.0	1917.9	10429.39		
129	621170.5	180257.0	732981.20		
130	11937.5	3018.6	14683.12	252698	339616
131	963887.0	135302.4	1224136.49		
132	36315.0	12272.8	45393.79		
133	675782.4	54496.6	885274.97	718268	495498
134	1745690.9	276338.4	2182113.61		
135	52566.9	36817.5	65708.57		
136	4937217.0	2816127.1	6171521.31	2806448	2531479
MIV	8.28E+12	1.32E+12			
MV	4.59E+12	6.25E+11		6.44E+12	1.84E+12

ErmF

ErmF soil & manure data calculation/summary 2010-2011

Label	Average Copy	StDev	Copy/gram dry	Average Copy/gram dw	StDev
1A	2673	2266	2099		
2A	7434	3339	5887		
3A	821	1162	640	2875	1838
4	4151	5871	3206		
5	617	872	480		
6	6542	4996	5095	2927	1606
7	14991	7757	11774		
8	26708	31343	20800		
9	12682	5823	9877	14150	4053
10	5321	1799	4144		
11	7888	2189	6196		
12	88631	60786	69026	26455	25530
13A	6483592533333	8273058589722	5263380418560		
14A	765733610667	927210993200	641837912461		
15A	47449772800	48695888721	38832894060	1981350408360	1900598525917
16	171952035556	228068767544	139590662464		
17(10)	1924057600	802005379	1549251180		
18(10)	19035940216178	26919691714885	15453376267493	5198172060379	5895723888269
19	22583	15150	17692		
20	5706	2232	4429		
21	17967	9965	13992	12038	4700
22	12440	5690	9631		
23	15816	13343	12171		
24	30867	21109	24182	15328	5287
25	14741969	20800938	11510235		
26	93919	131900	72958		
27	77671	87944	60952	3881382	4559933
28	4367837	5897366	3488154		
29	2669460	3663717	2124784		
30	77405255	109093252	60794087	22135675	22992671
31	8771605	6210173	6825537		
32	1407010916	1799844974	1101351864		
33	11913872711	16796457451	9482966123	3530381175	3506779644
34	3007011151	4235310420	2393460596		
35	1789477760	1238345605	1412542165		
36	96862364	35038936	76075701	1294026154	787596939
M1	2.24E+06	1.31E+05			
M11	1.22E+08	5.16E+07			
M111	1.35E+07	5.81E+06		4.58.E+07	53827158

ErmF water data calculation/summary 2011 and 2012

Date	Days After Manure Application	Chisel Manure	Chisel Control	No Till Manure	No Till Control
4/27/2011	181	735971	809816	760881	784740.352
5/3/2011	187	5023726	306158	148400	0
5/18/2011	202	0	0	0	179274.304
5/23/2011	207	0	0	0	0
5/26/2011	210	1212565	0	1658	0
6/1/2011	216	40582	8150	15176	39528.6496
6/8/2011	223	0	0	0	0
6/15/2011	230	0	0	213740	0
6/22/2011	237	0	0	0	0
6/29/2011	244	0	0	0	0
7/6/2011	251		0	0	0
4/15/2012	170				
4/19/2012	174				
4/20/2012	175.2917		1699.0		
4/28/2012	183				
5/2/2012	187	4076.3	5168.5	6425.159296	5152.456587
5/3/2012	188.7917	2584.3	5520.8	11249.53913	4763.804059
5/5/2012	190				2609.855262
5/6/2012	191.625				
5/7/2012	192		4022.5		9194.219712
5/9/2012	194				
5/15/2012	200				
5/23/2012	208				
5/26/2012	211.4375				
5/29/2012	214				
6/6/2012	222				
6/11/2012	227				

ErmF soil data calculation/summary 2011-2012

Label	Average Copy	StDev	Copy/gram dry	Average Copy/gram dw	StDev
37	1803.3	1112.2			1803.3
38					
39			1803		
40					
41					
42					
43					
44	3309.9	2312.5			3309.9
45			3310		
46	2372.7	2087.1			2372.7
47	1767.6	1074.1			1767.6
48	675.7	799.6	1605	702	675.7
49					
50	2903.6	721.5			2903.6
51			2904		
52					
53	31602.4	13671.2			31602.4
54	294261.7	336382.5	162932		294261.7
55					
56					
57					
58					
59					
60					
61					
62					
63					
64					
65					
66					

ErmF soil & manure data calculation/summary 2010-2011

Label	Average Copy	StDev	Copy/gram dry	Average Copy/gram dw	StDev
101					
102	21255.2	15436.8			21255.2
103			21255		
104					
105					
106					
107	20549378.7	3359001.9			20549378.7
108	6826.0	1727.6			6826.0
109			10278102		
110	15051.6	2191.8			15051.6
111	25964.7	31118.3			25964.7
112			20508		
113	24672762.9	12606101.0			24672762.9
114	190749991.2	26045810.9			190749991.2
115	459748330.7	122526100.3	225057028	179267802	459748330.7
116	75529974.0	16223926.5			75529974.0
117	552938332.8	124649548.0			552938332.8
118	1638471646.3	234635371.2	755646651	653969757	1638471646.3
119					
120					
121					
122					
123					
124					
125					
126					
127					
128	5287.3	586.5			5287.3
129	514907.7	105176.3			514907.7
130			260097	254810	
131	1697305.3	168500.1			1697305.3
132	18996.9	2903.0			18996.9
133	2017821.0	284734.4	1244708	876530	2017821.0
134	5495765.2	1027648.0			5495765.2
135					
136	8383062.7	2287329.2	6939414	1443649	8383062.7
MIV	21255.2	15436.8			21255.2
MV			21255		

Tylosin

Tylosin concentrations in manure in ppb/g from 2011 and 2012.

2011		2012	
Label	Tylosin A	Label	Tylosin A
NST-MI B	14.09858	MIV A	20.62595
NST-MI C	21.28609	MIV B	20.62595
NST-MI D	22.94475	MIV C	20.62595
NST-MII A	19.94604	MV A	20.60023
NST-MII B	11.73297	MV B	20.60023
NST-MII C	15.95683	MV C	20.60023
NST-MIII B	21.51080		
NST-MIII C	12.80405		
NST-MIII D	15.36486		

Fall 2010 and Spring 2011 Soil Samples

LAB CODE	Label	Sample Concentration (ppb/g dw)
1	NST-1A	0.00000
1	NST-1B	0.29867
2	NST-2A	0.00000
2	NST-2B	0.00000
3	NST-3A	0.00000
3	NST-3B	0.21928
4	NST-4	0.29583
5	NST-5	0.34356
6	NST-6	0.20275
7	NST-7	0.18890
8	NST-8	0.21771
9	NST-9	0.20376
10	NST-10	0.00000
11	NST-11	0.35901
12	NST-12	0.50061
13	NST-13A	0.46982
13	NST-13B	0.37262
14	NST-14A	0.66433
14	NST-14B	1.14597
15	NST-15A	0.59117
15	NST-15B	0.54191
16	NST-16	0.55299
17	NST-17	0.30581
18	NST-18	0.29237
19	NST-19	0.00000
20	NST-20	0.00000
21	NST-21	0.00000
22	NST-22	0.00000
23	NST-23	0.00000
24	NST-24	0.00000
25	NST-25	0.00000
26	NST-26	0.00000
27	NST-27	0.23433
28	NST-28	0.23981
29	NST-29	0.58582
30	NST-30	0.00000
31	NST-31	0.00000
32	NST-32	0.56498
33	NST-33	7.55955
34	NST-34	0.00000
35	NST-35	1.05929
36	NST-36	1.14885

Fall 2011 and Spring 2012 Soil Samples

LAB CODE	Label	Sample Concentration (ppb/g dw)
37	NST-37	0.83458
38	NST-38	1.05518
39	NST-39	0.34431
40	NST-40	0.00000
41	NST-41	0.00000
42	NST-42	0.92640
43	NST-43	0.99827
44	NST-44	0.00000
45	NST-45	0.00000
46	NST-46	0.00000
47	NST-47	0.64297
48	NST-48	1.17918
49	NST-49	0.62661
50	NST-50	2.71449
51	NST-51	0.00000
52	NST-52	0.44387
53	NST-53	1.30887
54	NST-54	0.00000
55	NST-55	0.00000
56	NST-56	0.00000
57	NST-57	0.00000
58	NST-58	5.08231
59	NST-59	0.92876
60	NST-60	3.47875
61	NST-61	2.85089
62	NST-62	9.78780
63	NST-63	0.81395
64	NST-64	0.00000
65	NST-65	0.00000
66	NST-66	0.00000

Fall 2011 and Spring 2012 Soil Samples

LAB CODE	Label	Sample Concentration (ppb/g dw)
101	NST-101	2.83371
102	NST-102	0.00000
103	NST-103	0.00000
104	NST-104	0.00000
105	NST-105	0.81503
106	NST-106	0.00000
107	NST-107	0.00000
108	NST-108	0.00000
109	NST-109	0.00000
110	NST-110	0.00000
111	NST-111	0.00000
112	NST-112	0.90996
113	NST-113	4.72628
114	NST-114	0.00000
115	NST-115	0.94426
116	NST-116	1.03511
117	NST-117	0.94118
118	NST-118	1.56958
119	NST-119	0.00000
120	NST-120	0.00000
121	NST-121	0.00000
122	NST-122	0.87962
123	NST-123	0.00000
124	NST-124	0.00000
125	NST-125	0.00000
126	NST-126	0.00000
127	NST-127	0.40675
128	NST-128	1.75408
129	NST-129	0.00000
130	NST-130	1.07975
131	NST-131	0.89901
132	NST-132	0.53164
133	NST-133	0.00000
134	NST-134	0.00000
135	NST-135	0.58210
136	NST-136	0.46486

Tylosin concentrations in tile water in ppb from summer 2011.

	Plot 23		Plot 24		Plot 25		Plot 34	
	Label	Tylosin A	Label	Tylosin A	Label	Tylosin A	Label	Tylosin A
4/29/2011	NT-23-2	0.0216	NT-24-2	0.3352	NT-25-2	0.0384	NT-34-2	0.024
5/3/2011	NT-23-3	0.8872	NT-24-3	<0.016	NT-25-3	<0.016	NT-34-3	0.068
5/18/2011	NT-23-4	<0.016	NT-24-4	0.0168	NT-25-4	<0.016	NT-34-4	0.0192
5/23/2011	NT-23-5	0.0784	NT-24-5	0.736	NT-25-5	<0.016	NT-34-5	<0.016
5/26/2011	NT-23-6	0.0168	NT-24-6	0.2824	NT-25-6	<0.016	NT-34-6	<0.016
6/1/2011	NT-23-7	<0.016	NT-24-7	0.0592	NT-25-7	<0.016	NT-34-7	<0.016
6/8/2011	NT-23-8	0.0112	NT-24-8	0.0376	NT-25-8	<0.0096	NT-34-8	<0.0096
6/8/2011	NT-23-9	<0.0096	NT-24-9	<0.0096	NT-25-9	<0.0096	NT-34-9	<0.0096
6/10/2011	NT-23-10	<0.0096	NT-24-10	<0.0096	NT-25-10	<0.0096	NT-34-10	<0.0096
6/10/2011	NT-23-11	<0.0096	NT-24-11	<0.0096	NT-25-11	<0.0096	NT-34-11	<0.0096
6/15/2011	NT-23-12	<0.0096	NT-24-12	<0.0096	NT-25-12	<0.0096	NT-34-12	<0.0096
6/22/2011	NT-23-13	<0.0096	NT-24-13	<0.0096	NT-25-13	<0.0096	NT-34-13	<0.0096
6/29/2011	NT-23-14	<0.0096	NT-24-14	<0.0096	NT-25-14	<0.0096	NT-34-14	<0.0096
7/6/2011	NT-23-16	<0.0096	NT-24-16	<0.0096	NT-25-16	<0.0096	NT-34-16	<0.0096

Tylosin concentrations in tile water in ppb from summer 2012.

	Plot 19		Plot 20		Plot 29		Plot 30	
Sample Date	Label	Tylosin A	Label	Tylosin A	Label	Tylosin A	Label	Tylosin A
4/15/2012			NT 20-0	< 0.0022			NT 30-0	< 0.0022
4/19/2012			NT 20-1	< 0.0003			NT 30-1	< 0.0003
4/20/2012			NT-E1-12	< 0.0003	NT-E1-24	< 0.0003	NT-E1-36	< 0.0003
4/28/2012			NT 20-2	< 0.3			NT 30-2	< 0.3
5/2/2012	NT 19-3	F	NT 20-3	< 0.3			NT 30-3	< 0.3
5/3/2012	NT E2 4	< 0.3	NT E2 8	< 0.3	NT E2 12	< 0.3	NT E2 16	< 0.3
5/5/2012			NT 20-4	< 0.3	NT 29-4	< 0.3	NT 30-4	< 0.3
5/6/2012	NT E3 4	< 0.3			N E3 9	< 0.3		
5/7/2012			N-E3-5	< 0.3			NT-E3-13	< 0.3
5/9/2012	NT 19-5	< 0.3	NT 20-5	< 0.3	NT 29-5	< 0.3	NT 30-5	< 0.3
5/15/2012	NT 19-6	< 0.3	NT 20-6	< 0.3	NT 29-6	< 0.3	NT 30-6	< 0.3
5/23/2012	NT 19-7	< 0.3	NT 20-7	< 0.3	NT 29-7	< 0.3	NT 30-7	< 0.3
5/26/2012	NT E4 4	< 0.3	NT E4 8	< 0.3	NT E4 12	< 0.3	NT E4 16	< 0.3
5/29/2012	NT 19-8	< 0.3	NT 20-8	< 0.3	NT 29-8	< 0.3	NT 30-8	< 0.3
6/6/2012	NT-19-9	< 0.3	NT-20-9	< 0.3			NT-30-9	< 0.3
6/11/2012	NT-19-10	0.004					NT-30-10	< 0.3

R Statistical Analysis

ANOVA Tables

ANOVA tables were generated using Akaike's Information Criterion (AIC). This method penalizes the model when additional terms are incorporated that doesn't have a large effect on the residual sum of squares. Therefore, the final model for each dataset only includes the parameters that AIC retained using stepwise regression for the remainder of the analysis. Those parameters will be denoted with a 'NS'. The significance of the other parameters will be denoted as follows:

***	<0.001
**	0.001
*	0.05
.	0.1
' '	1

*erm*B in soil

Main Effect		Significance
Tillage	CP, NT	NS
Season	FALL, SPRING	NS
Location	B, NB, CON	***
Year	2010 & 2011	***
StudyYear	1,2	*
Season:Location		*
Tillage:Treatment		NS

*erm*F in soil

Main Effect		Significance
Tillage	CP, NT	NS
Season	FALL, SPRING	***
Location	B, NB, CON	***
Year	2010 & 2011	***
StudyYear	1,2	**
Season:Location		**
Tillage:Treatment		NS

ENT in soil

Main Effect		Significance
Tillage	CP, NT	NS
Season	FALL, SPRING	**
Location	B, NB, CON	***
Year	2010 & 2011	***
StudyYear	1,2	*
Season:Location		NS
Tillage:Treatment		NS

TYL in soil

Main Effect		Significance
Tillage	CP, NT	NS
Season	FALL, SPRING	NS
Location	B, NB, CON	**
Year	2010 & 2011	NS
StudyYear	1,2	NS
Season:Location		NS
Tillage:Treatment		NS

ENT in water

Main Effect		Significance
Tillage	CP, NT	**
Treatment	MAN, CON	NS
Year	2010 & 2011	NS
StudyYear	1,2	NS
Season:Location		NS
Tillage:Treatment		NS

*erm*B in water

Main Effect		Significance
Tillage	CP, NT	NS
Treatment	MAN, CON	NS
Year	2010 & 2011	***
StudyYear	1,2	NS
Season:Location		NS
Tillage:Treatment		NS

*erm*F in water

Main Effect		Significance
Tillage	CP, NT	NS
Treatment	MAN, CON	NS
Year	2010 & 2011	***
StudyYear	1,2	NS
Season:Location		NS
Tillage:Treatment		NS

R Code and Tukey Data

Soil ENT

```
> ######First Year After Manure
> dat=read.csv("Soil ENT.csv", header=T)
> group=subset(dat,StudyYear=="1" & Year=="2010" | StudyYear=="2")
> modG=lm((Concentration)~Location:as.factor(Year):Season,group)
> summary(modG)

Call:
lm(formula = (Concentration) ~ Location:as.factor(Year):Season,
    data = group)

Residuals:
    Min      1Q  Median      3Q     Max
-313.33  -19.29   -4.00   11.42  482.17

Coefficients: (1 not defined because of singularities)
                                                Estimate Std. Error t value Pr(>|t|)
(Intercept)                                      78.5000    51.9658   1.511 0.136137
LocationBAND:as.factor(Year)2010:SeasonFALL     747.1667    73.4907  10.167 1.14e-14 ***
LocationCONTROL:as.factor(Year)2010:SeasonFALL  -54.8333    73.4907  -0.746 0.458505
LocationNO-BAND:as.factor(Year)2010:SeasonFALL   -0.3333    73.4907  -0.005 0.996396
LocationBAND:as.factor(Year)2011:SeasonFALL     266.8333    73.4907   3.631 0.000586 ***
LocationCONTROL:as.factor(Year)2011:SeasonFALL  -63.3333    73.4907  -0.862 0.392236
LocationNO-BAND:as.factor(Year)2011:SeasonFALL  123.0000    73.4907   1.674 0.099399 .
LocationBAND:as.factor(Year)2010:SeasonSPRING   167.3333    73.4907   2.277 0.026370 *
LocationCONTROL:as.factor(Year)2010:SeasonSPRING -44.1667   73.4907  -0.601 0.550115
LocationNO-BAND:as.factor(Year)2010:SeasonSPRING -42.6667   73.4907  -0.581 0.563703
LocationBAND:as.factor(Year)2011:SeasonSPRING   -72.5000    73.4907  -0.987 0.327840
LocationCONTROL:as.factor(Year)2011:SeasonSPRING -70.3333   73.4907  -0.957 0.342389
LocationNO-BAND:as.factor(Year)2011:SeasonSPRING       NA         NA      NA       NA
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 127.3 on 60 degrees of freedom
Multiple R-squared: 0.7921,     Adjusted R-squared: 0.754
F-statistic: 20.78 on 11 and 60 DF,  p-value: < 2.2e-16

> TukeyHSD(aov(modG),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modG)

$`Location:as.factor(Year):Season`
                                                  diff           lwr        upr     p adj
CONTROL:2010:FALL-BAND:2010:FALL          -802.0000000 -1031.7308466 -572.26915 0.0000000
NO-BAND:2010:FALL-BAND:2010:FALL          -747.5000000  -977.2308466 -517.76915 0.0000000
BAND:2011:FALL-BAND:2010:FALL             -480.3333333  -710.0641799 -250.60249 0.0000010
CONTROL:2011:FALL-BAND:2010:FALL          -810.5000000 -1040.2308466 -580.76915 0.0000000
NO-BAND:2011:FALL-BAND:2010:FALL          -624.1666667  -853.8975132 -394.43582 0.0000000
BAND:2010:SPRING-BAND:2010:FALL           -579.8333333  -809.5641799 -350.10249 0.0000000
CONTROL:2010:SPRING-BAND:2010:FALL        -791.3333333 -1021.0641799 -561.60249 0.0000000
NO-BAND:2010:SPRING-BAND:2010:FALL        -789.8333333 -1019.5641799 -560.10249 0.0000000
BAND:2011:SPRING-BAND:2010:FALL           -819.6666667 -1049.3975132 -589.93582 0.0000000
CONTROL:2011:SPRING-BAND:2010:FALL        -817.5000000 -1047.2308466 -587.76915 0.0000000
NO-BAND:2011:SPRING-BAND:2010:FALL        -747.1666667  -976.8975132 -517.43582 0.0000000
NO-BAND:2010:FALL-CONTROL:2010:FALL         54.5000000  -175.2308466  284.23085 0.9998206
BAND:2011:FALL-CONTROL:2010:FALL           321.6666667    91.9358201  551.39751 0.0026441
CONTROL:2011:FALL-CONTROL:2010:FALL         -8.5000000  -238.2308466  221.23085 1.0000000
NO-BAND:2011:FALL-CONTROL:2010:FALL        177.8333333   -51.8975132  407.56418 0.4093678
BAND:2010:SPRING-CONTROL:2010:FALL         222.1666667    -7.5641799  451.89751 0.1272908
CONTROL:2010:SPRING-CONTROL:2010:FALL       10.6666667  -219.0641799  240.39751 1.0000000
NO-BAND:2010:SPRING-CONTROL:2010:FALL       12.1666667  -217.5641799  241.89751 1.0000000
BAND:2011:SPRING-CONTROL:2010:FALL         -17.6666667  -247.3975132  212.06418 1.0000000
CONTROL:2011:SPRING-CONTROL:2010:FALL      -15.5000000  -245.2308466  214.23085 1.0000000
NO-BAND:2011:SPRING-CONTROL:2010:FALL       54.8333333  -174.8975132  284.56418 0.9998097
BAND:2011:FALL-NO-BAND:2010:FALL           267.1666667    37.4358201  496.89751 0.0261717
CONTROL:2011:FALL-NO-BAND:2010:FALL        -63.0000000  -292.7308466  166.73085 0.9992895
NO-BAND:2011:FALL-NO-BAND:2010:FALL        123.3333333  -106.3975132  353.06418 0.8708263
BAND:2010:SPRING-NO-BAND:2010:FALL         167.6666667   -62.0641799  397.39751 0.5004376
CONTROL:2010:SPRING-NO-BAND:2010:FALL      -43.8333333  -273.5641799  185.89751 0.9999794
NO-BAND:2010:SPRING-NO-BAND:2010:FALL      -42.3333333  -272.0641799  187.39751 0.9999855
```

```
BAND:2011:SPRING-NO-BAND:2010:FALL       -72.1666667  -301.8975132  157.56418 0.9975696
CONTROL:2011:SPRING-NO-BAND:2010:FALL    -70.0000000  -299.7308466  159.73085 0.9981450
NO-BAND:2011:SPRING-NO-BAND:2010:FALL      0.3333333  -229.3975132  230.06418 1.0000000
CONTROL:2011:FALL-BAND:2011:FALL        -330.1666667  -559.8975132 -100.43582 0.0017984
NO-BAND:2011:FALL-BAND:2011:FALL        -143.8333333  -373.5641799   85.89751 0.7190603
BAND:2010:SPRING-BAND:2011:FALL          -99.5000000  -329.2308466  130.23085 0.9677042
CONTROL:2010:SPRING-BAND:2011:FALL      -311.0000000  -540.7308466  -81.26915 0.0042487
NO-BAND:2010:SPRING-BAND:2011:FALL      -309.5000000  -539.2308466  -79.76915 0.0045376
BAND:2011:SPRING-BAND:2011:FALL         -339.3333333  -569.0641799 -109.60249 0.0011785
CONTROL:2011:SPRING-BAND:2011:FALL      -337.1666667  -566.8975132 -107.43582 0.0013031
NO-BAND:2011:SPRING-BAND:2011:FALL      -266.8333333  -496.5641799  -37.10249 0.0265114
NO-BAND:2011:FALL-CONTROL:2011:FALL      186.3333333   -43.3975132  416.06418 0.3390981
BAND:2010:SPRING-CONTROL:2011:FALL       230.6666667     0.9358201  460.39751 0.0969857
CONTROL:2010:SPRING-CONTROL:2011:FALL     19.1666667  -210.5641799  248.89751 1.0000000
NO-BAND:2010:SPRING-CONTROL:2011:FALL     20.6666667  -209.0641799  250.39751 1.0000000
BAND:2011:SPRING-CONTROL:2011:FALL        -9.1666667  -238.8975132  220.56418 1.0000000
CONTROL:2011:SPRING-CONTROL:2011:FALL     -7.0000000  -236.7308466  222.73085 1.0000000
NO-BAND:2011:SPRING-CONTROL:2011:FALL     63.3333333  -166.3975132  293.06418 0.9992538
BAND:2010:SPRING-NO-BAND:2011:FALL        44.3333333  -185.3975132  274.06418 0.9999768
CONTROL:2010:SPRING-NO-BAND:2011:FALL   -167.1666667  -396.8975132   62.56418 0.5050511
NO-BAND:2010:SPRING-NO-BAND:2011:FALL   -165.6666667  -395.3975132   64.06418 0.5189408
BAND:2011:SPRING-NO-BAND:2011:FALL      -195.5000000  -425.2308466   34.23085 0.2713119
CONTROL:2011:SPRING-NO-BAND:2011:FALL   -193.3333333  -423.0641799   36.39751 0.2865172
NO-BAND:2011:SPRING-NO-BAND:2011:FALL   -123.0000000  -352.7308466  106.73085 0.8727981
CONTROL:2010:SPRING-BAND:2010:SPRING    -211.5000000  -441.2308466   18.23085 0.1754716
NO-BAND:2010:SPRING-BAND:2010:SPRING    -210.0000000  -439.7308466   19.73085 0.1832269
BAND:2011:SPRING-BAND:2010:SPRING       -239.8333333  -469.5641799  -10.10249 0.0712615
CONTROL:2011:SPRING-BAND:2010:SPRING    -237.6666667  -467.3975132   -7.93582 0.0767501
NO-BAND:2011:SPRING-BAND:2010:SPRING    -167.3333333  -397.0641799   62.39751 0.5035123
NO-BAND:2010:SPRING-CONTROL:2010:SPRING    1.5000000  -228.2308466  231.23085 1.0000000
BAND:2011:SPRING-CONTROL:2010:SPRING     -28.3333333  -258.0641799  201.39751 0.9999998
CONTROL:2011:SPRING-CONTROL:2010:SPRING  -26.1666667  -255.8975132  203.56418 0.9999999
NO-BAND:2011:SPRING-CONTROL:2010:SPRING   44.1666667  -185.5641799  273.89751 0.9999777
BAND:2011:SPRING-NO-BAND:2010:SPRING     -29.8333333  -259.5641799  199.89751 0.9999996
CONTROL:2011:SPRING-NO-BAND:2010:SPRING  -27.6666667  -257.3975132  202.06418 0.9999998
NO-BAND:2011:SPRING-NO-BAND:2010:SPRING   42.6666667  -187.0641799  272.39751 0.9999843
CONTROL:2011:SPRING-BAND:2011:SPRING       2.1666667  -227.5641799  231.89751 1.0000000
NO-BAND:2011:SPRING-BAND:2011:SPRING      72.5000000  -157.2308466  302.23085 0.9974691
NO-BAND:2011:SPRING-CONTROL:2011:SPRING   70.3333333  -159.3975132  300.06418 0.9980648

>
> ######First Year After Manure 2010 means by location
> mean1=subset(group,Year=="2010")
> modM1=lm(Concentration~Location,mean1)
> summary(modM1)

Call:
lm(formula = Concentration ~ Location, data = mean1)

Residuals:
   Min     1Q Median     3Q    Max
-472.8  -25.0   -3.5   40.0  335.2

Coefficients:
               Estimate Std. Error t value Pr(>|t|)
(Intercept)       535.75      58.50   9.158 1.40e-10 ***
LocationCONTROL  -506.75      82.73  -6.125 6.68e-07 ***
LocationNO-BAND  -478.75      82.73  -5.787 1.81e-06 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 202.7 on 33 degrees of freedom
Multiple R-squared: 0.5896,     Adjusted R-squared: 0.5647
F-statistic: 23.71 on 2 and 33 DF,  p-value: 4.146e-07

> TukeyHSD(aov(modM1),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modM1)

$Location
                    diff     lwr     upr     p adj
CONTROL-BAND    -506.75 -682.63 -330.87 0.0000020
NO-BAND-BAND    -478.75 -654.63 -302.87 0.0000053
NO-BAND-CONTROL   28.00 -147.88  203.88 0.9389297

>
```

```
> ######First Year After Manure 2011 means by location
> mean2=subset(group,Year=="2011")
> modM2=lm(Concentration~Location,mean2)
> summary(modM2)

Call:
lm(formula = Concentration ~ Location, data = mean2)

Residuals:
    Min      1Q  Median      3Q     Max
-175.67 -140.00  -11.67   36.08  534.00

Coefficients:
                Estimate Std. Error t value Pr(>|t|)
(Intercept)       175.67      50.81   3.457  0.00152 **
LocationCONTROL  -164.00      71.85  -2.282  0.02904 *
LocationNO-BAND   -35.67      71.85  -0.496  0.62292
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 176 on 33 degrees of freedom
Multiple R-squared: 0.1487,     Adjusted R-squared: 0.0971
F-statistic: 2.882 on 2 and 33 DF,  p-value: 0.07021

> TukeyHSD(aov(modM2),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modM2)

$Location
                      diff        lwr       upr     p adj
CONTROL-BAND    -164.00000 -316.75324 -11.24676 0.0724392
NO-BAND-BAND     -35.66667 -188.41991 117.08658 0.8735279
NO-BAND-CONTROL  128.33333  -24.41991 281.08658 0.1898916

>
> ######First Year After Manure means by location
> modM3=lm(Concentration~Location,group)
> summary(modM3)

Call:
lm(formula = Concentration ~ Location, data = group)

Residuals:
    Min      1Q  Median      3Q     Max
-355.71  -75.50  -16.33   11.17  575.50

Coefficients:
                Estimate Std. Error t value Pr(>|t|)
(Intercept)       355.71      43.95   8.094 1.35e-11 ***
LocationCONTROL  -335.38      62.15  -5.396 9.05e-07 ***
LocationNO-BAND  -257.21      62.15  -4.138 9.73e-05 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 215.3 on 69 degrees of freedom
Multiple R-squared: 0.316,      Adjusted R-squared: 0.2962
F-statistic: 15.94 on 2 and 69 DF,  p-value: 2.035e-06

> TukeyHSD(aov(modM3),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modM3)

$Location
                      diff        lwr       upr     p adj
CONTROL-BAND    -335.37500 -465.08636 -205.6636 0.0000027
NO-BAND-BAND    -257.20833 -386.91969 -127.4970 0.0002839
NO-BAND-CONTROL   78.16667  -51.54469  207.8780 0.4238436

>
> ######Two-Year Study
> dat=read.csv("Soil ENT.csv", header=T)
> group1=subset(dat,StudyYear=="1")
> modG1=lm((Concentration)~Location:as.factor(Year):Season,group1)
> summary(modG1)
```

```
Call:
lm(formula = (Concentration) ~ Location:as.factor(Year):Season,
    data = group1)

Residuals:
    Min      1Q  Median      3Q     Max
-182.83  -18.96   -5.50    8.67  482.17

Coefficients: (2 not defined because of singularities)
                                                 Estimate Std. Error t value Pr(>|t|)
(Intercept)                                         5.50      35.23   0.156    0.877
LocationBAND:as.factor(Year)2010:SeasonFALL       820.17      49.83  16.461  < 2e-16 ***
LocationCONTROL:as.factor(Year)2010:SeasonFALL     18.17      49.83   0.365    0.717
LocationNO-BAND:as.factor(Year)2010:SeasonFALL     72.67      49.83   1.458    0.150
LocationBAND:as.factor(Year)2011:SeasonFALL        46.17      49.83   0.927    0.358
LocationCONTROL:as.factor(Year)2011:SeasonFALL     24.00      49.83   0.482    0.632
LocationNO-BAND:as.factor(Year)2011:SeasonFALL      3.00      49.83   0.060    0.952
LocationBAND:as.factor(Year)2010:SeasonSPRING     240.33      49.83   4.823 1.16e-05 ***
LocationCONTROL:as.factor(Year)2010:SeasonSPRING   28.83      49.83   0.579    0.565
LocationNO-BAND:as.factor(Year)2010:SeasonSPRING   30.33      49.83   0.609    0.545
LocationBAND:as.factor(Year)2011:SeasonSPRING         NA         NA      NA       NA
LocationCONTROL:as.factor(Year)2011:SeasonSPRING    5.50      49.83   0.110    0.913
LocationNO-BAND:as.factor(Year)2011:SeasonSPRING      NA         NA      NA       NA
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 86.3 on 55 degrees of freedom
Multiple R-squared: 0.8962,     Adjusted R-squared: 0.8773
F-statistic: 47.49 on 10 and 55 DF,  p-value: < 2.2e-16

> TukeyHSD(aov(modG1),conf.level=0.9))
Error: unexpected ')' in "TukeyHSD(aov(modG1),conf.level=0.9))"
>
> ######Two-Year Study 2011 means by location
> mean4=subset(group1,Year=="2011")
> modM4=lm(Concentration~Location,mean4)
> summary(modM4)

Call:
lm(formula = Concentration ~ Location, data = mean4)

Residuals:
   Min     1Q Median     3Q    Max
-51.67 -20.25  -7.00   6.00 226.33

Coefficients:
                Estimate Std. Error t value Pr(>|t|)
(Intercept)        51.67      20.76   2.489   0.0193 *
LocationCONTROL   -31.42      25.42  -1.236   0.2272
LocationNO-BAND   -44.67      25.42  -1.757   0.0903 .
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 50.85 on 27 degrees of freedom
Multiple R-squared: 0.1028,     Adjusted R-squared: 0.03636
F-statistic: 1.547 on 2 and 27 DF,  p-value: 0.2311

> TukeyHSD(aov(modM4),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modM4)

$Location
                     diff       lwr       upr     p adj
CONTROL-BAND    -31.41667 -85.89528 23.061949 0.4430089
NO-BAND-BAND    -44.66667 -99.14528  9.811949 0.2031631
NO-BAND-CONTROL -13.25000 -57.73160 31.231604 0.8004497

>
> ######Two-Year Study means by location
> modM5=lm(Concentration~Location,group1)
> summary(modM5)

Call:
lm(formula = Concentration ~ Location, data = group1)
```

```
Residuals:
    Min      1Q  Median      3Q     Max
-374.39  -32.00  -10.31   26.00  496.61

Coefficients:
                Estimate Std. Error t value Pr(>|t|)
(Intercept)       374.39      45.79   8.176 1.76e-11 ***
LocationCONTROL  -349.76      60.58  -5.774 2.56e-07 ***
LocationNO-BAND  -342.39      60.58  -5.652 4.10e-07 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 194.3 on 63 degrees of freedom
Multiple R-squared: 0.3975,     Adjusted R-squared: 0.3783
F-statistic: 20.78 on 2 and 63 DF,  p-value: 1.173e-07

> TukeyHSD(aov(modM5),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modM5)

$Location
                    diff       lwr       upr     p adj
CONTROL-BAND    -349.7639 -476.3878 -223.1400 0.0000008
NO-BAND-BAND    -342.3889 -469.0128 -215.7650 0.0000012
NO-BAND-CONTROL    7.3750 -109.8559  124.6059 0.9905131

>

> ######Two-Year Study means by location and season and year
> modM6=lm(Concentration~Location:Season:as.factor(Year),group1)
> summary(modM6)

Call:
lm(formula = Concentration ~ Location:Season:as.factor(Year),
    data = group1)

Residuals:
    Min      1Q  Median      3Q     Max
-182.83  -18.96   -5.50    8.67  482.17

Coefficients: (2 not defined because of singularities)
                                                 Estimate Std. Error t value Pr(>|t|)
(Intercept)                                         5.50      35.23   0.156    0.877
LocationBAND:SeasonFALL:as.factor(Year)2010        820.17      49.83  16.461  < 2e-16 ***
LocationCONTROL:SeasonFALL:as.factor(Year)2010      18.17      49.83   0.365    0.717
LocationNO-BAND:SeasonFALL:as.factor(Year)2010      72.67      49.83   1.458    0.150
LocationBAND:SeasonSPRING:as.factor(Year)2010      240.33      49.83   4.823 1.16e-05 ***
LocationCONTROL:SeasonSPRING:as.factor(Year)2010    28.83      49.83   0.579    0.565
LocationNO-BAND:SeasonSPRING:as.factor(Year)2010    30.33      49.83   0.609    0.545
LocationBAND:SeasonFALL:as.factor(Year)2011         46.17      49.83   0.927    0.358
LocationCONTROL:SeasonFALL:as.factor(Year)2011      24.00      49.83   0.482    0.632
LocationNO-BAND:SeasonFALL:as.factor(Year)2011       3.00      49.83   0.060    0.952
LocationBAND:SeasonSPRING:as.factor(Year)2011          NA         NA      NA       NA
LocationCONTROL:SeasonSPRING:as.factor(Year)2011    5.50      49.83   0.110    0.913
LocationNO-BAND:SeasonSPRING:as.factor(Year)2011       NA         NA      NA       NA
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 86.3 on 55 degrees of freedom
Multiple R-squared: 0.8962,     Adjusted R-squared: 0.8773
F-statistic: 47.49 on 10 and 55 DF,  p-value: < 2.2e-16

> TukeyHSD(aov(modM6),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modM6)

$`Location:Season:as.factor(Year)`
                                                      diff        lwr        upr     p adj
CONTROL:FALL:2010-BAND:FALL:2010                -802.000000 -958.19432 -645.80568 0.0000000
NO-BAND:FALL:2010-BAND:FALL:2010                -747.500000 -903.69432 -591.30568 0.0000000
BAND:SPRING:2010-BAND:FALL:2010                 -579.833333 -736.02765 -423.63902 0.0000000
CONTROL:SPRING:2010-BAND:FALL:2010              -791.333333 -947.52765 -635.13902 0.0000000
NO-BAND:SPRING:2010-BAND:FALL:2010              -789.833333 -946.02765 -633.63902 0.0000000
BAND:FALL:2011-BAND:FALL:2010                   -774.000000 -930.19432 -617.80568 0.0000000
```

CONTROL:FALL:2011-BAND:FALL:2010	-796.166667	-952.36098	-639.97235	0.0000000
NO-BAND:FALL:2011-BAND:FALL:2010	-817.166667	-973.36098	-660.97235	0.0000000
BAND:SPRING:2011-BAND:FALL:2010	NA	NA	NA	NA
CONTROL:SPRING:2011-BAND:FALL:2010	-814.666667	-970.86098	-658.47235	0.0000000
NO-BAND:SPRING:2011-BAND:FALL:2010	-820.166667	-976.36098	-663.97235	0.0000000
NO-BAND:FALL:2010-CONTROL:FALL:2010	54.500000	-101.69432	210.69432	0.9937647
BAND:SPRING:2010-CONTROL:FALL:2010	222.166667	65.97235	378.36098	0.0022221
CONTROL:SPRING:2010-CONTROL:FALL:2010	10.666667	-145.52765	166.86098	1.0000000
NO-BAND:SPRING:2010-CONTROL:FALL:2010	12.166667	-144.02765	168.36098	1.0000000
BAND:FALL:2011-CONTROL:FALL:2010	28.000000	-128.19432	184.19432	0.9999884
CONTROL:FALL:2011-CONTROL:FALL:2010	5.833333	-150.36098	162.02765	1.0000000
NO-BAND:FALL:2011-CONTROL:FALL:2010	-15.166667	-171.36098	141.02765	1.0000000
BAND:SPRING:2011-CONTROL:FALL:2010	NA	NA	NA	NA
CONTROL:SPRING:2011-CONTROL:FALL:2010	-12.666667	-168.86098	143.52765	1.0000000
NO-BAND:SPRING:2011-CONTROL:FALL:2010	-18.166667	-174.36098	138.02765	0.9999999
BAND:SPRING:2010-NO-BAND:FALL:2010	167.666667	11.47235	323.86098	0.0565356
CONTROL:SPRING:2010-NO-BAND:FALL:2010	-43.833333	-200.02765	112.36098	0.9990774
NO-BAND:SPRING:2010-NO-BAND:FALL:2010	-42.333333	-198.52765	113.86098	0.9993312
BAND:FALL:2011-NO-BAND:FALL:2010	-26.500000	-182.69432	129.69432	0.9999934
CONTROL:FALL:2011-NO-BAND:FALL:2010	-48.666667	-204.86098	107.52765	0.9976356
NO-BAND:FALL:2011-NO-BAND:FALL:2010	-69.666667	-225.86098	86.52765	0.9590197
BAND:SPRING:2011-NO-BAND:FALL:2010	NA	NA	NA	NA
CONTROL:SPRING:2011-NO-BAND:FALL:2010	-67.166667	-223.36098	89.02765	0.9683450
NO-BAND:SPRING:2011-NO-BAND:FALL:2010	-72.666667	-228.86098	83.52765	0.9453916
CONTROL:SPRING:2010-BAND:SPRING:2010	-211.500000	-367.69432	-55.30568	0.0044220
NO-BAND:SPRING:2010-BAND:SPRING:2010	-210.000000	-366.19432	-53.80568	0.0048626
BAND:FALL:2011-BAND:SPRING:2010	-194.166667	-350.36098	-37.97235	0.0128616
CONTROL:FALL:2011-BAND:SPRING:2010	-216.333333	-372.52765	-60.13902	0.0032461
NO-BAND:FALL:2011-BAND:SPRING:2010	-237.333333	-393.52765	-81.13902	0.0008074
BAND:SPRING:2011-BAND:SPRING:2010	NA	NA	NA	NA
CONTROL:SPRING:2011-BAND:SPRING:2010	-234.833333	-391.02765	-78.63902	0.0009564
NO-BAND:SPRING:2011-BAND:SPRING:2010	-240.333333	-396.52765	-84.13902	0.0006581
NO-BAND:SPRING:2010-CONTROL:SPRING:2010	1.500000	-154.69432	157.69432	1.0000000
BAND:FALL:2011-CONTROL:SPRING:2010	17.333333	-138.86098	173.52765	0.9999999
CONTROL:FALL:2011-CONTROL:SPRING:2010	-4.833333	-161.02765	151.36098	1.0000000
NO-BAND:FALL:2011-CONTROL:SPRING:2010	-25.833333	-182.02765	130.36098	0.9999949
BAND:SPRING:2011-CONTROL:SPRING:2010	NA	NA	NA	NA
CONTROL:SPRING:2011-CONTROL:SPRING:2010	-23.333333	-179.52765	132.86098	0.9999982
NO-BAND:SPRING:2011-CONTROL:SPRING:2010	-28.833333	-185.02765	127.36098	0.9999844
BAND:FALL:2011-NO-BAND:SPRING:2010	15.833333	-140.36098	172.02765	1.0000000
CONTROL:FALL:2011-NO-BAND:SPRING:2010	-6.333333	-162.52765	149.86098	1.0000000
NO-BAND:FALL:2011-NO-BAND:SPRING:2010	-27.333333	-183.52765	128.86098	0.9999910
BAND:SPRING:2011-NO-BAND:SPRING:2010	NA	NA	NA	NA
CONTROL:SPRING:2011-NO-BAND:SPRING:2010	-24.833333	-181.02765	131.36098	0.9999966
NO-BAND:SPRING:2011-NO-BAND:SPRING:2010	-30.333333	-186.52765	125.86098	0.9999739
CONTROL:FALL:2011-BAND:FALL:2011	-22.166667	-178.36098	134.02765	0.9999990
NO-BAND:FALL:2011-BAND:FALL:2011	-43.166667	-199.36098	113.02765	0.9991988
BAND:SPRING:2011-BAND:FALL:2011	NA	NA	NA	NA
CONTROL:SPRING:2011-BAND:FALL:2011	-40.666667	-196.86098	115.52765	0.9995407
NO-BAND:SPRING:2011-BAND:FALL:2011	-46.166667	-202.36098	110.02765	0.9985215
NO-BAND:FALL:2011-CONTROL:FALL:2011	-21.000000	-177.19432	135.19432	0.9999994
BAND:SPRING:2011-CONTROL:FALL:2011	NA	NA	NA	NA
CONTROL:SPRING:2011-CONTROL:FALL:2011	-18.500000	-174.69432	137.69432	0.9999998
NO-BAND:SPRING:2011-CONTROL:FALL:2011	-24.000000	-180.19432	132.19432	0.9999976
BAND:SPRING:2011-NO-BAND:FALL:2011	NA	NA	NA	NA
CONTROL:SPRING:2011-NO-BAND:FALL:2011	2.500000	-153.69432	158.69432	1.0000000
NO-BAND:SPRING:2011-NO-BAND:FALL:2011	-3.000000	-159.19432	153.19432	1.0000000
CONTROL:SPRING:2011-BAND:SPRING:2011	NA	NA	NA	NA
NO-BAND:SPRING:2011-BAND:SPRING:2011	NA	NA	NA	NA
NO-BAND:SPRING:2011-CONTROL:SPRING:2011	-5.500000	-161.69432	150.69432	1.0000000

Soil ENT+TYL

```
> ######First Year After Manure
> dat=read.csv("Soil ENT-TYL.csv", header=T)
> group=subset(dat,StudyYear=="1" & Year=="2010" | StudyYear=="2")
> modG=lm((Concentration)~Location:as.factor(Year):Season,group)
> summary(modG)

Call:
lm(formula = (Concentration) ~ Location:as.factor(Year):Season,
    data = group)

Residuals:
     Min       1Q   Median       3Q      Max
-27.1667  -0.1117   0.0000   0.0000  11.1633

Coefficients: (1 not defined because of singularities)
                                                Estimate Std. Error t value Pr(>|t|)
(Intercept)                                   -2.931e-15  1.933e+00   0.000   1.0000
LocationBAND:as.factor(Year)2010:SeasonFALL    3.667e+00  2.733e+00   1.342   0.1848
LocationCONTROL:as.factor(Year)2010:SeasonFALL 8.976e-15  2.733e+00   0.000   1.0000
LocationNO-BAND:as.factor(Year)2010:SeasonFALL 2.293e-15  2.733e+00   0.000   1.0000
LocationBAND:as.factor(Year)2011:SeasonFALL    3.317e+01  2.733e+00  12.135   <2e-16 ***
LocationCONTROL:as.factor(Year)2011:SeasonFALL -2.180e-16  2.733e+00   0.000   1.0000
LocationNO-BAND:as.factor(Year)2011:SeasonFALL 5.550e-01  2.733e+00   0.203   0.8398
LocationBAND:as.factor(Year)2010:SeasonSPRING  6.057e+00  2.733e+00   2.216   0.0305 *
LocationCONTROL:as.factor(Year)2010:SeasonSPRING 3.302e-18 2.733e+00   0.000   1.0000
LocationNO-BAND:as.factor(Year)2010:SeasonSPRING 3.734e-17 2.733e+00   0.000   1.0000
LocationBAND:as.factor(Year)2011:SeasonSPRING  1.388e-17  2.733e+00   0.000   1.0000
LocationCONTROL:as.factor(Year)2011:SeasonSPRING 1.117e-01 2.733e+00   0.041   0.9675
LocationNO-BAND:as.factor(Year)2011:SeasonSPRING       NA         NA      NA       NA
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 4.734 on 60 degrees of freedom
Multiple R-squared: 0.8158,     Adjusted R-squared: 0.782
F-statistic: 24.15 on 11 and 60 DF,  p-value: < 2.2e-16

> TukeyHSD(aov(modG),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modG)

$`Location:as.factor(Year):Season`
                                                    diff        lwr        upr     p adj
CONTROL:2010:FALL-BAND:2010:FALL          -3.666667e+00 -12.210556   4.877222 0.9697733
NO-BAND:2010:FALL-BAND:2010:FALL          -3.666667e+00 -12.210556   4.877222 0.9697733
BAND:2011:FALL-BAND:2010:FALL              2.950000e+01  20.956111  38.043889 0.0000000
CONTROL:2011:FALL-BAND:2010:FALL          -3.666667e+00 -12.210556   4.877222 0.9697733
NO-BAND:2011:FALL-BAND:2010:FALL          -3.111667e+00 -11.655556   5.432222 0.9914730
BAND:2010:SPRING-BAND:2010:FALL            2.390000e+00  -6.153889  10.933889 0.9991462
CONTROL:2010:SPRING-BAND:2010:FALL        -3.666667e+00 -12.210556   4.877222 0.9697733
NO-BAND:2010:SPRING-BAND:2010:FALL        -3.666667e+00 -12.210556   4.877222 0.9697733
BAND:2011:SPRING-BAND:2010:FALL           -3.666667e+00 -12.210556   4.877222 0.9697733
CONTROL:2011:SPRING-BAND:2010:FALL        -3.555000e+00 -12.098889   4.988889 0.9759068
NO-BAND:2011:SPRING-BAND:2010:FALL        -3.666667e+00 -12.210556   4.877222 0.9697733
NO-BAND:2010:FALL-CONTROL:2010:FALL       -1.487699e-14  -8.543889   8.543889 1.0000000
BAND:2011:FALL-CONTROL:2010:FALL           3.316667e+01  24.622778  41.710556 0.0000000
CONTROL:2011:FALL-CONTROL:2010:FALL       -1.280457e-14  -8.543889   8.543889 1.0000000
NO-BAND:2011:FALL-CONTROL:2010:FALL        5.550000e-01  -7.988889   9.098889 1.0000000
BAND:2010:SPRING-CONTROL:2010:FALL         6.056667e+00  -2.487222  14.600556 0.5451412
CONTROL:2010:SPRING-CONTROL:2010:FALL     -1.191639e-14  -8.543889   8.543889 1.0000000
NO-BAND:2010:SPRING-CONTROL:2010:FALL     -1.147230e-14  -8.543889   8.543889 1.0000000
BAND:2011:SPRING-CONTROL:2010:FALL        -1.147230e-14  -8.543889   8.543889 1.0000000
CONTROL:2011:SPRING-CONTROL:2010:FALL      1.116667e-01  -8.432222   8.655556 1.0000000
NO-BAND:2011:SPRING-CONTROL:2010:FALL     -1.147230e-14  -8.543889   8.543889 1.0000000
BAND:2011:FALL-NO-BAND:2010:FALL           3.316667e+01  24.622778  41.710556 0.0000000
CONTROL:2011:FALL-NO-BAND:2010:FALL        2.072416e-15  -8.543889   8.543889 1.0000000
NO-BAND:2011:FALL-NO-BAND:2010:FALL        5.550000e-01  -7.988889   9.098889 1.0000000
BAND:2010:SPRING-NO-BAND:2010:FALL         6.056667e+00  -2.487222  14.600556 0.5451412
CONTROL:2010:SPRING-NO-BAND:2010:FALL      2.960595e-15  -8.543889   8.543889 1.0000000
NO-BAND:2010:SPRING-NO-BAND:2010:FALL      3.404684e-15  -8.543889   8.543889 1.0000000
BAND:2011:SPRING-NO-BAND:2010:FALL         3.404684e-15  -8.543889   8.543889 1.0000000
CONTROL:2011:SPRING-NO-BAND:2010:FALL      1.116667e-01  -8.432222   8.655556 1.0000000
NO-BAND:2011:SPRING-NO-BAND:2010:FALL      3.404684e-15  -8.543889   8.543889 1.0000000
CONTROL:2011:FALL-BAND:2011:FALL          -3.316667e+01 -41.710556 -24.622778 0.0000000
NO-BAND:2011:FALL-BAND:2011:FALL          -3.261167e+01 -41.155556 -24.067778 0.0000000
```

```
BAND:2010:SPRING-BAND:2011:FALL          -2.711000e+01 -35.653889 -18.566111 0.0000000
CONTROL:2010:SPRING-BAND:2011:FALL       -3.316667e+01 -41.710556 -24.622778 0.0000000
NO-BAND:2010:SPRING-BAND:2011:FALL       -3.316667e+01 -41.710556 -24.622778 0.0000000
BAND:2011:SPRING-BAND:2011:FALL          -3.316667e+01 -41.710556 -24.622778 0.0000000
CONTROL:2011:SPRING-BAND:2011:FALL       -3.305500e+01 -41.598889 -24.511111 0.0000000
NO-BAND:2011:SPRING-BAND:2011:FALL       -3.316667e+01 -41.710556 -24.622778 0.0000000
NO-BAND:2011:FALL-CONTROL:2011:FALL       5.550000e-01  -7.988889   9.098889 1.0000000
BAND:2010:SPRING-CONTROL:2011:FALL        6.056667e+00  -2.487222  14.600556 0.5451412
CONTROL:2010:SPRING-CONTROL:2011:FALL     8.881784e-16  -8.543889   8.543889 1.0000000
NO-BAND:2010:SPRING-CONTROL:2011:FALL     1.332268e-15  -8.543889   8.543889 1.0000000
BAND:2011:SPRING-CONTROL:2011:FALL        1.332268e-15  -8.543889   8.543889 1.0000000
CONTROL:2011:SPRING-CONTROL:2011:FALL     1.116667e-01  -8.432222   8.655556 1.0000000
NO-BAND:2011:SPRING-CONTROL:2011:FALL     1.332268e-15  -8.543889   8.543889 1.0000000
BAND:2010:SPRING-NO-BAND:2011:FALL        5.501667e+00  -3.042222  14.045556 0.6830510
CONTROL:2010:SPRING-NO-BAND:2011:FALL    -5.550000e-01  -9.098889   7.988889 1.0000000
NO-BAND:2010:SPRING-NO-BAND:2011:FALL    -5.550000e-01  -9.098889   7.988889 1.0000000
BAND:2011:SPRING-NO-BAND:2011:FALL       -5.550000e-01  -9.098889   7.988889 1.0000000
CONTROL:2011:SPRING-NO-BAND:2011:FALL    -4.433333e-01  -8.987222   8.100556 1.0000000
NO-BAND:2011:SPRING-NO-BAND:2011:FALL    -5.550000e-01  -9.098889   7.988889 1.0000000
CONTROL:2010:SPRING-BAND:2010:SPRING     -6.056667e+00 -14.600556   2.487222 0.5451412
NO-BAND:2010:SPRING-BAND:2010:SPRING     -6.056667e+00 -14.600556   2.487222 0.5451412
BAND:2011:SPRING-BAND:2010:SPRING        -6.056667e+00 -14.600556   2.487222 0.5451412
CONTROL:2011:SPRING-BAND:2010:SPRING     -5.945000e+00 -14.488889   2.598889 0.5732192
NO-BAND:2011:SPRING-BAND:2010:SPRING     -6.056667e+00 -14.600556   2.487222 0.5451412
NO-BAND:2010:SPRING-CONTROL:2010:SPRING   4.440892e-16  -8.543889   8.543889 1.0000000
BAND:2011:SPRING-CONTROL:2010:SPRING      4.440892e-16  -8.543889   8.543889 1.0000000
CONTROL:2011:SPRING-CONTROL:2010:SPRING   1.116667e-01  -8.432222   8.655556 1.0000000
NO-BAND:2011:SPRING-CONTROL:2010:SPRING   4.440892e-16  -8.543889   8.543889 1.0000000
BAND:2011:SPRING-NO-BAND:2010:SPRING      0.000000e+00  -8.543889   8.543889 1.0000000
CONTROL:2011:SPRING-NO-BAND:2010:SPRING   1.116667e-01  -8.432222   8.655556 1.0000000
NO-BAND:2011:SPRING-NO-BAND:2010:SPRING   0.000000e+00  -8.543889   8.543889 1.0000000
CONTROL:2011:SPRING-BAND:2011:SPRING      1.116667e-01  -8.432222   8.655556 1.0000000
NO-BAND:2011:SPRING-BAND:2011:SPRING      0.000000e+00  -8.543889   8.543889 1.0000000
NO-BAND:2011:SPRING-CONTROL:2011:SPRING  -1.116667e-01  -8.655556   8.432222 1.0000000

>
> ######First Year After Manure 2010 means by location
> mean1=subset(group,Year=="2010")
> modM1=lm(Concentration~Location,mean1)
> summary(modM1)

Call:
lm(formula = Concentration ~ Location, data = mean1)

Residuals:
   Min     1Q Median     3Q    Max
-4.862  0.000  0.000  0.000  9.808

Coefficients:
                Estimate Std. Error t value Pr(>|t|)
(Intercept)       4.8617     0.8281   5.871 1.41e-06 ***
LocationCONTROL  -4.8617     1.1711  -4.151 0.000219 ***
LocationNO-BAND  -4.8617     1.1711  -4.151 0.000219 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 2.869 on 33 degrees of freedom
Multiple R-squared: 0.4105,     Adjusted R-squared: 0.3747
F-statistic: 11.49 on 2 and 33 DF,  p-value: 0.0001634

> TukeyHSD(aov(modM1),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modM1)

$Location
                         diff       lwr       upr     p adj
CONTROL-BAND    -4.861667e+00 -7.351370 -2.371964 0.000625
NO-BAND-BAND    -4.861667e+00 -7.351370 -2.371964 0.000625
NO-BAND-CONTROL  4.070818e-16 -2.489703  2.489703 1.000000

>
> ######First Year After Manure 2011 means by location
> mean2=subset(group,Year=="2011")
> modM2=lm(Concentration~Location,mean2)
> summary(modM2)
```

```
Call:
lm(formula = Concentration ~ Location, data = mean2)

Residuals:
     Min       1Q   Median       3Q      Max
-16.5833  -0.2775  -0.0558  -0.0558  27.7467

Coefficients:
                Estimate Std. Error t value Pr(>|t|)
(Intercept)       16.583      3.330   4.980 1.96e-05 ***
LocationCONTROL  -16.527      4.709  -3.509  0.00132 **
LocationNO-BAND  -16.306      4.709  -3.462  0.00150 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 11.54 on 33 degrees of freedom
Multiple R-squared: 0.3293,     Adjusted R-squared: 0.2887
F-statistic: 8.102 on 2 and 33 DF,  p-value: 0.001372

> TukeyHSD(aov(modM2),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modM2)

$Location
                       diff        lwr       upr     p adj
CONTROL-BAND     -16.5275000 -26.539172 -6.515828 0.0036819
NO-BAND-BAND     -16.3058333 -26.317505 -6.294162 0.0041752
NO-BAND-CONTROL    0.2216667  -9.790005 10.233338 0.9987793

>
> ######First Year After Manure means by location
> modM3=lm(Concentration~Location,group)
> summary(modM3)

Call:
lm(formula = Concentration ~ Location, data = group)

Residuals:
    Min      1Q  Median      3Q     Max
-10.723  -0.139  -0.139  -0.028  33.608

Coefficients:
                Estimate Std. Error t value Pr(>|t|)
(Intercept)       10.722      1.820   5.890 1.27e-07 ***
LocationCONTROL  -10.695      2.574  -4.154 9.20e-05 ***
LocationNO-BAND  -10.584      2.574  -4.111 0.000107 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 8.918 on 69 degrees of freedom
Multiple R-squared: 0.2481,     Adjusted R-squared: 0.2264
F-statistic: 11.39 on 2 and 69 DF,  p-value: 5.328e-05

> TukeyHSD(aov(modM3),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modM3)

$Location
                       diff       lwr       upr     p adj
CONTROL-BAND     -10.6945833 -16.06732 -5.321850 0.0002687
NO-BAND-BAND     -10.5837500 -15.95648 -5.211017 0.0003118
NO-BAND-CONTROL    0.1108333  -5.26190  5.483567 0.9989787

>
> ######Two-Year Study
> dat=read.csv("Soil ENT-TYL.csv", header=T)
> group1=subset(dat,StudyYear=="1")
> modG1=lm((Concentration)~Location:as.factor(Year):Season,group1)
> summary(modG1)

Call:
lm(formula = (Concentration) ~ Location:as.factor(Year):Season,
    data = group1)
```

```
Residuals:
   Min     1Q Median     3Q    Max
-6.057  0.000  0.000  0.000  8.613

Coefficients: (2 not defined because of singularities)
                                                  Estimate Std. Error t value Pr(>|t|)
(Intercept)                                      8.746e-16  8.782e-01   0.000  1.00000
LocationBAND:as.factor(Year)2010:SeasonFALL       3.667e+00  1.242e+00   2.952  0.00463 **
LocationCONTROL:as.factor(Year)2010:SeasonFALL   -2.437e-15  1.242e+00   0.000  1.00000
LocationNO-BAND:as.factor(Year)2010:SeasonFALL   -4.131e-17  1.242e+00   0.000  1.00000
LocationBAND:as.factor(Year)2011:SeasonFALL      -1.159e-15  1.242e+00   0.000  1.00000
LocationCONTROL:as.factor(Year)2011:SeasonFALL   -1.635e-15  1.242e+00   0.000  1.00000
LocationNO-BAND:as.factor(Year)2011:SeasonFALL    5.500e-02  1.242e+00   0.044  0.96484
LocationBAND:as.factor(Year)2010:SeasonSPRING     6.057e+00  1.242e+00   4.877  9.6e-06 ***
LocationCONTROL:as.factor(Year)2010:SeasonSPRING -2.083e-29  1.242e+00   0.000  1.00000
LocationNO-BAND:as.factor(Year)2010:SeasonSPRING -2.180e-29  1.242e+00   0.000  1.00000
LocationBAND:as.factor(Year)2011:SeasonSPRING            NA         NA      NA       NA
LocationCONTROL:as.factor(Year)2011:SeasonSPRING -2.004e-29  1.242e+00   0.000  1.00000
LocationNO-BAND:as.factor(Year)2011:SeasonSPRING         NA         NA      NA       NA
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 2.151 on 55 degrees of freedom
Multiple R-squared: 0.4941,     Adjusted R-squared: 0.4022
F-statistic: 5.373 on 10 and 55 DF,  p-value: 1.688e-05

> TukeyHSD(aov(modG1),conf.level=0.9))
Error: unexpected ')' in "TukeyHSD(aov(modG1),conf.level=0.9))"
>
> ######Two-Year Study 2011 means by location
> mean4=subset(group1,Year=="2011")
> modM4=lm(Concentration~Location,mean4)
> summary(modM4)

Call:
lm(formula = Concentration ~ Location, data = mean4)

Residuals:
    Min      1Q  Median      3Q     Max
-0.0275 -0.0275  0.0000  0.0000  0.3025

Coefficients:
                 Estimate Std. Error t value Pr(>|t|)
(Intercept)     -2.871e-18  2.482e-02   0.000    1.000
LocationCONTROL  1.034e-17  3.040e-02   0.000    1.000
LocationNO-BAND  2.750e-02  3.040e-02   0.905    0.374

Residual standard error: 0.0608 on 27 degrees of freedom
Multiple R-squared: 0.05172,    Adjusted R-squared: -0.01852
F-statistic: 0.7364 on 2 and 27 DF,  p-value: 0.4882

> TukeyHSD(aov(modM4),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modM4)

$Location
                       diff         lwr        upr     p adj
CONTROL-BAND    1.416691e-17 -0.06514793 0.06514793 1.0000000
NO-BAND-BAND    2.750000e-02 -0.03764793 0.09264793 0.6421463
NO-BAND-CONTROL 2.750000e-02 -0.02569307 0.08069307 0.5176068

>
> ######Two-Year Study means by location
> modM5=lm(Concentration~Location,group1)
> summary(modM5)

Call:
lm(formula = Concentration ~ Location, data = group1)

Residuals:
    Min      1Q  Median      3Q     Max
-3.2411 -0.0137 -0.0137  0.0000 11.4289

Coefficients:
               Estimate Std. Error t value Pr(>|t|)
(Intercept)      3.2411     0.5683   5.703 3.37e-07 ***
```

```
LocationCONTROL  -3.2411     0.7518  -4.311 5.81e-05 ***
LocationNO-BAND  -3.2274     0.7518  -4.293 6.20e-05 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 2.411 on 63 degrees of freedom
Multiple R-squared: 0.2722,     Adjusted R-squared: 0.2491
F-statistic: 11.78 on 2 and 63 DF,  p-value: 4.51e-05

> TukeyHSD(aov(modM5),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modM5)

$Location
                     diff       lwr       upr     p adj
CONTROL-BAND    -3.241111 -4.812526 -1.669696 0.0001701
NO-BAND-BAND    -3.227361 -4.798776 -1.655946 0.0001812
NO-BAND-CONTROL  0.013750 -1.441098  1.468598 0.9997848

> ######Two-Year Study means by location and season and year
> modM6=lm(Concentration~Location:Season:as.factor(Year),group1)
> summary(modM6)

Call:
lm(formula = Concentration ~ Location:Season:as.factor(Year),
    data = group1)

Residuals:
   Min     1Q Median     3Q    Max
-6.057  0.000  0.000  0.000  8.613

Coefficients: (2 not defined because of singularities)
                                                  Estimate Std. Error t value Pr(>|t|)
(Intercept)                                       2.733e-16  8.782e-01   0.000  1.00000
LocationBAND:SeasonFALL:as.factor(Year)2010       3.667e+00  1.242e+00   2.952  0.00463 **
LocationCONTROL:SeasonFALL:as.factor(Year)2010   -1.836e-15  1.242e+00   0.000  1.00000
LocationNO-BAND:SeasonFALL:as.factor(Year)2010    6.730e-16  1.242e+00   0.000  1.00000
LocationBAND:SeasonSPRING:as.factor(Year)2010     6.057e+00  1.242e+00   4.877  9.6e-06 ***
LocationCONTROL:SeasonSPRING:as.factor(Year)2010 -1.158e-17  1.242e+00   0.000  1.00000
LocationNO-BAND:SeasonSPRING:as.factor(Year)2010 -4.274e-18  1.242e+00   0.000  1.00000
LocationBAND:SeasonFALL:as.factor(Year)2011       1.742e-17  1.242e+00   0.000  1.00000
LocationCONTROL:SeasonFALL:as.factor(Year)2011    0.000e+00  1.242e+00   0.000  1.00000
LocationNO-BAND:SeasonFALL:as.factor(Year)2011    5.500e-02  1.242e+00   0.044  0.96484
LocationBAND:SeasonSPRING:as.factor(Year)2011             NA         NA      NA       NA
LocationCONTROL:SeasonSPRING:as.factor(Year)2011 -1.543e-29  1.242e+00   0.000  1.00000
LocationNO-BAND:SeasonSPRING:as.factor(Year)2011          NA         NA      NA       NA
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 2.151 on 55 degrees of freedom
Multiple R-squared: 0.4941,     Adjusted R-squared: 0.4022
F-statistic: 5.373 on 10 and 55 DF,  p-value: 1.688e-05

> TukeyHSD(aov(modM6),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modM6)

$`Location:Season:as.factor(Year)`
                                                 diff       lwr        upr     p adj
CONTROL:FALL:2010-BAND:FALL:2010        -3.666667e+00 -7.560044  0.2267106 0.1515743
NO-BAND:FALL:2010-BAND:FALL:2010        -3.666667e+00 -7.560044  0.2267106 0.1515743
BAND:SPRING:2010-BAND:FALL:2010          2.390000e+00 -1.503377  6.2833773 0.7392257
CONTROL:SPRING:2010-BAND:FALL:2010      -3.666667e+00 -7.560044  0.2267106 0.1515743
NO-BAND:SPRING:2010-BAND:FALL:2010      -3.666667e+00 -7.560044  0.2267106 0.1515743
BAND:FALL:2011-BAND:FALL:2010           -3.666667e+00 -7.560044  0.2267106 0.1515743
CONTROL:FALL:2011-BAND:FALL:2010        -3.666667e+00 -7.560044  0.2267106 0.1515743
NO-BAND:FALL:2011-BAND:FALL:2010        -3.611667e+00 -7.505044  0.2817106 0.1667892
BAND:SPRING:2011-BAND:FALL:2010                    NA        NA         NA        NA
CONTROL:SPRING:2011-BAND:FALL:2010      -3.666667e+00 -7.560044  0.2267106 0.1515743
NO-BAND:SPRING:2011-BAND:FALL:2010      -3.666667e+00 -7.560044  0.2267106 0.1515743
NO-BAND:FALL:2010-CONTROL:FALL:2010      1.221245e-15 -3.893377  3.8933773 1.0000000
BAND:SPRING:2010-CONTROL:FALL:2010       6.056667e+00  2.163289  9.9500440 0.0005489
CONTROL:SPRING:2010-CONTROL:FALL:2010   -4.440892e-16 -3.893377  3.8933773 1.0000000
NO-BAND:SPRING:2010-CONTROL:FALL:2010   -4.440892e-16 -3.893377  3.8933773 1.0000000
```

BAND:FALL:2011-CONTROL:FALL:2010	-4.440892e-16	-3.893377	3.8933773	1.0000000
CONTROL:FALL:2011-CONTROL:FALL:2010	-4.440892e-16	-3.893377	3.8933773	1.0000000
NO-BAND:FALL:2011-CONTROL:FALL:2010	5.500000e-02	-3.838377	3.9483773	1.0000000
BAND:SPRING:2011-CONTROL:FALL:2010	NA	NA	NA	NA
CONTROL:SPRING:2011-CONTROL:FALL:2010	-3.330669e-16	-3.893377	3.8933773	1.0000000
NO-BAND:SPRING:2011-CONTROL:FALL:2010	-3.330669e-16	-3.893377	3.8933773	1.0000000
BAND:SPRING:2010-NO-BAND:FALL:2010	6.056667e+00	2.163289	9.9500440	0.0005489
CONTROL:SPRING:2010-NO-BAND:FALL:2010	-1.665335e-15	-3.893377	3.8933773	1.0000000
NO-BAND:SPRING:2010-NO-BAND:FALL:2010	-1.665335e-15	-3.893377	3.8933773	1.0000000
BAND:FALL:2011-NO-BAND:FALL:2010	-1.665335e-15	-3.893377	3.8933773	1.0000000
CONTROL:FALL:2011-NO-BAND:FALL:2010	-1.665335e-15	-3.893377	3.8933773	1.0000000
NO-BAND:FALL:2011-NO-BAND:FALL:2010	5.500000e-02	-3.838377	3.9483773	1.0000000
BAND:SPRING:2011-NO-BAND:FALL:2010	NA	NA	NA	NA
CONTROL:SPRING:2011-NO-BAND:FALL:2010	-1.554312e-15	-3.893377	3.8933773	1.0000000
NO-BAND:SPRING:2011-NO-BAND:FALL:2010	-1.554312e-15	-3.893377	3.8933773	1.0000000
CONTROL:SPRING:2010-BAND:SPRING:2010	-6.056667e+00	-9.950044	-2.1632894	0.0005489
NO-BAND:SPRING:2010-BAND:SPRING:2010	-6.056667e+00	-9.950044	-2.1632894	0.0005489
BAND:FALL:2011-BAND:SPRING:2010	-6.056667e+00	-9.950044	-2.1632894	0.0005489
CONTROL:FALL:2011-BAND:SPRING:2010	-6.056667e+00	-9.950044	-2.1632894	0.0005489
NO-BAND:FALL:2011-BAND:SPRING:2010	-6.001667e+00	-9.895044	-2.1082894	0.0006385
BAND:SPRING:2011-BAND:SPRING:2010	NA	NA	NA	NA
CONTROL:SPRING:2011-BAND:SPRING:2010	-6.056667e+00	-9.950044	-2.1632894	0.0005489
NO-BAND:SPRING:2011-BAND:SPRING:2010	-6.056667e+00	-9.950044	-2.1632894	0.0005489
NO-BAND:SPRING:2010-CONTROL:SPRING:2010	0.000000e+00	-3.893377	3.8933773	1.0000000
BAND:FALL:2011-CONTROL:SPRING:2010	0.000000e+00	-3.893377	3.8933773	1.0000000
CONTROL:FALL:2011-CONTROL:SPRING:2010	0.000000e+00	-3.893377	3.8933773	1.0000000
NO-BAND:FALL:2011-CONTROL:SPRING:2010	5.500000e-02	-3.838377	3.9483773	1.0000000
BAND:SPRING:2011-CONTROL:SPRING:2010	NA	NA	NA	NA
CONTROL:SPRING:2011-CONTROL:SPRING:2010	1.110223e-16	-3.893377	3.8933773	1.0000000
NO-BAND:SPRING:2011-CONTROL:SPRING:2010	1.110223e-16	-3.893377	3.8933773	1.0000000
BAND:FALL:2011-NO-BAND:SPRING:2010	0.000000e+00	-3.893377	3.8933773	1.0000000
CONTROL:FALL:2011-NO-BAND:SPRING:2010	0.000000e+00	-3.893377	3.8933773	1.0000000
NO-BAND:FALL:2011-NO-BAND:SPRING:2010	5.500000e-02	-3.838377	3.9483773	1.0000000
BAND:SPRING:2011-NO-BAND:SPRING:2010	NA	NA	NA	NA
CONTROL:SPRING:2011-NO-BAND:SPRING:2010	1.110223e-16	-3.893377	3.8933773	1.0000000
NO-BAND:SPRING:2011-NO-BAND:SPRING:2010	1.110223e-16	-3.893377	3.8933773	1.0000000
CONTROL:FALL:2011-BAND:FALL:2011	0.000000e+00	-3.893377	3.8933773	1.0000000
NO-BAND:FALL:2011-BAND:FALL:2011	5.500000e-02	-3.838377	3.9483773	1.0000000
BAND:SPRING:2011-BAND:FALL:2011	NA	NA	NA	NA
CONTROL:SPRING:2011-BAND:FALL:2011	1.110223e-16	-3.893377	3.8933773	1.0000000
NO-BAND:SPRING:2011-BAND:FALL:2011	1.110223e-16	-3.893377	3.8933773	1.0000000
NO-BAND:FALL:2011-CONTROL:FALL:2011	5.500000e-02	-3.838377	3.9483773	1.0000000
BAND:SPRING:2011-CONTROL:FALL:2011	NA	NA	NA	NA
CONTROL:SPRING:2011-CONTROL:FALL:2011	1.110223e-16	-3.893377	3.8933773	1.0000000
NO-BAND:SPRING:2011-CONTROL:FALL:2011	1.110223e-16	-3.893377	3.8933773	1.0000000
BAND:SPRING:2011-NO-BAND:FALL:2011	NA	NA	NA	NA
CONTROL:SPRING:2011-NO-BAND:FALL:2011	-5.500000e-02	-3.948377	3.8383773	1.0000000
NO-BAND:SPRING:2011-NO-BAND:FALL:2011	-5.500000e-02	-3.948377	3.8383773	1.0000000
CONTROL:SPRING:2011-BAND:SPRING:2011	NA	NA	NA	NA
NO-BAND:SPRING:2011-BAND:SPRING:2011	NA	NA	NA	NA
NO-BAND:SPRING:2011-CONTROL:SPRING:2011	0.000000e+00	-3.893377	3.8933773	1.0000000

Soil ermB

```
>
> ######First Year After Manure
> dat=read.csv("Soil ermBa.csv", header=T)
> group=subset(dat,StudyYear=="1" & Year=="2010" | StudyYear=="2")
> modG=lm((Concentration)~Location:as.factor(Year):Season,group)
> summary(modG)

Call:
lm(formula = (Concentration) ~ Location:as.factor(Year):Season,
    data = group)

Residuals:
       Min         1Q     Median         3Q        Max
-594958344    -660403      -3314     370713 1218620718

Coefficients: (1 not defined because of singularities)
                                                  Estimate Std. Error t value Pr(>|t|)
(Intercept)                                         138982   82115476   0.002 0.998655
LocationBAND:as.factor(Year)2010:SeasonFALL      1009263543  116128820   8.691 3.28e-12 ***
LocationCONTROL:as.factor(Year)2010:SeasonFALL       152909  116128820   0.001 0.998954
LocationNO-BAND:as.factor(Year)2010:SeasonFALL      1603817  116128820   0.014 0.989027
LocationBAND:as.factor(Year)2011:SeasonFALL       461750668  116128820   3.976 0.000191 ***
LocationCONTROL:as.factor(Year)2011:SeasonFALL      -138982  116128820  -0.001 0.999049
LocationNO-BAND:as.factor(Year)2011:SeasonFALL       533680  116128820   0.005 0.996349
LocationBAND:as.factor(Year)2010:SeasonSPRING      86865913  116128820   0.748 0.457374
LocationCONTROL:as.factor(Year)2010:SeasonSPRING     209177  116128820   0.002 0.998569
LocationNO-BAND:as.factor(Year)2010:SeasonSPRING     2748581  116128820   0.024 0.981196
LocationBAND:as.factor(Year)2011:SeasonSPRING        1623376  116128820   0.014 0.988893
LocationCONTROL:as.factor(Year)2011:SeasonSPRING     -135909  116128820  -0.001 0.999070
LocationNO-BAND:as.factor(Year)2011:SeasonSPRING          NA         NA      NA       NA
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 201100000 on 60 degrees of freedom
Multiple R-squared: 0.719,      Adjusted R-squared: 0.6675
F-statistic: 13.96 on 11 and 60 DF,  p-value: 8.664e-13

> TukeyHSD(aov(modG),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modG)

$`Location:as.factor(Year):Season`
                                                    diff         lwr         upr     p adj
CONTROL:2010:FALL-BAND:2010:FALL          -1.009111e+09 -1372127697 -646093570 0.0000000
NO-BAND:2010:FALL-BAND:2010:FALL          -1.007660e+09 -1370676790 -644642662 0.0000000
BAND:2011:FALL-BAND:2010:FALL             -5.475129e+08  -910529939 -184495811 0.0008435
CONTROL:2011:FALL-BAND:2010:FALL          -1.009403e+09 -1372419588 -646385461 0.0000000
NO-BAND:2011:FALL-BAND:2010:FALL          -1.008730e+09 -1371746927 -645712799 0.0000000
BAND:2010:SPRING-BAND:2010:FALL           -9.223976e+08 -1285414693 -559380566 0.0000000
CONTROL:2010:SPRING-BAND:2010:FALL        -1.009054e+09 -1372071429 -646037302 0.0000000
NO-BAND:2010:SPRING-BAND:2010:FALL        -1.006515e+09 -1369532026 -643497898 0.0000000
BAND:2011:SPRING-BAND:2010:FALL           -1.007640e+09 -1370657230 -644623103 0.0000000
CONTROL:2011:SPRING-BAND:2010:FALL        -1.009399e+09 -1372416515 -646382387 0.0000000
NO-BAND:2011:SPRING-BAND:2010:FALL        -1.009264e+09 -1372280606 -646246479 0.0000000
NO-BAND:2010:FALL-CONTROL:2010:FALL        1.450908e+06  -361566156  364467971 1.0000000
BAND:2011:FALL-CONTROL:2010:FALL           4.615978e+08    98580695  824614823 0.0095608
CONTROL:2011:FALL-CONTROL:2010:FALL       -2.918908e+05  -363308955  362725173 1.0000000
NO-BAND:2011:FALL-CONTROL:2010:FALL        3.807708e+05  -362636293  363397835 1.0000000
BAND:2010:SPRING-CONTROL:2010:FALL         8.671300e+07  -276304059  449730068 0.9998083
CONTROL:2010:SPRING-CONTROL:2010:FALL      5.626800e+04  -362960796  363073332 1.0000000
NO-BAND:2010:SPRING-CONTROL:2010:FALL      2.595672e+06  -360421392  365612736 1.0000000
BAND:2011:SPRING-CONTROL:2010:FALL         1.470467e+06  -361546596  364487531 1.0000000
CONTROL:2011:SPRING-CONTROL:2010:FALL     -2.888174e+05  -363305881  362728246 1.0000000
NO-BAND:2011:SPRING-CONTROL:2010:FALL     -1.529089e+05  -363169973  362864155 1.0000000
BAND:2011:FALL-NO-BAND:2010:FALL           4.601469e+08    97129787  823163915 0.0099354
CONTROL:2011:FALL-NO-BAND:2010:FALL       -1.742799e+06  -364759862  361274265 1.0000000
NO-BAND:2011:FALL-NO-BAND:2010:FALL       -1.070137e+06  -364087201  361946927 1.0000000
BAND:2010:SPRING-NO-BAND:2010:FALL         8.526210e+07  -277754967  448279160 0.9998372
CONTROL:2010:SPRING-NO-BAND:2010:FALL     -1.394640e+06  -364411703  361622424 1.0000000
NO-BAND:2010:SPRING-NO-BAND:2010:FALL      1.144764e+06  -361872300  364161828 1.0000000
BAND:2011:SPRING-NO-BAND:2010:FALL         1.955963e+04  -362997504  363036623 1.0000000
CONTROL:2011:SPRING-NO-BAND:2010:FALL     -1.739725e+06  -364756789  361277339 1.0000000
NO-BAND:2011:SPRING-NO-BAND:2010:FALL     -1.603817e+06  -364620880  361413247 1.0000000
CONTROL:2011:FALL-BAND:2011:FALL          -4.618896e+08  -824906713  -98872586 0.0094871
```

```
NO-BAND:2011:FALL-BAND:2011:FALL         -4.612170e+08  -824234052  -98199924 0.0096579
BAND:2010:SPRING-BAND:2011:FALL          -3.748848e+08  -737901818  -11867691 0.0778692
CONTROL:2010:SPRING-BAND:2011:FALL       -4.615415e+08  -824558555  -98524427 0.0095751
NO-BAND:2010:SPRING-BAND:2011:FALL       -4.590021e+08  -822019151  -95985023 0.0102406
BAND:2011:SPRING-BAND:2011:FALL          -4.601273e+08  -823144355  -97110228 0.0099406
CONTROL:2011:SPRING-BAND:2011:FALL       -4.618866e+08  -824903640  -98869512 0.0094879
NO-BAND:2011:SPRING-BAND:2011:FALL       -4.617507e+08  -824767732  -98733604 0.0095221
NO-BAND:2011:FALL-CONTROL:2011:FALL       6.726617e+05  -362344402  363689725 1.0000000
BAND:2010:SPRING-CONTROL:2011:FALL        8.700490e+07  -276012169  450021959 0.9998019
CONTROL:2010:SPRING-CONTROL:2011:FALL     3.481588e+05  -362668905  363365223 1.0000000
NO-BAND:2010:SPRING-CONTROL:2011:FALL     2.887563e+06  -360129501  365904626 1.0000000
BAND:2011:SPRING-CONTROL:2011:FALL        1.762358e+06  -361254706  364779422 1.0000000
CONTROL:2011:SPRING-CONTROL:2011:FALL     3.073450e+03  -363013990  363020137 1.0000000
NO-BAND:2011:SPRING-CONTROL:2011:FALL     1.389819e+05  -362878082  363156046 1.0000000
BAND:2010:SPRING-NO-BAND:2011:FALL        8.633223e+07  -276684830  449349297 0.9998163
CONTROL:2010:SPRING-NO-BAND:2011:FALL    -3.245028e+05  -363341567  362692561 1.0000000
NO-BAND:2010:SPRING-NO-BAND:2011:FALL     2.214901e+06  -360802163  365231965 1.0000000
BAND:2011:SPRING-NO-BAND:2011:FALL        1.089696e+06  -361927367  364106760 1.0000000
CONTROL:2011:SPRING-NO-BAND:2011:FALL    -6.695882e+05  -363686652  362347476 1.0000000
NO-BAND:2011:SPRING-NO-BAND:2011:FALL    -5.336797e+05  -363550744  362483384 1.0000000
CONTROL:2010:SPRING-BAND:2010:SPRING     -8.665674e+07  -449673800  276360327 0.9998095
NO-BAND:2010:SPRING-BAND:2010:SPRING     -8.411733e+07  -447134396  278899731 0.9998573
BAND:2011:SPRING-BAND:2010:SPRING        -8.524254e+07  -448259601  277774527 0.9998376
CONTROL:2011:SPRING-BAND:2010:SPRING     -8.700182e+07  -450018885  276015242 0.9998020
NO-BAND:2011:SPRING-BAND:2010:SPRING     -8.686591e+07  -449882977  276151151 0.9998050
NO-BAND:2010:SPRING-CONTROL:2010:SPRING   2.539404e+06  -360477660  365556468 1.0000000
BAND:2011:SPRING-CONTROL:2010:SPRING      1.414199e+06  -361602864  364431263 1.0000000
CONTROL:2011:SPRING-CONTROL:2010:SPRING  -3.450854e+05  -363362149  362671978 1.0000000
NO-BAND:2011:SPRING-CONTROL:2010:SPRING  -2.091769e+05  -363226241  362807887 1.0000000
BAND:2011:SPRING-NO-BAND:2010:SPRING     -1.125205e+06  -364142268  361891859 1.0000000
CONTROL:2011:SPRING-NO-BAND:2010:SPRING  -2.884489e+06  -365901553  360132575 1.0000000
NO-BAND:2011:SPRING-NO-BAND:2010:SPRING  -2.748581e+06  -365765645  360268483 1.0000000
CONTROL:2011:SPRING-BAND:2011:SPRING     -1.759285e+06  -364776348  361257779 1.0000000
NO-BAND:2011:SPRING-BAND:2011:SPRING     -1.623376e+06  -364640440  361393688 1.0000000
NO-BAND:2011:SPRING-CONTROL:2011:SPRING   1.359084e+05  -362881155  363152972 1.0000000

>
> ######First Year After Manure 2010 means by location
> mean1=subset(group,Year=="2010")
> modM1=lm(Concentration~Location,mean1)
> summary(modM1)

Call:
lm(formula = Concentration ~ Location, data = mean1)

Residuals:
       Min         1Q     Median         3Q        Max
-532114071    -950353     -47316     166583  873293179

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)      548203710   89200773   6.146 6.29e-07 ***
LocationCONTROL -547883685  126148943  -4.343 0.000126 ***
LocationNO-BAND -545888529  126148943  -4.327 0.000132 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 3.09e+08 on 33 degrees of freedom
Multiple R-squared: 0.4316,     Adjusted R-squared: 0.3972
F-statistic: 12.53 on 2 and 33 DF,  p-value: 8.945e-05

> TukeyHSD(aov(modM1),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modM1)

$Location
                     diff        lwr        upr     p adj
CONTROL-BAND    -547883685 -816064565 -279702805 0.0003618
NO-BAND-BAND    -545888529 -814069409 -277707649 0.0003785
NO-BAND-CONTROL    1995156 -266185724  270176036 0.9998621

>
> ######First Year After Manure 2011 means by location
> mean2=subset(group,Year=="2011")
> modM2=lm(Concentration~Location,mean2)
> summary(modM2)
```

```
Call:
lm(formula = Concentration ~ Location, data = mean2)

Residuals:
       Min         1Q     Median         3Q        Max
-231780610    -401046    -178828      -1537 1448684364

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)      231826004   78884054   2.939  0.00597 **
LocationCONTROL -231824467  111558899  -2.078  0.04556 *
LocationNO-BAND -231420182  111558899  -2.074  0.04592 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 273300000 on 33 degrees of freedom
Multiple R-squared: 0.1483,     Adjusted R-squared: 0.09672
F-statistic: 2.874 on 2 and 33 DF,  p-value: 0.0707

> TukeyHSD(aov(modM2),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modM2)

$Location
                       diff        lwr       upr     p adj
CONTROL-BAND     -231824467 -468988276   5339342 0.1100012
NO-BAND-BAND     -231420182 -468583991   5743627 0.1107909
NO-BAND-CONTROL     404285 -236759524 237568094 0.9999928

> ######First Year After Manure means by location
> modM3=lm(Concentration~Location,group)
> summary(modM3)

Call:
lm(formula = Concentration ~ Location, data = group)

Residuals:
       Min         1Q     Median         3Q        Max
-389969463   -1355726    -160781     229306 1290495511

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)      390014857   61265156   6.366 1.84e-08 ***
LocationCONTROL -389854076   86642015  -4.500 2.69e-05 ***
LocationNO-BAND -388654356   86642015  -4.486 2.82e-05 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 300100000 on 69 degrees of freedom
Multiple R-squared: 0.2806,     Adjusted R-squared: 0.2597
F-statistic: 13.46 on 2 and 69 DF,  p-value: 1.163e-05

> TukeyHSD(aov(modM3),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modM3)

$Location
                       diff        lwr        upr     p adj
CONTROL-BAND     -389854076 -570673006 -209035146 0.0000790
NO-BAND-BAND     -388654356 -569473286 -207835426 0.0000831
NO-BAND-CONTROL     1199720 -179619209  182018650 0.9998943
```

Soil ermF

```
> ######First Year After Manure
> dat=read.csv("Soil ermF.csv", header=T)
> group=subset(dat,StudyYear=="1" & Year=="2010" | StudyYear=="2")
> modG=lm((Concentration)~Location:as.factor(Year):Season,group)
> summary(modG)

Call:
lm(formula = (Concentration) ~ Location:as.factor(Year):Season,
    data = group)

Residuals:
       Min         1Q     Median         3Q        Max
-3.588e+12 -3.434e+06 -8.892e+03  3.060e+02  1.186e+13

Coefficients: (1 not defined because of singularities)
                                                 Estimate Std. Error t value Pr(>|t|)
(Intercept)                                     8.673e+04  7.258e+11   0.000 1.000000
LocationBAND:as.factor(Year)2010:SeasonFALL     3.590e+12  1.026e+12   3.497 0.000891 ***
LocationCONTROL:as.factor(Year)2010:SeasonFALL -8.383e+04  1.026e+12   0.000 1.000000
LocationNO-BAND:as.factor(Year)2010:SeasonFALL -6.643e+04  1.026e+12   0.000 1.000000
LocationBAND:as.factor(Year)2011:SeasonFALL     4.904e+08  1.026e+12   0.000 0.999620
LocationCONTROL:as.factor(Year)2011:SeasonFALL -8.319e+04  1.026e+12   0.000 1.000000
LocationNO-BAND:as.factor(Year)2011:SeasonFALL  3.347e+06  1.026e+12   0.000 0.999997
LocationBAND:as.factor(Year)2010:SeasonSPRING   2.412e+09  1.026e+12   0.002 0.998133
LocationCONTROL:as.factor(Year)2010:SeasonSPRING -7.305e+04  1.026e+12   0.000 1.000000
LocationNO-BAND:as.factor(Year)2010:SeasonSPRING  1.292e+07  1.026e+12   0.000 0.999990
LocationBAND:as.factor(Year)2011:SeasonSPRING   2.850e+06  1.026e+12   0.000 0.999998
LocationCONTROL:as.factor(Year)2011:SeasonSPRING -8.673e+04  1.026e+12   0.000 1.000000
LocationNO-BAND:as.factor(Year)2011:SeasonSPRING         NA         NA      NA       NA
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 1.778e+12 on 60 degrees of freedom
Multiple R-squared: 0.272,      Adjusted R-squared: 0.1386
F-statistic: 2.038 on 11 and 60 DF,  p-value: 0.03993

> TukeyHSD(aov(modG),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modG)

$`Location:as.factor(Year):Season`
                                                      diff           lwr           upr     p adj
CONTROL:2010:FALL-BAND:2010:FALL                -3.589761e+12 -6.798232e+12 -381289597380 0.0384594
NO-BAND:2010:FALL-BAND:2010:FALL                -3.589761e+12 -6.798232e+12 -381289579979 0.0384594
BAND:2011:FALL-BAND:2010:FALL                   -3.589270e+12 -6.797741e+12 -380799070505 0.0385097
CONTROL:2011:FALL-BAND:2010:FALL                -3.589761e+12 -6.798232e+12 -381289596738 0.0384594
NO-BAND:2011:FALL-BAND:2010:FALL                -3.589757e+12 -6.798228e+12 -381286166166 0.0384597
BAND:2010:SPRING-BAND:2010:FALL                 -3.587348e+12 -6.795820e+12 -378877396617 0.0387074
CONTROL:2010:SPRING-BAND:2010:FALL              -3.589761e+12 -6.798232e+12 -381289586599 0.0384594
NO-BAND:2010:SPRING-BAND:2010:FALL              -3.589748e+12 -6.798219e+12 -381276591753 0.0384607
BAND:2011:SPRING-BAND:2010:FALL                 -3.589758e+12 -6.798229e+12 -381286663724 0.0384597
CONTROL:2011:SPRING-BAND:2010:FALL              -3.589761e+12 -6.798232e+12 -381289600282 0.0384594
NO-BAND:2011:SPRING-BAND:2010:FALL              -3.589761e+12 -6.798232e+12 -381289513551 0.0384594
NO-BAND:2010:FALL-CONTROL:2010:FALL              1.740145e+04 -3.208471e+12 3208471104660 1.0000000
BAND:2011:FALL-CONTROL:2010:FALL                 4.905269e+08 -3.207981e+12 3208961614133 1.0000000
CONTROL:2011:FALL-CONTROL:2010:FALL              6.425721e+02 -3.208471e+12 3208471087901 1.0000000
NO-BAND:2011:FALL-CONTROL:2010:FALL              3.431215e+06 -3.208468e+12 3208474518473 1.0000000
BAND:2010:SPRING-CONTROL:2010:FALL               2.412201e+09 -3.206059e+12 3210883288021 1.0000000
CONTROL:2010:SPRING-CONTROL:2010:FALL            1.078154e+04 -3.208471e+12 3208471098040 1.0000000
NO-BAND:2010:SPRING-CONTROL:2010:FALL            1.300563e+07 -3.208458e+12 3208484092885 1.0000000
BAND:2011:SPRING-CONTROL:2010:FALL               2.933656e+06 -3.208468e+12 3208474020914 1.0000000
CONTROL:2011:SPRING-CONTROL:2010:FALL           -2.901251e+03 -3.208471e+12 3208471084357 1.0000000
NO-BAND:2011:SPRING-CONTROL:2010:FALL            8.382937e+04 -3.208471e+12 3208471171088 1.0000000
BAND:2011:FALL-NO-BAND:2010:FALL                 4.905095e+08 -3.207981e+12 3208961596732 1.0000000
CONTROL:2011:FALL-NO-BAND:2010:FALL             -1.675888e+04 -3.208471e+12 3208471070499 1.0000000
NO-BAND:2011:FALL-NO-BAND:2010:FALL              3.413813e+06 -3.208468e+12 3208474501071 1.0000000
BAND:2010:SPRING-NO-BAND:2010:FALL               2.412183e+09 -3.206059e+12 3210883270620 1.0000000
CONTROL:2010:SPRING-NO-BAND:2010:FALL           -6.619910e+03 -3.208471e+12 3208471080638 1.0000000
NO-BAND:2010:SPRING-NO-BAND:2010:FALL            1.298823e+07 -3.208458e+12 3208484075484 1.0000000
BAND:2011:SPRING-NO-BAND:2010:FALL               2.916254e+06 -3.208468e+12 3208474003513 1.0000000
CONTROL:2011:SPRING-NO-BAND:2010:FALL           -2.030270e+04 -3.208471e+12 3208471066956 1.0000000
NO-BAND:2011:SPRING-NO-BAND:2010:FALL            6.642792e+04 -3.208471e+12 3208471153686 1.0000000
CONTROL:2011:FALL-BAND:2011:FALL                -4.905262e+08 -3.208962e+12 3207980561026 1.0000000
NO-BAND:2011:FALL-BAND:2011:FALL                -4.870957e+08 -3.208958e+12 3207983991598 1.0000000
```

```
BAND:2010:SPRING-BAND:2011:FALL           1.921674e+09 -3.206549e+12 3210392761147 1.0000000
CONTROL:2010:SPRING-BAND:2011:FALL       -4.905161e+08 -3.208962e+12 3207980571165 1.0000000
NO-BAND:2010:SPRING-BAND:2011:FALL       -4.775212e+08 -3.208949e+12 3207993566010 1.0000000
BAND:2011:SPRING-BAND:2011:FALL          -4.875932e+08 -3.208959e+12 3207983494039 1.0000000
CONTROL:2011:SPRING-BAND:2011:FALL       -4.905298e+08 -3.208962e+12 3207980557482 1.0000000
NO-BAND:2011:SPRING-BAND:2011:FALL       -4.904430e+08 -3.208962e+12 3207980644213 1.0000000
NO-BAND:2011:FALL-CONTROL:2011:FALL       3.430572e+06 -3.208468e+12 3208474517830 1.0000000
BAND:2010:SPRING-CONTROL:2011:FALL        2.412200e+09 -3.206059e+12 3210883287379 1.0000000
CONTROL:2010:SPRING-CONTROL:2011:FALL     1.013897e+04 -3.208471e+12 3208471097397 1.0000000
NO-BAND:2010:SPRING-CONTROL:2011:FALL     1.300498e+07 -3.208458e+12 3208484092243 1.0000000
BAND:2011:SPRING-CONTROL:2011:FALL        2.933013e+06 -3.208468e+12 3208474020272 1.0000000
CONTROL:2011:SPRING-CONTROL:2011:FALL    -3.543823e+03 -3.208471e+12 3208471083714 1.0000000
NO-BAND:2011:SPRING-CONTROL:2011:FALL     8.318680e+04 -3.208471e+12 3208471170445 1.0000000
BAND:2010:SPRING-NO-BAND:2011:FALL        2.408770e+09 -3.206062e+12 3210879856807 1.0000000
CONTROL:2010:SPRING-NO-BAND:2011:FALL    -3.420433e+06 -3.208475e+12 3208467666825 1.0000000
NO-BAND:2010:SPRING-NO-BAND:2011:FALL     9.574412e+06 -3.208462e+12 3208480661671 1.0000000
BAND:2011:SPRING-NO-BAND:2011:FALL       -4.975588e+05 -3.208472e+12 3208470589699 1.0000000
CONTROL:2011:SPRING-NO-BAND:2011:FALL    -3.434116e+06 -3.208475e+12 3208467653142 1.0000000
NO-BAND:2011:SPRING-NO-BAND:2011:FALL    -3.347385e+06 -3.208474e+12 3208467739873 1.0000000
CONTROL:2010:SPRING-BAND:2010:SPRING     -2.412190e+09 -3.210883e+12 3206058897277 1.0000000
NO-BAND:2010:SPRING-BAND:2010:SPRING     -2.399195e+09 -3.210870e+12 3206071892122 1.0000000
BAND:2011:SPRING-BAND:2010:SPRING        -2.409267e+09 -3.210880e+12 3206061820151 1.0000000
CONTROL:2011:SPRING-BAND:2010:SPRING     -2.412204e+09 -3.210883e+12 3206058883594 1.0000000
NO-BAND:2011:SPRING-BAND:2010:SPRING     -2.412117e+09 -3.210883e+12 3206058970324 1.0000000
NO-BAND:2010:SPRING-CONTROL:2010:SPRING   1.299485e+07 -3.208458e+12 3208484082104 1.0000000
BAND:2011:SPRING-CONTROL:2010:SPRING      2.922874e+06 -3.208468e+12 3208474010133 1.0000000
CONTROL:2011:SPRING-CONTROL:2010:SPRING -1.368279e+04 -3.208471e+12 3208471073575 1.0000000
NO-BAND:2011:SPRING-CONTROL:2010:SPRING   7.304783e+04 -3.208471e+12 3208471160306 1.0000000
BAND:2011:SPRING-NO-BAND:2010:SPRING     -1.007197e+07 -3.208481e+12 3208461015287 1.0000000
CONTROL:2011:SPRING-NO-BAND:2010:SPRING -1.300853e+07 -3.208484e+12 3208458078730 1.0000000
NO-BAND:2011:SPRING-NO-BAND:2010:SPRING -1.292180e+07 -3.208484e+12 3208458165461 1.0000000
CONTROL:2011:SPRING-BAND:2011:SPRING     -2.936557e+06 -3.208474e+12 3208468150701 1.0000000
NO-BAND:2011:SPRING-BAND:2011:SPRING     -2.849826e+06 -3.208474e+12 3208468237432 1.0000000
NO-BAND:2011:SPRING-CONTROL:2011:SPRING   8.673062e+04 -3.208471e+12 3208471173989 1.0000000
>
> ######First Year After Manure 2010 means by location
> mean1=subset(group,Year=="2010")
> modM1=lm(Concentration~Location,mean1)
> summary(modM1)

Call:
lm(formula = Concentration ~ Location, data = mean1)

Residuals:
       Min         1Q     Median         3Q        Max
-1.796e+12 -1.280e+12 -5.416e+06 -1.469e+03  1.366e+13

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)      1.796e+12  7.592e+11   2.366    0.024 *
LocationCONTROL -1.796e+12  1.074e+12  -1.673    0.104
LocationNO-BAND -1.796e+12  1.074e+12  -1.673    0.104
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 2.63e+12 on 33 degrees of freedom
Multiple R-squared: 0.1016,     Adjusted R-squared: 0.04714
F-statistic: 1.866 on 2 and 33 DF,  p-value: 0.1707

> TukeyHSD(aov(modM1),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modM1)

$Location
                        diff           lwr          upr     p adj
CONTROL-BAND    -1.796086e+12 -4.078528e+12 4.863553e+11 0.2305674
NO-BAND-BAND    -1.796080e+12 -4.078522e+12 4.863618e+11 0.2305698
NO-BAND-CONTROL  6.506123e+06 -2.282435e+12 2.282448e+12 1.0000000

>
> ######First Year After Manure 2011 means by location
> mean2=subset(group,Year=="2011")
> modM2=lm(Concentration~Location,mean2)
> summary(modM2)

Call:
```

```
lm(formula = Concentration ~ Location, data = mean2)

Residuals:
       Min         1Q     Median         3Q        Max
-246733167  -15298807   -1739908      -1772 1392333040

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)      246733167   79720454   3.095  0.00399 **
LocationCONTROL -246731395  112741747  -2.188  0.03582 *
LocationNO-BAND -244972743  112741747  -2.173  0.03707 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 276200000 on 33 degrees of freedom
Multiple R-squared: 0.1612,     Adjusted R-squared: 0.1103
F-statistic:  3.17 on 2 and 33 DF,  p-value: 0.05503

> TukeyHSD(aov(modM2),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modM2)

$Location
                      diff        lwr       upr     p adj
CONTROL-BAND    -246731395 -486409828  -7052961 0.0880794
NO-BAND-BAND    -244972743 -484651177  -5294310 0.0909339
NO-BAND-CONTROL    1758651 -237919782 241437085 0.9998659

>
> ######First Year After Manure means by location
> modM3=lm(Concentration~Location,group)
> summary(modM3)

Call:
lm(formula = Concentration ~ Location, data = group)

Residuals:
       Min         1Q     Median         3Q        Max
-8.982e+11 -8.905e+11 -4.114e+06 -4.912e+03  1.456e+13

Coefficients:
                  Estimate Std. Error t value Pr(>|t|)
(Intercept)      8.982e+11  3.867e+11   2.323   0.0231 *
LocationCONTROL -8.982e+11  5.468e+11  -1.643   0.1050
LocationNO-BAND -8.982e+11  5.468e+11  -1.643   0.1050
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 1.894e+12 on 69 degrees of freedom
Multiple R-squared: 0.04955,    Adjusted R-squared: 0.022
F-statistic: 1.799 on 2 and 69 DF,  p-value: 0.1732

> TukeyHSD(aov(modM3),conf=0.9)
  Tukey multiple comparisons of means
    90% family-wise confidence level

Fit: aov(formula = modM3)

$Location
                        diff           lwr           upr     p adj
CONTROL-BAND    -898166584352 -2.039361e+12 2.430282e+11 0.2349201
NO-BAND-BAND    -898162451965 -2.039357e+12 2.430323e+11 0.2349232
NO-BAND-CONTROL       4132387 -1.141191e+12 1.141199e+12 1.0000000

>
```

APPENDIX D: STANDARD OPERATING PROCEDURES

Sample Collection: Soil

Summary
Soil samples are collected from control plots, manure plots, and from within manure bands of the manure plots. Each sample is a composite of 3 cores to 15 cm depth. A total of six composite soil samples will be collected from each manure plot, three from the manure band and three from area between the manure bands. Three composite samples are collected from the control plots.

Materials
36-1 gallon bags
Lab notebook
Ice chest with ice
Gloves
70% ethanol
Paper towels
Soil spatula
Permanent marker
Soil core sampler

Procedures

1. Pace off plot so that sampling takes place as show in Figure 11. Note for manure bands, take samples in the same approximate location as the manure plot samples.
2. Spray soil core sampler with 70% ethanol and wipe down to sterilize the surface
3. At 10 paces in, take the first soil core. Place in sample bag
4. Take another 10 paces and take another core. Repeat a total of 3 times
5. Spray soil core sampler and soil spatula with 70% ethanol and wipe down to sterilize the surface
6. Put the filled sample bags on ice immediately.
7. Repeat and sample the manure bands for manure plots (skip this step in control plots)
8. Repeat steps 3-7 for all eight plots.
9. Transport samples back to lab (on ice). Bacterial samples must be analyzed within 24 hours of sample collection. Antibiotics must be extracted within 72 hours. Store at 4°C.
10. Perform a moisture content analysis for each sample.
11. Freeze samples if antibiotic or DNA extraction is to be performed at a later date

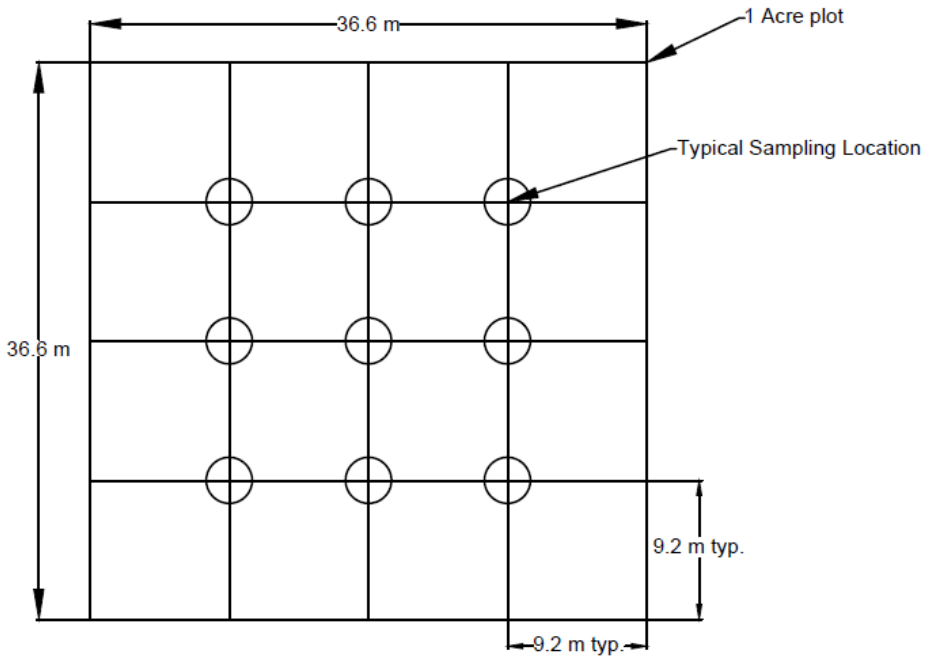


Figure 11: Diagram of typical plot for soil sampling locations at the ISU Northeast Research Farm.

Figure 12: Typical soil probe to collect 15cm samples

Sample Collection: Tile Water

Summary

Tile water samples are collected from the tile drain inflow in the drainage sump for each plot.

Materials

Sample Bottles

2-1 L Plastic Sample Bottles per plot

1-0.5 L Wide-Mouthed Amber Glass Bottles per plot +extras

Lab notebook
Ice chest with ice
Gloves
Permanent marker
Tape
Paper towels
Pre-made bottle labels
Stop watch
Flow-measurement bottle
Grab-sample extension

Procedures

Carefully open the lid from the top of the sump (

1. Figure 13)
2. Fit the flow-measurement bottle into the apparatus
3. Extend the bottle and apparatus (Figure 15) down the sump, placing opening below drainage outlet
4. Record the amount of time (in seconds) that it takes to fill to the marked 1 L line
5. Discard the sample by carefully pouring on the grassed buffer strip between the plots
6. Fit each of the sample bottles into the apparatus. Put the lid back on each sample bottle immediately after sample collection. Use care with the amber glass bottle.
7. Put the filled sample bottles on ice immediately.
8. Repeat steps 3-7 for all eight plots.
9. Transport samples back to lab (on ice). Bacterial samples must be analyzed within 24 hours of sample collection. Antibiotics must be extracted within 72 hours. Store at 4°C.

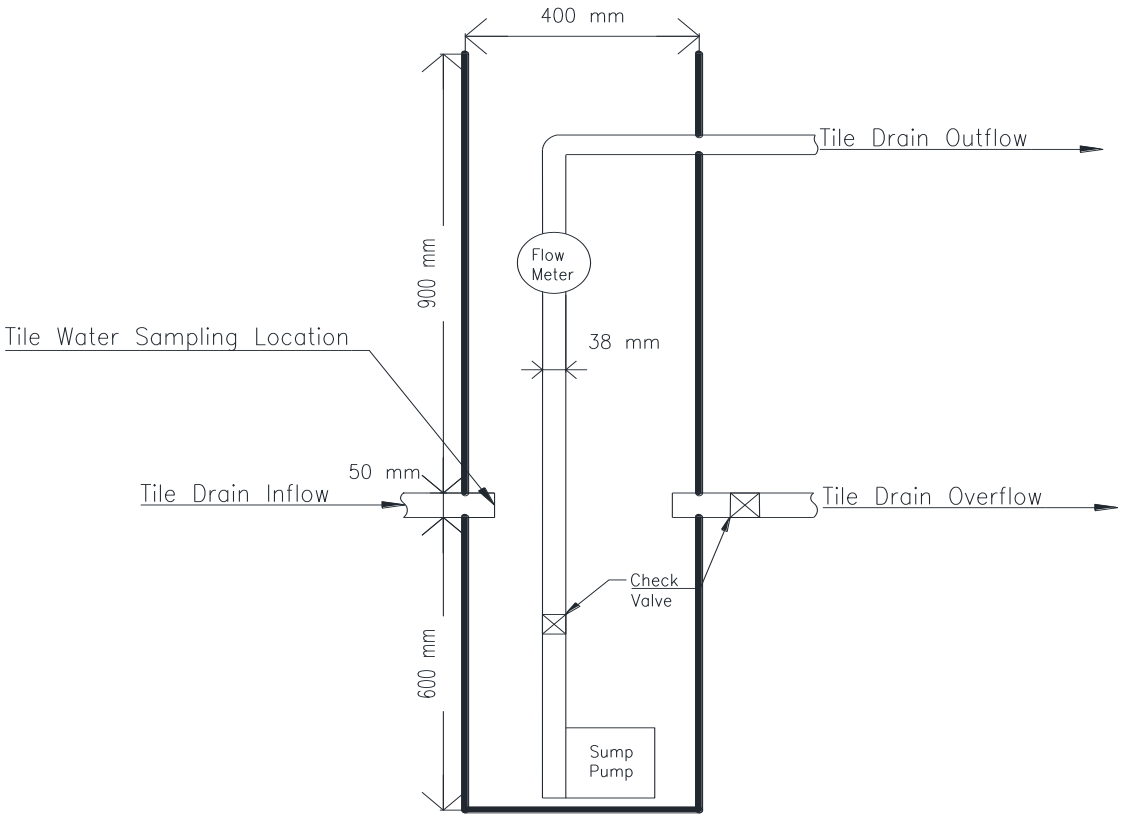


Figure 13: Schematic diagram of sump at the ISU Northeast Research Farm. Water samples were taken where the tile drain discharged into the sump. The weekly tile flow measurements were made from the flow meter. Source: Kanwar et al. 1999.

Figure 14: Inside water sampling sump

Figure 15: Tile water sample collection apparatus

Figure 16: Tile water sample collection

Preparation of mEnterococcus Agar for Membrane Filtration

Summary
mEnterococcus (mE) Agar is used to detect enterococci in water samples using the membrane filtration technique.

Materials
1000 mL volumetric flask
Corning Hotplate/stirrer
Stir bars
Difco mE Agar

Precautions
Keep container tightly closed.
Irritating to eyes, respiratory system and skin.

Preparation of mEnterococcus Agar
1. Suspend 42 g of the powder in 1 L of purified water.
2. Mix thoroughly.
3. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. The media will turn pink.
4. Aliquot into sterile petri dishes
5. Do not autoclave
6. Test samples of the finished product for performance using stable, typical control cultures.

Waste Disposal
Any waste chemicals that remain following the preparation of mEnterococcus agar will be handled in accordance with ISU Environmental Health and Safety procedures. No chemicals are poured down the drain.

Preparation of mEnterococcus Agar infused with Tylosin for Membrane Filtration

Summary
Tylosin infused media is used to detect enterococci concentrations resistant to tylosin in manure, soil, and water samples using the membrane filtration technique. The MIC for this test is 35 µg/mL.

Materials
Methanol
Phosphate Buffered Water
Difco mE Agar
Tylosin Tartrate (Sigma)

Preparation of mEnterococcus Agar with Tylosin
1. Dissolve 410mg Tylosin in 10mL of 50% Methanol (this accounts for 85% purity)
2. Prepare 1 L of mEnterococcus agar following manufacturer's instructions
3. Allow agar to cool to 50°C before adding tylosin solution
4. Add 1 mL of the dissolved tylosin solution to 1 Liter of prepared Agar (concentration is at 35 µg/mL)
5. Aliquot into sterile petri dishes

Tips

- Allow mEnterococcus agar to get a pink tint before removing from the heat.
- Only make one liter of the mEnterococcus agar at a time.

Enterococci Analysis

Summary
The Membrane Filtration method provides a direct count of enterococci in water and soil based on the development of colonies that grow on the surface of a membrane filter. A water sample is filtered through the membrane, which retains the bacteria. After filtration, the membrane containing the bacteria is placed on a selective and differential medium, mEnterococcus Agar, incubated at 35 ± 0.5°C for 48 ± 3 h. The target colonies on mEnterococcus agar are dark red in color after the incubation period.

Materials
Pipettes
Graduated cylinders
Disposable pipette tips
Petri dishes, sterile, prepared with mEnterococcus agar
Filtration units (filter base and funnel)
Filter flask
Sterile, white gridded 0.45 µm membrane filters
Sterile forceps
Ethanol (for flame-sterilizing forceps)
Bunsen burner
Thermometer
Incubator maintained at 35±0.5°C

Precautions
Always wear gloves when handling water samples containing fecal bacteria.

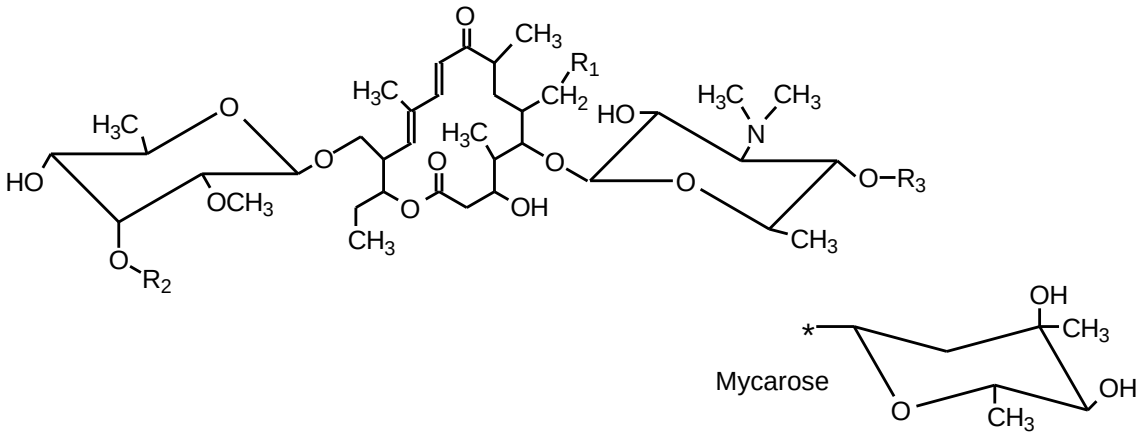
General Procedures for Membrane filtration

1. Sample volume will be governed by expected bacterial density.
2. Suggested sample volumes for lake samples ranges from 10 to 100 mL.
3. Suggested sample volumes for river samples range from 0.001 to 1 mL.
4. Suggested sample volumes for soil/manure samples was 9 mL water with 1g soil or manure slurry.
5. Sterile filtration units at the beginning of each filtration series to prevent accidental contamination. A filtration series is considered to be interrupted when an interval of 30 minutes or longer elapses between sample filtrations.
6. Using sterile forceps, place a sterile membrane filter over porous plate of receptacle.
7. Carefully place matched funnel unit over receptacle and lock it in place.
8. Filter sample under partial vacuum.
9. Rinse the interior surface of the funnel by filtering three 20 to 30 mL portions of sterile dilution water.
10. Immediately remove membrane filter with sterile forceps and place on culture dishes prepared with mEnterococcus agar.
11. Invert dish and incubate for 48 hours at 35±0.5°C.

Extraction and Analysis of Tylosin

Ver. 3 Feb 17, 2010

Overview: This method describes the extraction and analysis of tylosin, a veterinary antibiotic from (1) soil and stream sediment, (2) water and (3) manure. The extraction methods vary for the different environmental matrices. Extraction is followed by cleanup/concentration using solid phase extraction (SPE) columns. Limit of detection (LOD) and limit of quantification (LOQ) are set based on the signal to noise ratio where the LOD is based on a factor of 3 x the noise and the LOQ is based on a factor of 10 x noise (Choi et al., 2007)(Choi et al., 2007)(Choi et al., 2007)(Choi et al., 2007)(Choi et al., 2007)(Choi et al., 2007)(Choi et al., 2007)(Choi et al., 2007)(Choi et al., 2007)(Choi et al., 2007) where noise is the assessment of the irregularities in the baseline.



	Tylosin A	Tylosin B	Tylosin C	Tylosin D	Dihydrodesmycosin
R1	-CHO	-CHO	-CHO	$-CH_2OH$	$-CH_2OH$
R2	$-CH_3$	$-CH_3$	-H	$-CH_3$	$-CH_3$
R3	-Mycarose	-H	-Mycarose	-Mycarose	-H

Tylosin tartrate is produced from Streptomyces fermentations and when purchased from Sigma contains about 85% pure tylosin, with 85% being tylosin A, with lesser amounts of factors B, C, D.

Extraction from soil or sediment

1. Sieve moist soil (4 mm mesh) onto brown Kraft paper and mix. Weigh duplicate 15 ± 0.5 g into "50 mL" Teflon centrifuge tubes. Label the tube cap with lab tape and record the weight of the sample in lab book. Do not write on the tube.
2. If needed, weigh another sub-sample for soil moisture determination.
3. Extract the 15 g soil sample with 30 mL of acetonitrile:0.1 M ammonium acetate (85:15, v/v) at by shaking on a reciprocating shaker for 60 minutes. To prepare the extraction solution, dissolve 3.85 g NH_4CH_3COOH in 500 mL water. Mix 150 mL ammonium acetate with 850 mL acetonitrile to yield 1 L volume.
4. Allowed samples to stand overnight at room temperature. Shake for 15 minutes and centrifuge using the Sorvall SS-34 fixed angle rotor at 5,000 rpm (approximately 3,000 x *g*) for 20 minutes. Pipet or pour the clear liquid extract into a clean 70 mL brown glass tube.
5. Add a second 30-mL aliquot of acetonitrile: 0.3 M ammonium acetate (85:15, v/v) to each sediment sample and shake for 30 minutes.
6. Centrifuge the sample and decant the supernatant into the same bottle containing the first extract. These extracts can be stored in the refrigerator.
7. Concentrate each sample extract to a volume of 9 - 10 mL by evaporating the acetonitrile with N_2 gas flow at 10 to 15 psi at 55^{o}C using the N-Evap. Add 50 mL Milli Q water. The resulting solution is primarily water and can be stored in the refrigerator. This step will take about an hour and 20 minutes.
8. Load the sample on an Oasis-HLB SPE column previously conditioned and proceed as described below.

Notes: Work by Ashley Jessick showed that this extraction solution was superior compared to others for the extraction of 14C-erythromycin, a macrolide antibiotic closely related to tylosin.

Solid Phase Extraction from Soil Extracts

1. Use the round SPE extraction chamber. Using lab tape label each cartridge with the sample ID. Attach sample funnel to the cartridge.
2. Add 2 mL of methanol to each cartridge (Oasis HLB cartridge 200 mg, Waters-Millipore) and draw through at 1mL/min rate. Very little vacuum is required at this point.
3. Add and draw through 2 mL of water.
4. Add sample to the SPE and draw through at 1-2 mL/min (start at 2-3 inches Hg vacuum and increase as needed). Rinse sample bottle with 5 mL water and add that to the SPE and draw through. This should take 30 to 60 minutes. Slower flow rates allow for complete adsorption of tylosin to the SPE cartridge.
5. Wash the SPE with 1 mL of 5% methanol. As each extraction is completed, the SPE can be removed and the red plug placed in the manifold port.
6. When all extractions are complete, release the vacuum and transfer the SPE cartridges to the square glass manifold. Remove the attached funnels. Place 2-mL conical volumetrics in the rack below the SPE to catch the eluted methanol containing the tylosin.

7. With no vacuum add 1 mL of methanol to each SPE. Apply very slight vacuum and pull through. Release vacuum and add a second 1 ML of methanol and pull through. Release the vacuum and remove the volumetrics and cap. Transfer tape labels from SPE to volumetrics as you go.
8. Add 0.1 mL of the Internal Standard working solution to the 2 mL methanol obtained after SPE elution in the tylosin extraction procedure. A total of 42 ng of simetone is added to each sample and this would result in a 21 ng/mL (21 ppb) concentration, assuming no losses during evaporation.
9. Dry this to 0.5 mL volume or less using the N-Evap. This may take up to an hour.
10. Bring to a total volume of 2 mL with 10 mM ammonium acetate. (This matches the mobile phase of the HPLC-MS/MS) Allow at least 1 hr. for tylosin to re-dissolve into the ammonium acetate. It is important that this 2 mL volume be established accurately. Gently vortex the volumetric to help dissolve the entire sample.
11. Using a 3 mL plastic syringe and long stainless steel needle transfer and filter using a 13 mm 0.45 µm pore filter into a second 3 mL syringe. Filter again using a 13 mm 0.2 µm pore filter transfer the extract to a HPLC auto-injector-compatible vial. The double filtering is done to remove any larger fines which may clog the 0.2 µm filter, and prevent the full 2mL from being transferred. Label and cap. Store in the refrigerator.

The 60 mg SPE may be suitable for soil extract clean-up.

Figure 17: Oasis HLB SPE cartridge, adapter and reservoir mounted on the round vacuum unit used for cartridge conditioning and sample loading.

Solid Phase Extraction from Tile Water

1. Use the glass round plastic SPE extraction chamber. Label cartridges and install sample funnels.
2. The sample should be 250 mL in volume. If it is smaller, measure and record the actual volume. If there are settled sediments in the sample measure out a defined volume (240 mL) for extraction, leaving the sediment behind.
3. Add 2 mL of methanol to each cartridge (Oasis HLB cartridge manufactured. by Waters-Millipore) and draw through at 1mL/min rate.
4. Add and draw through 2 mL of water.
5. Add sample to the SPE and draw through at 2 mL/min (start at 2-3 inches Hg vacuum and increase as needed).Rinse sample bottle with 5 mL water and add that to the SPE and draw through. This takes about 90 minutes.
6. Wash the SPE with 1 mL of 5% methanol.
7. Release the vacuum, transfer the SPE cartridges to the square manifold and position 2 mL conical volumetric tubes to catch the eluted methanol.
8. With no vacuum add 1 mL of methanol to each SPE. Apply very slight vacuum and pull through. Release vacuum and add a second 1 ML of methanol and pull through. Release the vacuum and remove the volumetrics and cap. Transfer tape labels from SPE to volumetrics as you go. The volume of methanol in the volumetrics should be very close to 2 mL. These can be stored in the refrigerator.
9. Add 0.1 mL of the Internal Standard working solution to the 2 mL methanol obtained after SPE elution in the tylosin extraction procedure. A total of 42 ng of simetone is added to each sample and this would result in a 21 ng/mL (21 ppb) concentration, assuming no losses during evaporation.
10. Dry this to 0.5 mL volume or less using the N-Evap. This may take up to an hour.
11. Bring to a total volume of 2 mL with 10 mM ammonium acetate. (This matches the mobile phase of the HPLC-MS/MS) Allow at least 30 min for tylosin to re-dissolve into the ammonium acetate.
12. Using a 3 mL plastic syringe and long stainless steel needle transfer and filter (13 mmm, 0.2 µm pore) the extract to a HPLC auto-injector-compatible vial. Label and cap. Store in the refrigerator.

Extraction from Manure

1. Transfer 30g of liquid manure into 50 mL HDPE centrifuge tubes. Label the tube cap with lab tape and record the exact weight of the sample in lab book. Do not write on the tube.
2. Centrifuge at 12,500 x g (approx. 15,000 RPM) on the Sorvall SS-34 fixed angle rotor for 30 minutes.
3. Transfer the liquid supernatant to a 70 mL brown glass tube. Cap and label.
4. Add 15 mL of Extraction Solution 1 (85% acetonitrile + 15% ammonium acetate) to the centrifuge tube, vortex, and shake for 1 hour.
5. Centrifuge at 12,500 x g (approx. 15,000 RPM) for 30 minutes.
6. Transfer supernatant to a new 70 mL brown glass tube. Cap and label.
7. Add 15 mL of Extraction Solution 2 (95% acetonitrile + 5% isopropyl alcohol) to the centrifuge tube, vortex, and shake for 1 hour.
8. Centrifuge at 12,500 x g for 30 minutes.
9. Transfer supernatant to the same 70 mL brown glass tube. This should result in a total volume of 30 mL in the tube.
10. Evaporate the solvent extracts under N_2 gas to 5 mL using the N-Evap. This may take up to an hour.
11. Combine the extracted solvents and the supernatant from step 3 into a 50 mL HDPE centrifuge tube. Centrifuge again for 30 minutes at 12,500 x g.
12. The sample will still contain many suspended particles. Filter the sample twice, first though a 0.7µm filter and then though a 0.45 µm filter. This should prevent the SPE from clogging.
13. Add 80 mL of water. This should bring the total volume of the sample up to 100-110 mL.
14. Pass the sample though an Oasis HLB SPE, as described below.

Solid Phase Extraction from Manure

1. Use the round SPE extraction chamber. Using lab tape, label each cartridge with the sample ID. Attach sample funnel to the cartridge.
2. Add 2 mL of methanol to each cartridge (Oasis HLB cartridge 200 mg manufactured by Waters-Millipore) and draw through at 1mL/min rate. Very little vacuum is required at this point.
3. Add and draw through 2 mL of water.
4. Add sample to the SPE and draw through at 1-2 mL/min (start at 2-3 inches Hg vacuum and increase as needed). Rinse sample bottle with 5 mL water and add that to the SPE and draw through. This should take 30 to 60 minutes. Slower flow rates allow for complete adsorption of tylosin to the SPE cartridge.
5. Wash the SPE with 3 mL of 60% methanol + 38% water + 2% ammonium hydroxide. As each extraction is completed, the SPE can be removed and the red plug placed in the manifold port.
6. When all extractions are complete, release the vacuum and transfer the SPE cartridges to the square glass manifold. Remove the attached funnels. Place 2-mL conical volumetrics in the rack below the SPE to catch the eluted methanol containing the tylosin.

7. With no vacuum add 1 mL of methanol to each SPE. Apply very slight vacuum and pull through. Release vacuum and add a second 1 mL of methanol and pull through. Release the vacuum and remove the volumetrics and cap. Transfer tape labels from SPE to volumetrics as you go.
8. Add 0.1 mL of the Internal Standard working solution to the 2 mL methanol obtained after SPE elution in the tylosin extraction procedure. A total of 42 ng of simetone is added to each sample and this would result in a 21 ng/mL (21 ppb) concentration, assuming no losses during evaporation.
9. Dry this to 0.5 mL volume or less using the N-Evap. This may take up to an hour.
10. Bring to a total volume of 2 mL with 10 mM ammonium acetate. (This matches the mobile phase of the HPLC-MS/MS) Allow at least 1 hr. for tylosin to re-dissolve into the ammonium acetate. It is important that this 2 mL volume be established accurately. Gently vortex the volumetric to help dissolve the entire sample.
11. Using a 3 mL plastic syringe and long stainless steel needle transfer and filter using a 13 mm 0.45 µm pore filter into a second 3 mL syringe. Filter again using a 13 mm 0.2 µm pore filter transfer the extract to a HPLC auto-injector-compatible vial. The double filtering is done to remove any larger fines which may clog the 0.2 µm filter, and prevent the full 2 mL from being transferred. Label and cap. Store in the refrigerator.

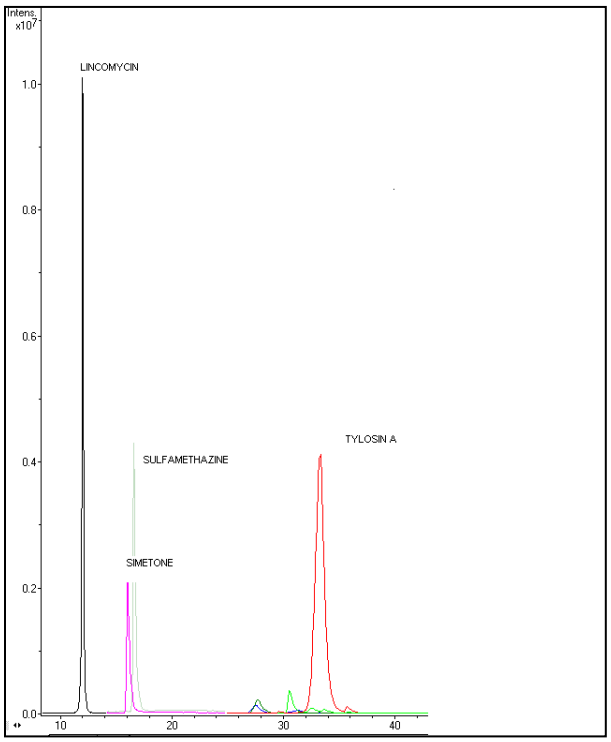


Figure 18: A representative result for tylosin analysis is shown below. Tylosin B, C, and D are seen in the three small peaks immediately preceding the tylosin A peak.

Extraction of DNA from Water Samples

Based on MoBio protocol for the MoBio Power Water DNA kit

Materials and Prep:

Wear gloves at all times
Ice bucket with ice
Set Centrifuge to 22 °C
Heat water bath so as to warm solutions PW1 and PW3 to dissolve precipitates. Both solutions can be used warm.
Set up: 1 bead tube
3 2-mL collection tubes
1 spin filter
2 2-mL collection tubes

Label all tubes. Be sure to label side and date the final centrifuge tube.

Procedure:

1. Filter 250 mL water samples using the disposable 0.22 um kit filters.
2. Remove the upper portion of the MoBio filter by snapping it off of the base.
3. Use surface-sterilized forceps and needle to facilitate rolling of the filter membrane prior to insertion into the 5-mL PowerWater Bead Tube.
4. Add 1.0 mL of Solution PW1 to the PowerWater Bead Tube, cap tightly and secure onto a MoBio Vortex adapter.
5. Vortex at maximum speed for 5 minutes.
6. Centrifuge the tubes at ~4000 g for 1 (3) minute at room temp.
7. Transfer all the supernatant to a clean 2-mL collection tube. Note: You must place the pipet tip down into the bead pellet. Expect 600-650 µl.
8. Centrifuge at 13,000 g (9000-9500 rpm) for 1 minute.
9. Avoiding the pellet, transfer the supernatant to a clean 2-mL collection tube.
10. Add 200 µl of Solution PW2 and vortex briefly to mix.
11. Incubate at 4 C for 5 minutes.
12. Centrifuge at 13,000 g (9000-9500 rpm) for 1 minute.
13. Avoiding the pellet, transfer the supernatant to a clean 2-mL collection tube.
14. Add 650 µl of Solution PW3 and vortex briefly to mix.
15. Load 650 µl of the mixture onto a Spin Filter and centrifuge at 13,000 g (9000-9500 rpm) for 1 minute. Discard flow through and repeat. Note: A total of 2-3 loads for each sample processed are required.
16. Place spin filter basket into a clean 2-mL collection tube.
17. Shake to mix Solution PW4 before use. Add 650 µl of Solution PW4 and centrifuge at 3,000g (9000-9500 rpm) for 1 minute.
18. Discard flow through and add 650 µl of Solution PW5 and centrifuge at 13,000 g (9000-9500 rpm) for 1 minute.

19. Discard flow through and centrifuge again at 13,000 g (9000-9500 rpm) for 3 minutes to remove residual wash.
20. Place the Spin Filter basket into a clean 2-mL collection tube.
21. Add 100 µl of Solution PW6 to the center of the white filter membrane,
22. Centrifuge at 13,000 g (9000-9500 rpm) for 1 minute.
23. Discard the Spin Filter basket. The DNA is now ready for any downstream application.
24. Determine DNA concentration by adding 5 µL of prepared DNA extract to 55 µL PCR grade water. Blank the Eppendorf Biophotometer. Enter dilution parameters using key pad. Place the entire aliquot into an Eppendorf UVette tube and measure the DNA concentration on biophotometer. Record the concentration in the lab book and onto tube of original extract along with ID and date.

Extraction of DNA from Soil Samples
Based on MoBio protocol for the MoBio UltraClean Soil DNA kit

Materials and Prep:
Wear gloves at all times
Ice bucket with ice
Set Centrifuge to 22 °C
Heat water bath so as to warm (60° C) solutions S1 to dissolve precipitates.
Set up 1 50 Mega Bead Tube
2 Centrifuge Tubes
1 Spin Filter
1 Centrifuge Tube
Label all tubes. Be sure to label side and date the final centrifuge tube.

Procedure:
1. Add 10 g (± 0.05) soil to Mega Bead Tube
2. Add 15 mL of Bead Solution to Mega Bead Tube (now called Bead Solution Tube)
3. Vortex vigorously for 1 minute
4. Add 1.2 mL of Solution S1 and vortex vigorously for 30 seconds
5. Add 4 mL of Solution IRS (inhibitor Removal Solution). (Only required for PCR)
6. Place the 50 mL Mega Bead Tube on the MO BIO Vortex Adaptor for a Genie 2 Vortex and vortex for 10 minutes at highest speed (cap facing in).
7. Centrifuge tubes at 2500 x g (5000 rpm) for 3 (5) minutes. Be sure to check balance.
8. Transfer supernatant to a clean Centrifuge Tube. Supernatant may still contain some soil particles.
9. Add 2 mL of Solution S2, invert twice to mix
10. Incubate at 4° C (ice bath) for 10 minutes
11. Centrifuge the tubes at 2500 x g (5000 rpm) for 4 (5) minutes
12. Avoiding the pellet, transfer the supernatant to a clean Centrifuge Tube
13. Shake to mix Solution S3. Add 30 mL of Solution S3 to supernatant and invert twice.
14. Fill a spin filter with solution from step 13. Centrifuge at 2500 x g (5000 rpm) for 2 minutes. Discard flow through and repeat 2-3 times until all solution has been filtered.
15. Add 6 mL of Solution S4 and centrifuge at 2500 x g (5000 rpm) for 3 (5) minutes
16. Discard flow through
17. Centrifuge again at 2500 x g (5000 rpm) for 5 minutes
18. Carefully place Spin Filter in a new clean Centrifuge Tube. Avoid splashing any Solution S4 onto the Spin Filter.
19. Add 8 mL of Solution S5 to the center of the Spin Filter Membrane
20. Centrifuge at 2500 x g (5000 rpm) for 3 (5) minutes
21. Discard Spin Filter. DNA is ready for any downstream application.
22. Determine DNA concentration by adding 5 µL of prepared DNA extract to 55 µL PCR grade water. Blank the Eppendorf Biophotometer. Enter dilution parameters using key pad. Place the entire aliquot into an Eppendorf UVette tube and measure the DNA concentration on biophotometer. Record the concentration in the lab book and onto tube of original extract along with ID and date.

Extraction of DNA from Manure Samples

Modified Ziemer Lab Method for Manure DNA Extraction combined with Qiagen Protocol: (Repeated Bead Beating Plus Column Extraction Method [RBBC] (Yu and Morrison, 2004) Developed by B. Douglass, T.B. Moorman Lab, Updated: 8/2011.

Preliminary Prep: Remove lysis buffer from MoBio bead tubes.
1. 0.25 mL sample into 2-mL MoBio Bead screw cap tube (record weights)
2. Add 1 mL Lysis Buffer.
3. Homogenize for 10 minutes with MoBio Vortex Genie tube adaptor
4. Incubate at 70^{o}C for 15 minutes, gently shaking on Eppendorf shaker block at 250-300 rpm.
5. Centrifuge at 4^{o}C for 5 minutes at 12,500 rpm (16,000 x g)
6. Transfer supernatant to a new 2-mL tube
7. Add 300 µL of fresh Lysis Buffer to original lysis tube
8. Repeat steps 3-5
9. Pool 2nd supernatant with first

10. Add 260 µL of 10 M ammonium acetate to each lysate tube
11. Vortex to mix
12. Incubate on ice 15 minutes
13. Centrifuge at 4^{o}C for 10 minutes at 14,000 rpm (20,817 x g)
14. Transfer supernatant (approx. 750 µL each) to two 1.5-mL tubes
15. Add one volume (750 µL) of isopropanol and mix well (or whatever volume will match half the amount of supernatant)
16. Incubate on ice for 30 minutes
17. Centrifuge at 4^{o}C for 15 minutes at 14,000 rpm (20,817 x g)
18. Remove supernatant (Z. recommends aspiration, I used a pipette tip)
19. Dry off. (Try under vacuum for 3 minutes; may also put under nitrogen stream for 15-30min)
20. Dissolve nucleic acid pellet in 100 µL of TE (Tris-EDTA) buffer
21. Pool the 2 aliquots
22. Add 2 µL of DNase-free RNase (optional)
23. Incubate at 37^{o}C for 15 minutes (optional)

Now switch back to Qiagen QIAamp DNA Stool source reagents and materials:
24. Add 15 µL of proteinase K and 200 µL of Buffer AL; invert gently to mix.
25. Incubate at 70^{o}C for 10 minutes
26. Add 200 µL 100% ethanol and invert gently to mix
27. Transfer to a QIAamp column
28. Centrifuge for 1 minute at 8000 rpm at room temp
29. Discard flow through
30. Add 500 µL Buffer AW1
31. Centrifuge for 1 minute at 8000 rpm at room temp
32. Discard flow through
33. Add 500 µL Buffer AW2
34. Centrifuge for 3 minutes at 14,000 rpm (21,817 x g) at room temp

35. Discard flow through
36. Centrifuge for 2 minutes at 14,000 rpm (21,817 x g) at room temp to dry column; Place column into clean microvial.
37. Add 100 µL H_2O or Buffer AE to elute DNA
38. Centrifuge for 1 minute at 8000 rpm at room temp to elute DNA
39. Repeat steps 37 and 38.
40. Determine DNA concentration by adding 5 µL of prepared DNA extract to 55 µL PCR grade water. Blank the Eppendorf Biophotometer. Enter dilution parameters using key pad. Place the entire aliquot into an Eppendorf UVette tube and measure the DNA concentration on biophotometer. Record the concentration in the lab book and onto tube of original extract along with ID and date.

Solutions for RBBC Extraction Procedure:

Lysis Buffer

To make 100 mL: Place 75 mL Milli-Q H2O into 150-mL beaker with small stir bar
Add: 2.922 g NaCl
5.0 mL 1M Tris-HCl, pH 8.0
1.861 g EDTA
4.0 g Sodium Dodecyl Sulfate (SDS)
Stir each component until dissolved. Bring to a final volume of 100 mL. Please note that the SDS will probably not go into solution completely. This did not appear to hinder the process.
This will give a solution which is: 500 mM NaCl
50 mM Tris-HCl, pH 8.0
50 mM EDTA, pH 8.0
4% SDS

10 mM Ammonium Acetate

Make 200 mL directly in a media bottle by adding 154.16 g ammonium acetate to 200 mL ddH2O.

TE Buffer

TE Buffer at a 10:1 with EDTA
Want pH=8

Stock solutions:
TRIS-base 100x (1 M): 8.17 g TRIS/50 mL ddH2O; filter.
EDTA 100x (0.1 M): 1.86 g Na2 EDTA/50 mL ddH2O; filter.

Working TE 1x (10 mM TRIS + 1 mM EDTA)
0.5 mL TRIS 100x + 45 mL ddH2O;
Adjust pH to 8 with HCl;
Add 0.5 mL EDTA 100x;
Add ddH2O up to 50 mL
Filter

APPENDIX E: QPCR ANALYSIS PROTOCOL

Introduction

This detailed protocol provides instructions for setting up and analyzing a real-time PCR reaction on the MJ Opticon2 Instrument for environmental samples. It includes helpful advice on how to set up the experimental design and provides a detailed QA/QC to ensure primer specificity, reproducibility, and check for inhibition.

Sample Collection

Sample collection for environmental samples can be collected in the same nature that samples are collected for other analysis, such as grab samples, ISCO automatic samples for water, standard soil collection using a sterilized soil probe, or grab samples of manure slurry. It is important, like in any sample collection, to use sterilized containers that have been acid washed or autoclaved to prevent contamination from an unclean container. Additionally, it is good to transport samples on ice and store at 4°C until processing to maintain a more constant microbial community and be representative of the time in which the sample was collected. DNA was usually extracted within 48 hours of sample collection for water, while manure and soil samples can be frozen after collection and extracted later.

DNA Extraction

There are many kits on the market that have streamlined the DNA extraction process from vendors such as Qiagen and MoBio. Each vendor has a wide array of kits targeting specific matrices. After a series of trials in the lab, the MoBio kits were selected for their performance. Step by step instructions are sent with each kit and can also be found on the vendor's website.

DNA Concentration

The concentration of the DNA is important when dealing with plasmid DNA and DNA from environmental samples. The concentration of the DNA extract in ng/ µL can be used for standard development and also to get a feel for the quality and quantity of DNA

present before analysis. The BioPhotometer and UVette®, shown in Figure 19, is one simple method to determine the quantity and quality of DNA. The DNA concentration can be determined by adding 5 µL of prepared DNA extract to 55 µL PCR grade water. This step dilutes the DNA to save as much as possible for qPCR analysis. Next, blank the Eppendorf BioPhotometer. Enter the previously stated dilution parameters using key pad. Place the entire aliquot into an Eppendorf UVette® tube and measure the DNA concentration on BioPhotometer. Record the concentration in ng/ µL and the quality reading, the fraction of 260/280, in the lab book and onto tube of original DNA extract along with ID and date.

Figure 19: Eppendorf BioPhotometer and UVette

Standard Development

Development of DNA standards for qPCR is perhaps one of the more challenging steps in this process, yet one of the most important. We constructed plasmids hosted in *E. coli* that contained portions of ermB and ermT targeted by primers we selected. Enterococcus strains Man T1-C and Soil T3-R described by Hoang (2010) were grown in broth and their

DNA was extracted. PCR products were cloned into pCR^{R}-4TOPO using the TOPO-TA cloning kit. Plasmids were mixed with competent E coli strains and selected using kanamycin, exploiting a kanamycin resistance marker on the plasmid. *E. coli* which have taken up the plasmids carry a gene coding for kanamycin resistance. Bacteria that have been transformed with pCR^{R}-4TOPO plasmid containing kanamycin resistance genes are plated on agar or broth infused with kanamycin at a rate of 50-100 µg/mL. Only the bacteria that have successfully taken up the kanamycin resistance gene become resistant and will grow under these conditions. The plasmid DNA of these bacteria can then be extracted and purified using kits and used as for PCR standards, and by creating a set of serial dilutions, make the standard curve to quantify the target gene in the run. Typically, these dilutions consist of 10µL of plasmid DNA + 100 of water. The use of plasmid DNA for standards has the advantage of a constant copy number, and the number of erm sequences can be estimated from plasmid DNA concentrations and plasmid base pair length.

Figure 20: Plasmid DNA for *erm*B, *erm*F, and *erm*T (left) and serial dilutions (right)

Sample storage

Extracted DNA should remain frozen until use. This will preserve the DNA. However, the quality of DNA will decrease over time. It is recommended, that if the DNA will be used multiple days in succession, to leave the DNA thawed in the refrigerator between uses.

Plate set up

The setting up of the plate is one of the most important steps in this process. An effective plate setup allows for easy pipetting and for result analysis. For each plate, determine the total number reactions to be run: # of standards + # of samples + 5 additional reactions. This is used to determine the total volume of the qPCR Reaction Mix required and allows room for error as the pipetted volumes are so small that some liquid might stick to the outside of the pipette tip each time. Next, complete the worksheet for the run. A blank and sample completed worksheet is shown in Figure 21 and Figure 22 respectively. Worksheet A can be used to calculate the dilutions for working stocks of primers. This plate layout was developed for simplicity, which will hopefully avoid errors in pipetting.

A standard sample plate setup is shown in Figure 22. For this set up, a series of 5-ten-fold dilutions of the 2.5 µL of standards in triplicate wells (going from left to right, top to bottom) in wells A1-E3. Label the original with a '1' or -1 (for 10^{-1}) and increase numerically for each standard dilution, i.e. 1, 2, 3, 4, 5. Always have the highest concentration of the standard in row A, and the lowest in E. Wells F1-F3 are designated as template with 2.5 µL of PCR-grade water as the sample. Wells G1-G3 consists of 2.5 µL a negative control DNA. Wells H1-H3 have 25 µL of PCR-grade RNase free water only.

The negative controls and water blanks play a key role in the analysis of the results. High concentrations of PCR products in the negative controls can indicate contamination in the primers of amplification of primer-primer dimers, for example. They also provide a limit of detection for the samples as a whole. For example, if an average of 1.1×10^2 copies/µL is reported in the negative controls, this would constitute the limit of the primers to accurately quantify the copies in the DNA. The water only blank should always return a zero, as its only PCR-grade water. If quantities are reported in these wells, there is a likely contamination in the water. Similarly, the wells with 2.5 µL of water with the master mix should have no quantity. If quantities are reported in these wells, there is contamination in the SYBR Green MasterMix that will likely affect the results in the other wells as well.

Plate Worksheet												
Date:					Filename:							
Primer Set:					Experiment:							
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Worksheet B	# of RXNS		Total Vol. for Rxn Mix				
QuantTect SYBR PCR Master Mix		X 12.5 uL	=				
Primer Forward:		X 5.0 uL	=				
Primer Reverse:		X 5.0 uL	=				
Template:		X 2.5 uL	=				

Add 5-10 additional rxns onto each of the totals above to ensure enough volume in case of error.

Figure 21: Sample Plate Worksheet

Plate Worksheet												
Date:	1/1/2012				**Filename:**	Jason erm B						
Primer Set:		Koike erm B			**Experiment**	Jason erm B						
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	STD 1	STD 1	STD 1	SAMPLE 1	SAMPLE 1	SAMPLE 1	SAMPLE 9	SAMPLE 9	SAMPLE 9	SAMPLE 17	SAMPLE 17	SAMPLE 17
B	STD 2	STD 2	STD 2	SAMPLE 2	SAMPLE 2	SAMPLE 2	SAMPLE 10	SAMPLE 10	SAMPLE 10	SAMPLE 18	SAMPLE 18	SAMPLE 18
C	STD 3	STD 3	STD 3	SAMPLE 3	SAMPLE 3	SAMPLE 3	SAMPLE 11	SAMPLE 11	SAMPLE 11	SAMPLE 19	SAMPLE 19	SAMPLE 19
D	STD 4	STD 4	STD 4	SAMPLE 4	SAMPLE 4	SAMPLE 4	SAMPLE 12	SAMPLE 12	SAMPLE 12	SAMPLE 20	SAMPLE 20	SAMPLE 20
E	STD 5	STD 5	STD 5	SAMPLE 5	SAMPLE 5	SAMPLE 5	SAMPLE 13	SAMPLE 13	SAMPLE 13	SAMPLE 21	SAMPLE 21	SAMPLE 21
F	2.5 ul WATER	2.5 ul WATER	2.5 ul WATER	SAMPLE 6	SAMPLE 6	SAMPLE 6	SAMPLE 14	SAMPLE 14	SAMPLE 14	SAMPLE 22	SAMPLE 22	SAMPLE 22
G	NEG CONT.	NEG CONT.	NEG CONT.	SAMPLE 7	SAMPLE 7	SAMPLE 7	SAMPLE 15	SAMPLE 15	SAMPLE 15	SAMPLE 23	SAMPLE 23	SAMPLE 23
H	WATER BLANK	WATER BLANK	WATER BLANK	SAMPLE 8	SAMPLE 8	SAMPLE 8	SAMPLE 16	SAMPLE 16	SAMPLE 16	SAMPLE 24	SAMPLE 24	SAMPLE 24

Worksheet B				**Total Vol. for Rxn Mix**				
	# of RXNS							
QuantTect SYBR PCR Master Mix	96+4	**X 12.5 uL**	=	1250 uL				
Primer Forward:	96+4	**X 5.0 uL**	=	50 uL				
Primer Reverse:	96+4	**X 5.0 uL**	=	50 uL				
Template:		**x 2.5 uL**	=					
Water:	3	X 25 uL	=	75 uL				

Add 5-10 additional rxns onto each of the totals above to ensure enough volume in case of error.

Figure 22: Sample Completed qPCR

Before starting this procedure, spray the bench and pipetters down with a 70% ethanol and wipe clean with a Kim wipe. Make up the reaction mix from DNA extracted from positive control organisms for genes of interest as follows:

1. Prepare the qPCR Reaction Mix by aliquoting the calculated total volumes from the worksheet shown in Figure 22 of QuantiTect PCR Master Mix (which includes DNTP , Taq SYBR Green, and buffer) forward primer and reverse primer into one vial and mix thoroughly. An additional 5-10 reactions should be added to the total in order to account for losses in pipetting. A full 96 well plate requires about 100 total reactions.
2. Dispense 22.5 µL of the qPCR reaction mix into each well that will requires the reaction mix. The only exception will be the complete PCR-grade water blanks, which will have 25 µL of PCR-grade water only. Be sure to have the plate or the strips on the cold block. Use a new pipette tip for each well to ensure accuracy in pipette. A good hint is to align the 96 well plate next to a 96 well pipette tip box and correlate each tip with a well. That way, as long as you put the aliquot into the same well on the plate that the pipette tip came from, there should be no reason to lose your place.
3. Dispense 2.5 µL of the DNA template into the proper well. Use a small strip of parafilm wax or a sanitary Kim wipe to cover the wells filled in order to keep track of progress. Pipette three columns across, and then work down the entire column. Move the wax/Kim wipe over, and repeat. Gently mix the template and reaction mix after adding template DNA. Discard and replace pipette tip after each well.

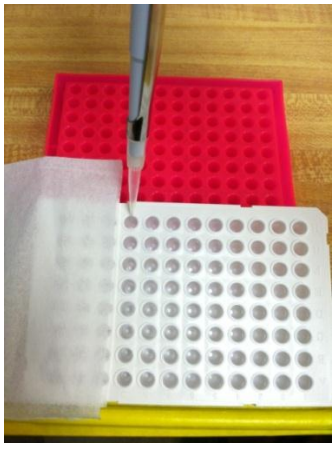


Figure 23: Plate with Kim wipe

4. Cap the strip tubes or cover the plates with adhesive plate seals, using the appropriate capping tool or roller. Be careful not to touch the cover as this might impact the ability of the MJ Opticon 2 to read the sample.

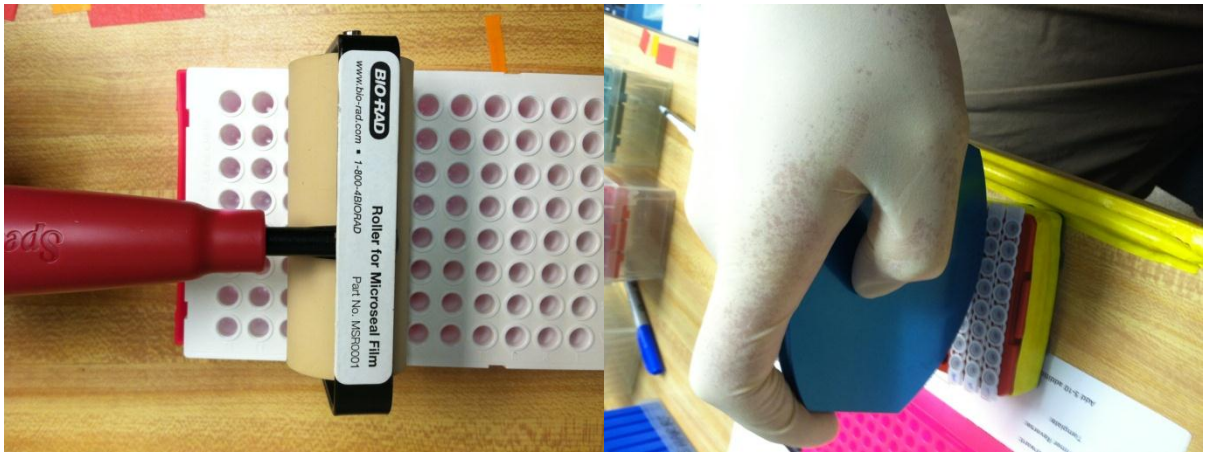


Figure 24: Roller (left) and capping tool (right) to seal plates or strips

5. Spin down the strip tubes using the 8-tube strip microfuge for 15 seconds. When using a full plate, use the larger rotor with the plate/slide adapter and centrifuge for 2 minutes at 3000 RPM. This removes the bubbles in the well which may interfere with the results. While is has been reported that plates can be made up ahead of time, it is best to run the qPCR assay immediately after preparation.

Figure 25: Microfuge and centrifuge rotor for strips and plates

6. Open the MJ Opticon 2 by squeezing the blue handle, pulling up on the heated lid, and pulling out the drawer. Place the tube strips or plate into the well grid and close drawer. When using a full plate, carefully place the rubber guard over the plate with the proper side facing up.

Figure 26: MJ Opticon 2 Instrument with door closed and open

Software Procedure

It will be easier to set up the protocol (for the first time) prior to creating plate. Subsequent plates do not require as much set up, and therefore can be set up right before the run.

1. Open up the Opticon Monitor 2 software by clicking on the icon:

Figure 27 shows the main page when opening up. This particular example shows a master file, plate setup, and protocol for ermB.

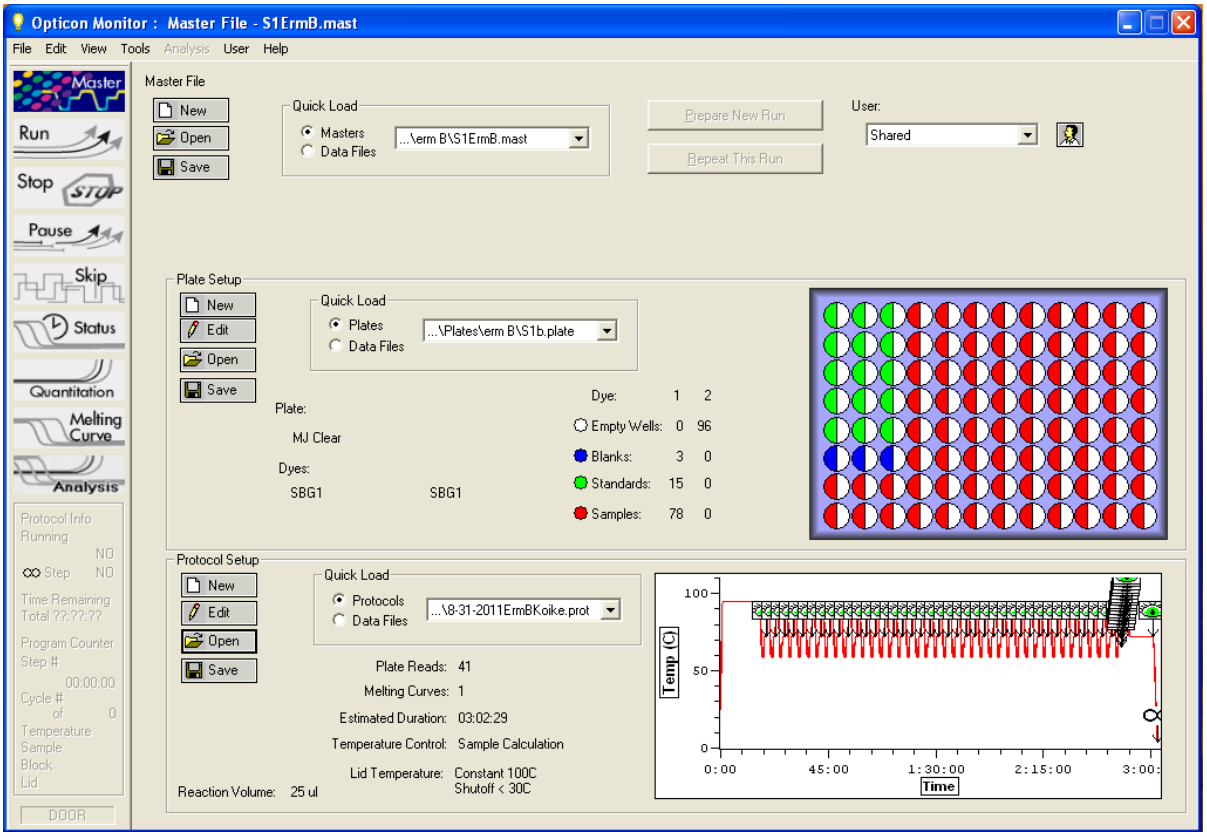


Figure 27: Main page of Opticon Software

2. At the top left of the page you will see a tab, 'Master File' click 'new' to create a new master file. Or, when working from a pre-existing master, click 'Open' and select your Master File (for example: users\jason\masters\ermB\ermB.mast). A master file in this program simply saves the plate setup and the cycling protocol. A complete master file will be created after setting up those parameters.

3. In order to set up the plate, in the 'Plate Setup' box, click 'Edit'. This allows you to specify within the plate what is going in each of the wells, set up the plate type, dye type, and specify the standards so that the output produces real-quantitative numbers. Start by filling in the plate by highlighting the wells and detonating the contents of each. Select the type of well contents and click the specific well and label as either 'Empty', 'Blank', 'Standard' or 'Sample'. Under the dyes category on the left, be sure to denote as 'Single'.

Next, select the plate type that will be used in the experiment. It will be either the ‘MJ White’ or ‘MJ Clear’. Select ‘SBG1’ (for SYBER Green 1) for both Dye 1 and Dye 2 and also select ‘Singleplex Experiment’ at the bottom.

Further down on the right-hand side of the page you will see a table where you can enter a description of your sample or standards. Label each well such that the output data is clear. As shown in the example, well H4 is Sample 8. This should match the plate setup previously designed for this experiment.

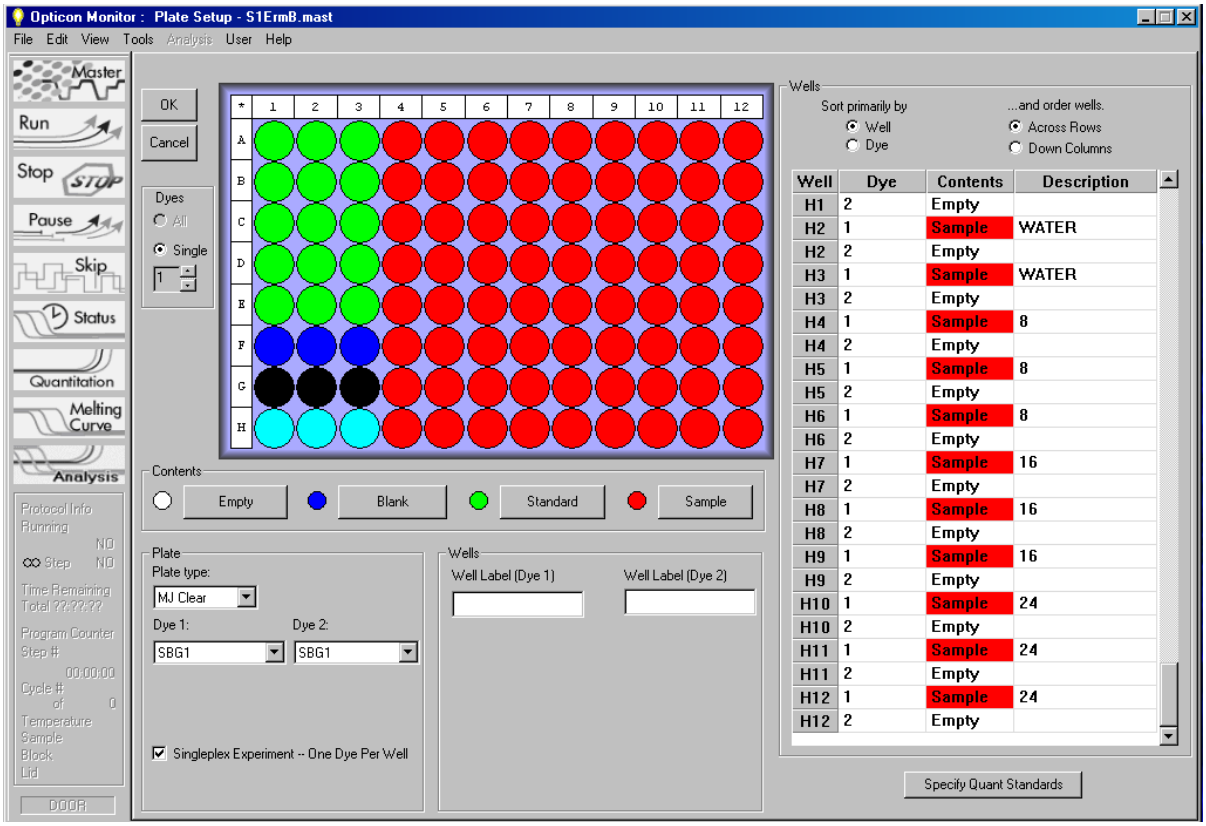


Figure 28: Plate Setup Screen

After editing the table, click on ‘Specify Quant Standards’ tab located at the bottom of the table, Figure 29. This step specifies the quantitative amount of DNA per reaction for each of the standard wells. This must be done in order to facilitate calculations of the unknown samples. For example, after reading the concentration of the standard original DNA using the microspectrometer or nano drop, you had a DNA extract concentration of 75.5 ng/µL.

Knowing that 2.5 µL of DNA template is added to each well, a simple calculation can be made to determine the quantification and dilution series: 75.5 ng/µL*2.5µL=188.75 ng/reaction. A 10-fold serial dilution of the original extract would then begin with this. Next, type the set of dilution values in under the appropriate standard label. When finished entering the values, check that the units designated are 'ng' and click 'OK'. Then save the plate set up under your master.

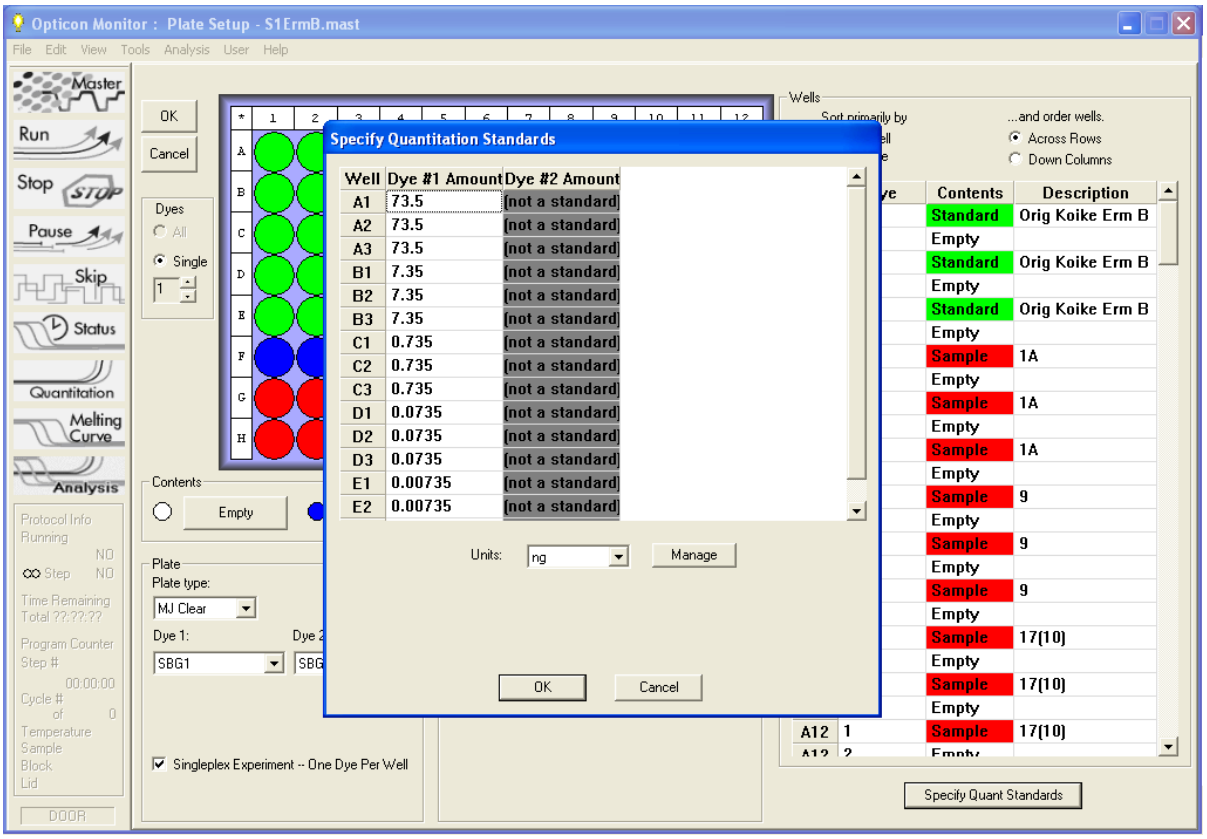


Figure 29: Specify Quantification Standards

4. Next, select the 'Protocol Setup' box below the plate. Each Master File will come with the saved protocol, as soon as it is created. The protocol file determines the thermocycling parameters for the analysis. To create a protocol, click 'Edit Setup' and specify the temperature and time for each step, as shown in Figure 27. These protocols will be unique with each target gene. A sample protocol and a graphic of the protocol for the entire run is shown in Figure 30. Also confirm that for the protocol that the reaction volume is correct by specifying 25uL (top right corner) unless a different volume is used. Once you have a Master

File with the protocol of choice in place, you need only review the protocol for correctness by clicking ‘Edit’ and confirming that the steps are correct.

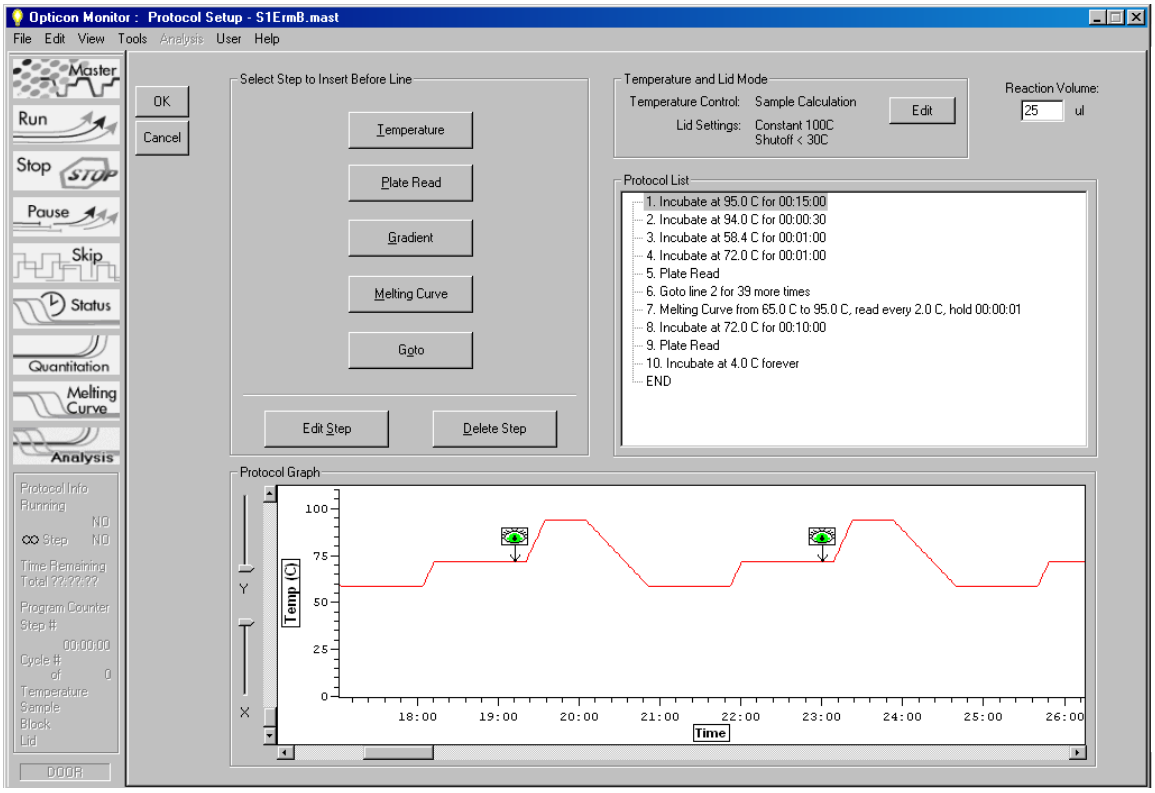


Figure 30: Thermal Profile and Protocol for qPCR Analysis

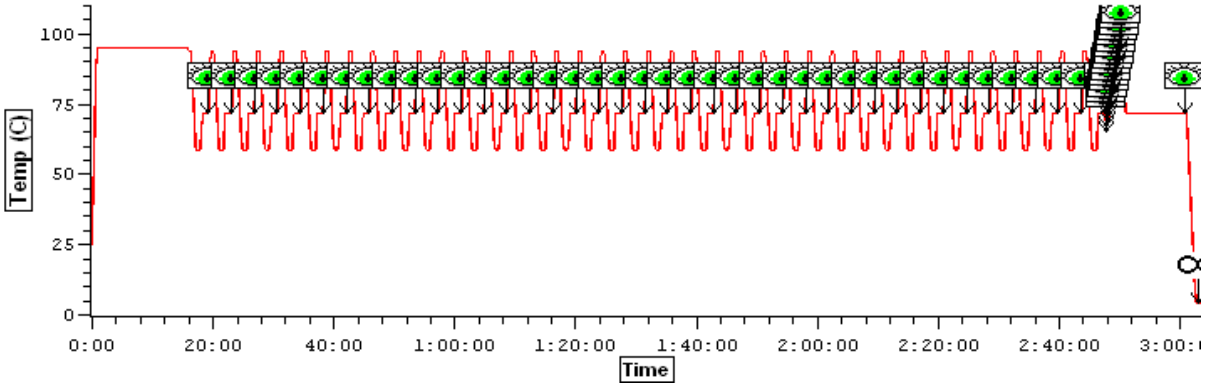


Figure 31: Thermal Profile for an Entire qPCR Run

5. The prepared strip tubes or plates can now be loaded on to the Opticon Monitor 2 as described in the plate setup step.

6. Start the qPCR process by selecting ‘Run’ on the left side of the Opticon Monitor 2 Operation page. Any of the tabs in dark blue/black indicate that you can select for that

particular activity. A blue light will turn on in the front of the instrument after clicking on the run tab.

7. When the run has finished, click on the ‘Quantitation’ button on the left to perform data analysis, shown in Figures Figure 32 and Figure 33 Figure 32. The graph located on the left of the figure is called the Amplification plot, which shows the fluorescence vs. cycle. The sooner the fluorescence in the sample, the higher the copy number of the target nucleic acid in that sample. The graph on the right is a plot of the standard curve generated. The dot clusters represent the triplicate standards in the assays, and show the serial dilution from left to right.

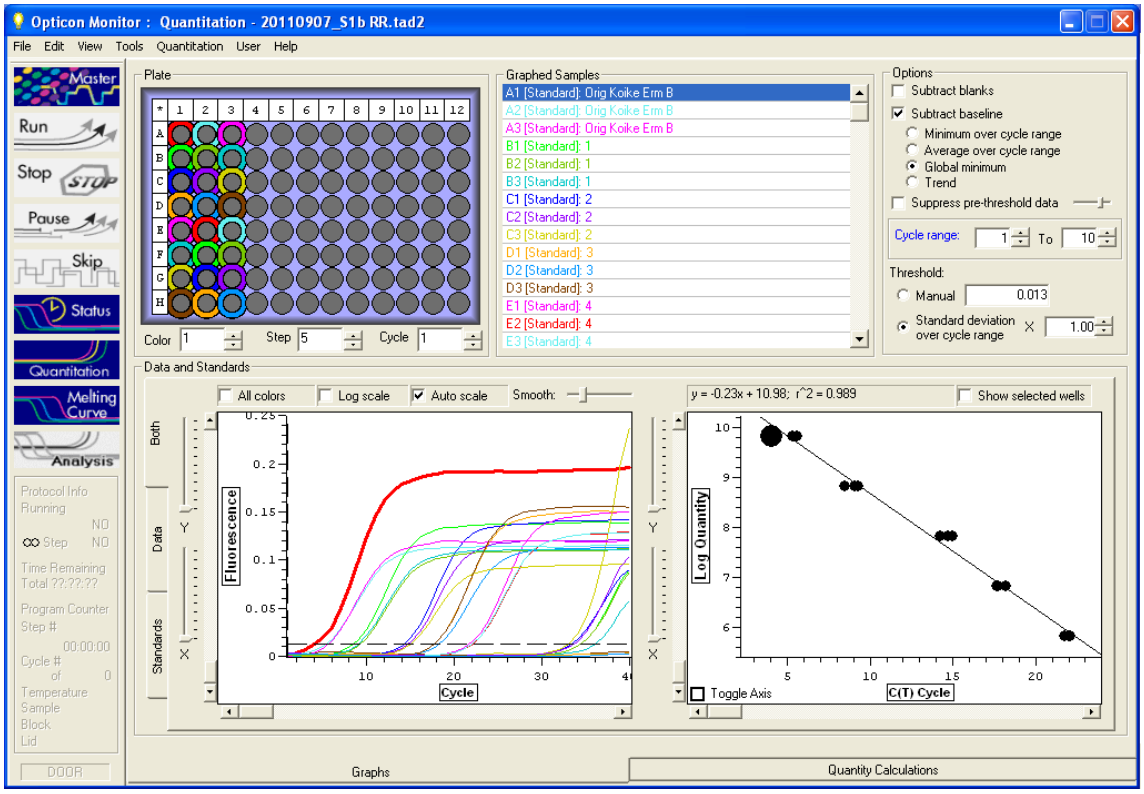


Figure 32: Quantitation Graphs

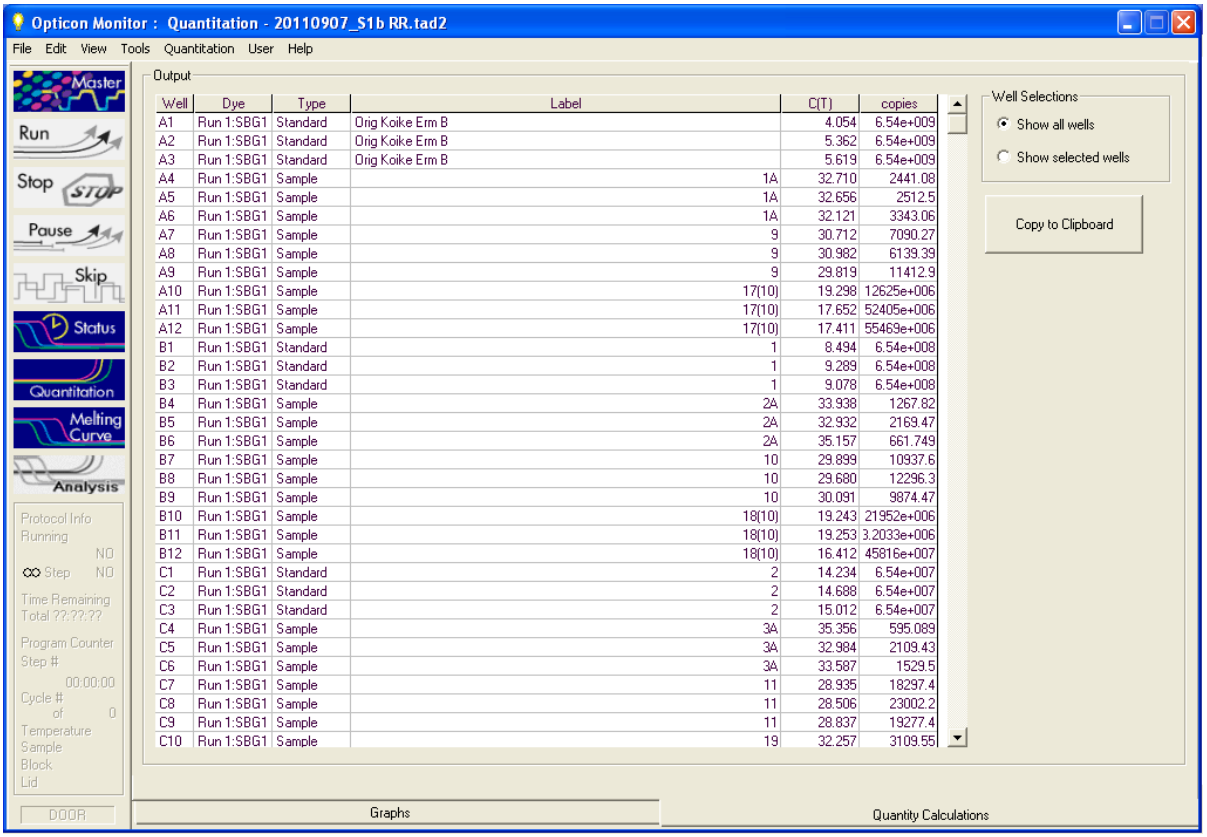


Figure 33: Quantity Calculations

7. Additionally, click on the 'Melting Curve' button on the left to verify that the samples melted at the proper and specified annealing temperature. As shown in Figure 34, the melting temperature was at 77°C.

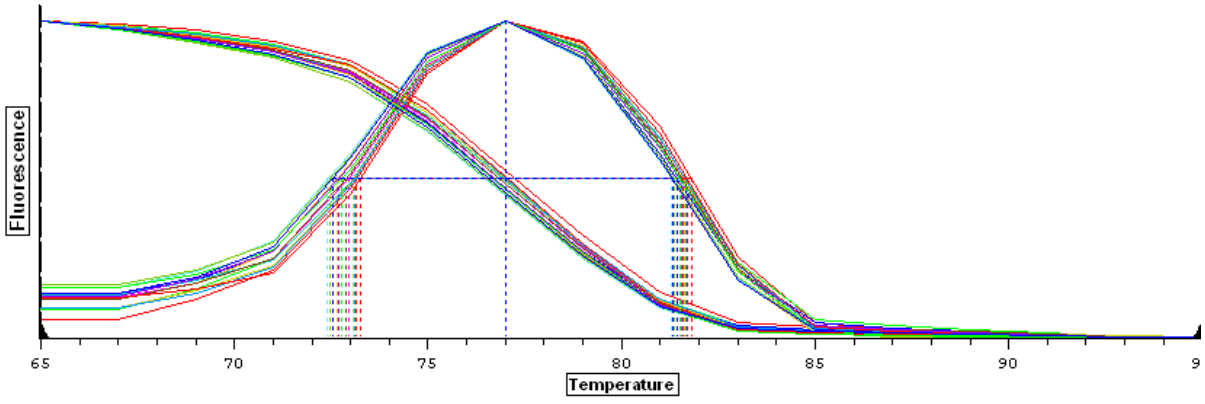


Figure 34: Melt Curve of Standards

Results and Data Analysis

To perform the quantification of the DNA in each well, start out by clicking the "Quantitation" button to perform data analysis. This will produce a set of results that show the well Id, dye, type, label, C_t and copies. qPCR results are based on the detection of an accumulated florescent signal over the course of the assay. A typical assay for qPCR has 40 cycles. The Cycle Threshold, or C_t, is the number of cycles that are required for the florescence to exceed the background or baseline levels of florescence. The C_t of a sample is inversely proportional to the amount of target gene in the sample. Therefore, the lower the C_t, the more target gene in the sample. C_t,'s that are 29 or less indicate an abundance of target nucleic acid in the sample whereas C_t,'s between 30 and approximately 37 indicate a medium level of nucleic acid. However, C_t,'s on the range of 38 to 40 indicate low levels of target nucleic acid and the possibility for contamination. Since all assays are run with negative controls and blanks to define the lower limit of the quantification, the hope is that the negative controls are 38 or greater.

The software package also allows for simple result analysis. The first thing (and default) that will be done will be to subtract the baseline amplification. Following this, the threshold values will be set, and the final quantification data will be exported to Excel for modification. Beyond the default, there are further modifications that can be performed which will help improve the standard curve, and therefore improve the results. These additional modifications are outlined below.

1. Using the mouse, select all wells with standards, samples, and negative controls. If you do not select all of the active wells, no data will be processed by the software.
2. Subtract the baseline by moving the cursor over the vicinity of the florescence vs. cycle graph, shown in Figure 32. This step requires experience and judgment. Some recommendations include the following:

- If amplification begins in the first 10 cycles, choose "min. over all data".
- If amplification begins after 10 cycles, choose a "cycle range" option. Start at cycle 3 and end at cycle 15 or 2 cycles before the start of amplification.

3. Set the threshold manually on the data graph. This should be set such that the threshold is above the baseline on linear portion of the curve. All samples should be parallel and linear at this point (excluding water blanks and negative controls).

4. Next, take a look at the standard curve which will automatically be plotted in the "standards" graph. Outliers may be eliminated by clicking on them, which will turn the spot red. Use caution when removing outliers, although they should be well defined since the standards were done in triplicate. Figure 35 shows an example of a standard curve plot. In theory, a perfectly efficient reaction will have standard curve with a slope of -0.3. High r^2 values indicate the closeness of the standards to the predicted value.

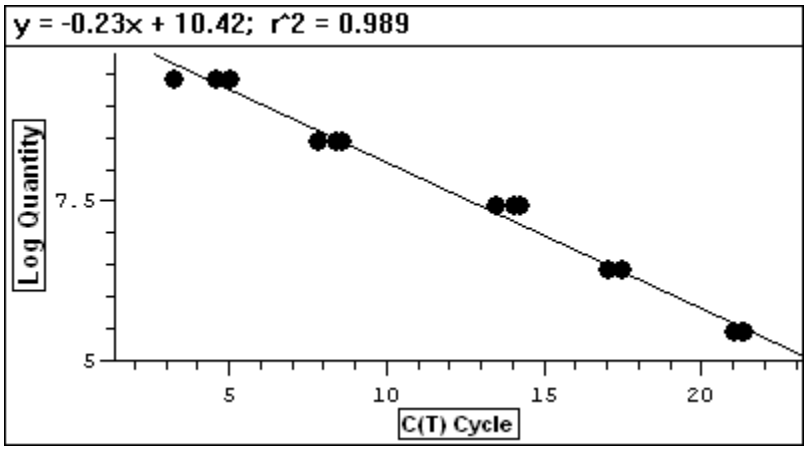


Figure 35: Example Standard Curve

5. Click on the 'Quantity Calculations' tab at the bottom of the screen to display the Ct and the copies for each well. The option also exists to export in terms of nanograms/well of the gene as well, if desired. Copy this data to an Excel sheet for further data manipulation. To do so, click 'Quantitation', 'Copy to Clipboard', 'Quantity Calculations.' Then paste in Excel file. It is also recommended to copy over the 'Data Graph,' 'Standard Graph,' plate setup, and melt curve in a similar fashion. This will aid in data analysis

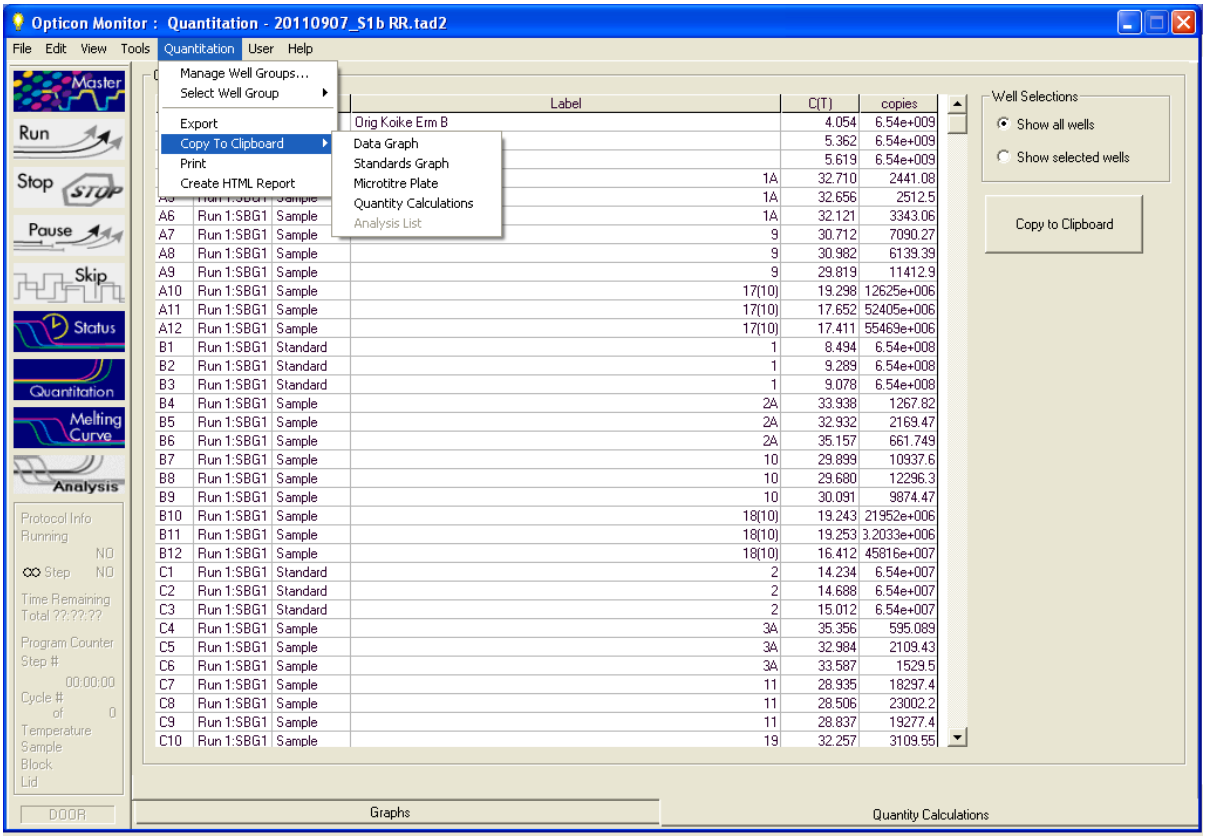


Figure 36: Quantity Calculations: exporting data

6. The Ct values in Excel can now be used to determine the quantities in each well. The target is to get the copies/well into units of copies/g soil (or manure) or copies/100 mL water. Paste the data into an Excel file, which will be similar to this example in Table 14 for the first row of the plate. Next, manipulate the data such all the standards are on top, followed by the water controls, negative controls and blanks shown in Table 14.

Table 14: Quantity Calculations Output in Excel

Well	Dye	Type	Label	C(T)	copies/well
A1	Run 1:SBG1	Standard	Orig Koike	3.241	2.61E+09
A2	Run 1:SBG1	Standard	Orig Koike	4.577	2.61E+09
A3	Run 1:SBG1	Standard	Orig Koike	5.027	2.61E+09
A4	Run 1:SBG1	Sample	1A	31.925	1111.15
A5	Run 1:SBG1	Sample	1A	31.979	1080.08
A6	Run 1:SBG1	Sample	1A	31.285	1561.82
A7	Run 1:SBG1	Sample	9	29.987	3113.86
A8	Run 1:SBG1	Sample	9	30.158	2842.71
A9	Run 1:SBG1	Sample	9	29.074	5058.35
A10	Run 1:SBG1	Sample	17(10)	18.6	1.33E+06
A11	Run 1:SBG1	Sample	17(10)	16.641	3.76E+06
A12	Run 1:SBG1	Sample	17(10)	15.809	5.84E+06

The next step is to calculate the concentration of the gene of interest from the Ct and the calculated copy/well that is given in the output from the software. Follow the following unit general conversion:

$$\left(\frac{copy}{well}\right)\left(\frac{well}{\mu L\ DNA}\right)\left(\frac{volume\ of\ DNA\ extracted}{mass\ (or\ volume) media}\right)$$

For the example shown in, Table 15, 8mL of DNA was extracted from 15 g of soil. Therefore:

$$\left(\frac{2.61\ x\ 10^{9} copy}{well}\right)\left(\frac{well}{2.5\ \mu L\ DNA}\right)\left(\frac{8000\ \mu L}{15\ g\ soil}\right) = \frac{5.57x\ 10^{11}\ copies}{g\ soil}$$

It is also possible to convert from ng to copies (ir vice versa) using a simple online tool, if you know the vector and the PCR size. This tool may be found at: http://www.finnzymes.fi/java_applets/copy_number_calculation.html.

Table 15: Sample Copy Number Calculation

Well	Dye	Type	Label	C(T)	copies/well	copy/ul	copies/g
A1	Run 1:SBG1	Standard	Orig Koike	3.241	2614000000	1045600000	5.5765E+11
A2	Run 1:SBG1	Standard	Orig Koike	4.577	2614000000	1045600000	5.5765E+11
A3	Run 1:SBG1	Standard	Orig Koike	5.027	2614000000	1045600000	5.5765E+11
B1	Run 1:SBG1	Standard	1	7.827	261400000	104560000	5.5765E+10
B2	Run 1:SBG1	Standard	1	8.634	261400000	104560000	5.5765E+10
B3	Run 1:SBG1	Standard	1	8.398	261400000	104560000	5.5765E+10
C1	Run 1:SBG1	Standard	2	13.51	26140000	10456000	5.5765E+09
C2	Run 1:SBG1	Standard	2	14.042	26140000	10456000	5.5765E+09
C3	Run 1:SBG1	Standard	2	14.258	26140000	10456000	5.5765E+09
D1	Run 1:SBG1	Standard	3	17.082	2614000	1045600	5.5765E+08
D2	Run 1:SBG1	Standard	3	17.507	2614000	1045600	5.5765E+08
D3	Run 1:SBG1	Standard	3	17.079	2614000	1045600	5.5765E+08
E1	Run 1:SBG1	Standard	4	21.05	261400	104560	5.5765E+07
E2	Run 1:SBG1	Standard	4	21.346	261400	104560	5.5765E+07
E3	Run 1:SBG1	Standard	4	21.294	261400	104560	5.5765E+07
H1	Run 1:SBG1	Sample	WATER	None	N/A	0	0.0000E+00
H2	Run 1:SBG1	Sample	WATER	None	N/A	0	0.0000E+00
H3	Run 1:SBG1	Sample	WATER	None	N/A	0	0.0000E+00
G1	Run 1:SBG1	Sample	Neg Contrc	32.225	947.737	379.0948	2.0218E+05
G2	Run 1:SBG1	Sample	Neg Contrc	32.575	786.825	314.73	1.6786E+05
G3	Run 1:SBG1	Sample	Neg Contrc	32.792	700.839	280.3356	1.4951E+05
F1	Run 1:SBG1	Blank	blank with	35.224	192.429	76.9716	4.1052E+04
F2	Run 1:SBG1	Blank	blank with	33.637	447.328	178.9312	9.5430E+04
F3	Run 1:SBG1	Blank	blank with	33.609	453.994	181.5976	9.6852E+04

For water samples, a similar conversion can be made is 100 µL of DNA was extracted from 250 mL of water. Therefore:

$$\left(\frac{2.61\ x\ 10^{9} copy}{well}\right)\left(\frac{well}{2.5\ \mu L\ DNA}\right)\left(\frac{100\ \mu L}{250\ mL\ water}\right)(100\ mL) = \frac{4.18x\ 10^{10}\ copies}{100\ mL\ water}$$

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APPENDIX E: ERM GENES QPCR PROTOCOL

Table 16: qPCR protocol for *erm*B on the MJ Research Opticon2.

Step	Procedure
1	Incubate at 95.0°C for 00:15:00
2	Incubate at 94.0°C for 00:00:30
3	Incubate at 58.4°C for 00:01:00
4	Incubate at 72.0°C for 00:01:00
5	Plate Read
6	Go to line 2 for 39 more times
7	Melting Curve from 65.0°C to 95.0°C, read every 2.0°C, hold 00:00:01
8	Incubate at 72.0°C for 00:10:00
9	Plate Read
10	Incubate at 4.0°C forever
	END

Table 17: qPCR protocol for *erm*F on the MJ Research Opticon2.

Step	Procedure
1	Incubate at 90.0°C for 00:15:00
2	Incubate at 95.0°C for 00:00:30
3	Incubate at 54.3°C for 00:01:00
4	Incubate at 70.0°C for 00:01:00
5	Plate Read
6	Go to line 2 for 39 more times
7	Melting Curve from 60.0°C to 90.0°C, read every 2.0°C, hold 00:00:01
8	Incubate at 70.0°C for 00:10:00
9	Plate Read
10	Incubate at 4.0°C forever
	END

Table 18: qPCR protocol for *erm*T on the MJ Research Opticon2.

Step	Procedure
1	Incubate at 90.0°C for 00:15:00
2	Incubate at 95.0°C for 00:00:30
3	Incubate at 51.0°C for 00:01:00
4	Incubate at 70.0°C for 00:01:00
5	Plate Read
6	Go to line 2 for 39 more times
7	Melting Curve from 60.0°C to 90.0°C, read every 2.0°C, hold 00:00:01
8	Incubate at 70.0°C for 00:10:00
9	Plate Read
10	Incubate at 4.0°C forever
	END

APPENDIX G: QPCR PRODUCT SEQUENCING

This information and procedure were developed by Beth Douglass, USDA-ARS NLAE, and consultation with Todd Atherly, USDA-ARS NLAE, when PCR products from the 2011 samples including manure, soil and water were sent to the DNA Facility of the Iowa State University Office of Biotechnology.

Erm Gene Confirmation Procedure

Preparation

Preparation of PCR product samples submitted for sequencing began with running two sets of reactions, one using the *erm*B primers reported by Koike (2010) selecting for an amplicon 191 bp in size and one using *erm*F primer set used by Chen (2007) selecting for an amplicon 309 bp long, as shown in Table 19. There was no special change to methods, as described in the Chapter 3, but the procedure used is briefly outlined here. The primer annealing temperatures, as reported in both papers was adjusted to temperatures that were found to be most effective on the MJ Research Opticon2 instrument. The reported temperatures were 65°C for *erm*B (Koike et al., 2010) and 56°C for *erm*F (Chen et al., 2007). Table 19 shows the annealing temperatures that were actually used in the lab which were optimized.

Table 19: Primers used for PCR analysis of *erm* genes.

Primer	Class targeted	Primer Sequence (5'→3')	Amplicon Size (bp)	Primer annealing temp. (°C)	Reference
Erm B-FW *Erm* B-RV	*erm*B	GGTTGCTCTTGCACACTCAAG CAGTTGACGATATTCTCGATTG	191	58.4	Koike et al. 2010
Erm F-189f *Erm* F-497r	*erm*F	CGACACAGCTTTGGTTGAAC GGACCTACCTCATAGACAAG	309	54.3	Chen et al. 2007

Samples were selected to encompass soil and water matrices, both tillage types, and plots that received manure application, as these had the highest concentrations of extractable DNA (

Table 20). DNA samples were amplified with both forward and reverse primers (without SYBER Green) using the standard procedure outlined in Table 16 and Table 17.The reaction products, from the samples listed in Table 20, were subsampled by selecting the well of each set of three that exhibited the strongest reaction based on the amount of calculated product following the run and purified using Qiagen's QIAquick PCR Purification Kit (Cat. No. 28104). The purified product subsamples were then submitted to the DNA Facility of the Iowa State University Office of Biotechnology. Details of this processing are outlined below.

Table 20: Samples chosen for PCR product sequencing

Treatment	Tillage	Matrix	Plot	Sample Label
Manure Band	Chisel Plow	Soil	23	13A
Manure Band	No Till	Soil	25	16
Manure Band	Chisel Plow	Soil	23	32
No-Band	Chisel Plow	Soil	23	7
No-Band	No Till	Soil	25	12
No-Band	No Till	Soil	25	28
Manure	Chisel Plow	Water	23	23(5/19/2011)
Manure	No Till	Water	25	25(5/19/2011)
Manure	Chisel Plow	Water	23	23(5/24/2011)

qPCR Runs

DNA samples were amplified with both forward and reverse primers (with and without SYBER Green) using the standard procedure. Separate analyses for e*rm*B and *erm*F were performed due to different annealing temperatures. Each sample was run in triplicate. 50 µL reaction volumes were used instead of 25 µL, as used during the qPCR analysis, to increase the amount of product for purification. Therefore, each reaction mixture contained 25 µL 2X HotStart Taq, 10.0 µL of each primer (forward and reverse) at 2.5 µM concentration, and 5.0 µL template or control or water for blank. The MJ Research Opticon procedure for the *erm*B and *erm*F is as shown in Table 16 and Table 17.

Based on the melting curve analysis, the melting temperature for *erm*B = 77°C and for *erm*F = 74°C. From the triplicate qPCR wells, the wells exhibiting the strongest reaction based on the amount of calculated product following the run were selected. Qiagen's QIAquick PCR Purification Kit (Cat. No. 28104) was used to purify the PCR products. A 40 µL aliquot containing the PCR reaction product was taken from each well and mixed with

200 µL of Buffer PB from the kit. (1:5 sample: buffer). The protocol was then followed per manufacturer's instructions. The purified DNA samples were then submitted to the DNA Facility of the Iowa State University Office of Biotechnology

The concentration of the purified product DNA was then determined by mixing 3 µL of sample with 61 µL H_2O and read on an Eppendorf Biophotometer. The ISU DNA Facility requested that primers and samples be made up to specified concentrations and volumes prior to submission for their sequencing protocol. The concentration of the samples is dependent upon the length of the fragment and the source of the DNA. For our purposes, we submitted PCR products which require that there be 2.5 ng /100 bases / µL. So, for a 309 bp product, samples needed to be mixed to a DNA concentration of 7.7 ng/ µL. 50 µL of each sample in 1.5 mL microfuge tubes. The primers were made up to a concentration of 5 pmol/µL and 25 µL were submitted of each. For each sample and gene there were 2 reactions; 22 samples were for a total of 44 reactions. See the following worksheets for *erm*B and *erm*F as shown in Table 21 and

Table 22. The primer name shown includes the gene, whether the primer is forward (FW) or reverse (RV), and the source of the primer, either Koike (Ko) or Chen (C).

Table 21: Worksheet for preparation of templates/primers for *erm*B submitted for sequencing

Template ID	[Product DNA] (ng/µL)	DNA to dilute (µL)	[DNA] for ISU (ng/µL)	Vol. for ISU (µL)	H_2O Vol (µL)	Primer Name	
						Forward	Reverse
ErmBSTD1	102.8	2.3	4.7	50	47.7	ErmB FW Ko	ErmB RV Ko
ErmBSTD4	143.1	1.6	4.7	50	48.4	ErmB FW Ko	ErmB RV Ko
ErmBS7	78	3	4.7	50	47	ErmB FW Ko	ErmB RV Ko
ErmBS12	74	3.2	4.7	50	46.8	ErmB FW Ko	ErmB RV Ko
ErmBS13	103.6	2.3	4.7	50	47.7	ErmB FW Ko	ErmB RV Ko
ErmBS16	69.8	3.4	4.7	50	46.6	ErmB FW Ko	ErmB RV Ko
ErmBS28	62.1	3.8	4.7	50	46.2	ErmB FW Ko	ErmB RV Ko
ErmBS32	80.3	2.9	4.7	50	47.1	ErmB FW Ko	ErmB RV Ko
EBP23519	98.6	2.4	4.7	50	47.6	ErmB FW Ko	ErmB RV Ko
EBP25519	128	1.8	4.7	50	48.2	ErmB FW Ko	ErmB RV Ko
EBP23524	86.8	2.7	4.7	50	47.3	ErmB FW Ko	ErmB RV Ko

Table 22: Worksheet for preparation of templates/primers for *erm*F submitted for sequencing

Template ID	[Product DNA] (ng/µL)	DNA to dilute (µL)	[DNA] for ISU (ng/µL)	Vol. for ISU (µL)	H2O Vol (µL)	Primer Name	
						Forward	Reverse
ErmFSTD1	90.3	4.3	7.7	50	45.7	ErmF189f C	EmF497r C
ErmFSTD3	101.4	3.8	7.7	50	46.2	EmF189fC	EmF497r C
ErmFS7	25.2	15.3	7.7	50	34.7	EmF189fC	EmF497r C
ErmFS12	33.6	11.5	7.7	50	38.5	EmF189fC	EmF497r C
ErmFS13	32	12	7.7	50	38	EmF189fC	EmF497r C
ErmFS16	29.7	13	7.7	50	37	EmF189fC	EmF497r C
ErmFS28	20.5	18.8	7.7	50	31.2	EmF189fC	EmF497r C
ErmFS32	22.2	17.3	7.7	50	32.7	EmF189fC	EmF497r C
EFP23Mix[1]	90.4	4.3	7.7	50	45.7	EmF189fC	EmF497r C
EBP23519	70.6	5.5	7.7	50	44.5	EmF189fC	EmF497r C
EFP25519	65.7	5.9	7.7	50	44.1	EmF189fC	EmF497r C

[1]DNA was combined for plot 23 water from 5/19/2011 and 5/24/2011 as there was an insufficient quantity to submit for sequencing. This is denoted with 'mix.'

Results

The forward and reverse *erm*B and *erm*F PCR product sequences were aligned and consensus sequences were developed using Vector NTI software. The sequence size was 182-185 bp. The *erm*F fragment was 310 bp. According to (Koike et al., 2010) and (Chen et al., 2007), the primers used produced an *erm*B amplicon of 191 bp and an *erm*F amplicon of 309 bp, respectively. All of the standards and samples submitted for *erm*B were successfully sequenced by ISU. However, only the standards and the water samples (matrix) produced consensus sequences for *erm*F. Matches to the consensus sequences were identified using Mega BLAST searches of the NCBI nucleotide database.

***erm*B – soil**

Samples *erm*BS13, *erm*BS12, *erm*BS7, *erm*BS16, and *erm*BS28 yielded consensus sequences with 183 to 187 bp length. Blast searches produced 100 results for each search that were 100% matches. E values ranged from 5e-19 to 1e-90 (*erm*Bs7). The E value represents the number of matches expected per chance, thus a low score indicates the quality sequence matching. Inspection of the sequence matches also showed high quality, at least 99% matching with no gaps. The accessions identified by Blast showed a lot of communality for

*erm*B PCR products from soil. Among the matches were *erm*B rRNA methylase genes from *Enterococcus faecium* (e.g. Accessions JN899594.1, JN899582.1), *Clostridium difficile erm*B gene (Accession JN607214.1), *Streptococcus pneumonia* Tn916 element (Accession FR671418.1), *Streptococcus suis erm*B gene (Accession FN677479.1), and *Streptococcus pyrogenes erm*B gene (Accession FN677480.1). In addition, there were matches to 14 *erm*B sequences from uncultured bacteria in environmental samples taken for a study erythromycin-resistance genes associated with swine production (Koike et al., 2010). No matches to accessions not related to *erm*B were obtained. Comparison of matches to ermB standards produced similar results.

***erm*B – water**

Similar results were obtained for *erm*B PCR products from tile water samples. Sequences *erm*Bp23524 matched the same accession sequences described for matches to soil-derived PCR products See above). This includes the sequences in NCBI provided by Koike et al (2010).

***erm*F – soil and water**

Consensus DNA sequences were not obtained for PCR products for *erm*F derived from soil samples. For sequences, *erm*FP25519 and *erm*FP23519, Blast searches returned 18 matches. Matches included the *erm*F gene on R plasmid pBF4 from *Bacteriodes fragilis* (Acession M14730.1) and *Bacteriodes thetaiotamicron* (AJ31171.1), and a sequence from uncultured bacteria (Accession DQ887621.1) described by Chen et al. (2007) (AEM 73, 4407-4416), and *Capnocytophaga* (JQ707297), a peridontal isolate. Only one match was to an accession not related to *erm*F.

Table 23: DNA sequences (FASTA format) of amplicons from PCR reactions using standards or environmental DNA as templates.

Source	Sequence
ErmB – Standard	>ErmBstd1\con gctcttgcacactcaagtctcgattcagcaattgcttaagctgccagcggaatgctttcatcctaaaccaaaagtaaacagtgtcttaataaaact tacccgccataccacagatgttccagataaatattggaagctatatacgtactttgtttcaaaatgggtcaatcgagaatatcgtc
ErmB - Standard	>ErmBstd4 gctcttgcacactcaagtctcgattcagcaattgcttaagctgccagcggaatgctttcatcctaaaccaaaagtaaacagtgtcttaataaaact tacccgccataccacagatgttccagataaatattggaagctatatacgtactttgtttcaaaatgggtcaatcgagaatatcgtc
ErmB – Soil	>ErmBS7 tgctcttgcacactcaagtctcgattcagcaattgcttaagctgccagcggaatgctttcatcctaaaccaaaagtaaacagtgtcttaataaaac

Plot 23	ttacccgccataccacagatgttccagataaatattggaagctatatacgtactttgtttcaaaatgggtcaatcgagaatatcgtca
ErmB –	>ErmBS12
Soil	tggttgctcttgcacactcaagtctcgattcagcaattgcttaagctgccagcggaatgctttcatcctaaaccaaaagtaaacagtgtcttaata
Plot 12	aaacttacccgccataccacagatgttccagataaatattggaagctatatacgtactttgtttcaaaatgggtcaatcgagaatatcgtc
ErmB –	>ErmBS13
Soil	tgctcttgcacactcaagtctcgattcagcaattgcttaagctgccagcggaatgctttcatcctaaaccaaaagtaaacagtgtcttaataaaac
Soil	ttacccgccataccacagatgttccagataaatattggaagctatatacgtactttgtttcaaaatgggtcaatcgagaatatcgtc
Plot 13	
Erm B-	>ErmBS16
Soil	tgctcttgcacactcaagtctcgattcagcaattgcttaagctgccagcggaatgctttcatcctaaaccaaaagtaaacagtgtcttaataaaac
Plot 16	ttacccgccataccacagatgttccagataaatattggaagctatatacgtactttgtttcaaaatgggtcaatcgagaatatcgtca
Erm B	>ErmBS28
Soil	tggttgctcttgcacactcaagtctcgattcagcaattgcttaagctgccagcggaatgctttcatcctaaaccaaaagtaaacagtgtcttaata
Plot 28	aaacttacccgccataccacagatgttccagataaatattggaagctatatacgtactttgtttcaaaatgggtcaatcgagaatatcgtc
ErmB	>ErmBS32
Soil	tgctcttgcacactcaagtctcgattcagcaattgcttaagctgccagcggaatgctttcatcctaaaccaaaagtaaacagtgtcttaataaaac
Plot 32	ttacccgccataccacagatgttccagataaatattggaagctatatacgtactttgtttcaaaatgggtcaatcgagaatatcgtc
ErmB	>ErmBP23519
Water	tgctcttgcacactcaagtctcgattcagcaattgcttaagctgccagcggaatgctttcatcctaaaccaaaagtaaacagtgtcttaataaaac
Plot 23	ttacccgccataccacagatgttccagataaatattggaagctatatacgtactttgtttcaaaatgggtcaatcgagaatatcgtcaa
ermB-	>ErmBP25519
Water	tgctcttgcacactcaagtctcgattcagcaattgcttaagctgccagcggaatgctttcatcctaaaccaaaagtaaacagtgtcttaataaaac
Plot 25	ttacccgccataccacagatgttccagataaatattggaagctatatacgtactttgtttcaaaatgggtcaatcgagaatatcgtc
ErmB-	>ErmBP23524
Water	tgctcttgcacactcaagtctcgattcagcaattgcttaagctgccagcggaatgctttcatcctaaaccaaaagtaaacagtgtcttaataaaac
Plot 23	ttacccgccataccacagatgttccagataaatattggaagctatatacgtactttgtttcaaaatgggtcaatcgagaatatcgtc
ErmF-	>ErmFstd1
PCR	cgacacagctttggttgaacatttacgaaaattattttctgatgcccgaaatgttcaagttgtcggttgtgattttaggaattttgcagttccgaaattt
standard	cctttcaaagtggtgtcaaatattccttatggcattacttccgatattttcaaaatcctgatgtttgagagtcttggaaattttctgggaggttccattg
	tccttcaattagaacctacacaaaagttattttcgaggaagctttacaatccatataccgttttctatcatacttttttgatttgaaacttgtctatgag
	gtaggt
ErmF-	>ErmFstd3
PCR	tcgacacagctttggttgaacatttacgaaaattattttctgatgcccgaaatgttcaagttgtcggttgtgattttaggaattttgcagttccgaaatt
standard	tcctttcaaagtggtgtcaaatattccttatggcattacttccgatattttcaaaatcctgatgtttgagagtcttggaaattttctgggaggttccatt
	gtccttcaattagaacctacacaaaagttattttcgaggaagctttacaatccatataccgttttctatcatacttttttgatttgaaacttgtctatga
	ggtagg
ErmF	>ErmFP23Mix
Plot 23	tcgacacagctttggttgaacatttacgaaaattattttctgatgcccgaaatgttcaagttgtcggttgtgattttaggaattttgcagttccgaaatt
	tcctttcaaagtggtgtcaaatattccttatggcattacttccgatattttcaaaatcctgatgtttgagagtcttggaaattttctgggaggttccatt
	gtccttcaattagaacctacacaaaagttattttcgaggaagctttacaatccatataccgttttctatcatacttttttgatttgaaacttgtctatga
	ggtaggtcc
ErmF-	>ErmFP23519
Water	tcgacacagctttggttgaacatttacgaaaattattttctgatgcccgaaatgttcaagttgtcggttgtgattttaggaattttgcagttccgaaatt
Plot 23	tcctttcaaagtggtgtcaaatattccttatggcattacttccgatattttcaaaatcctgatgtttgagagtcttggaaattttctgggaggttccatt
	gtccttcaattagaacctacacaaaagttattttcgaggaagctttacaatccatataccgttttctatcatacttttttgatttgaaacttgtctatga
	ggtag
ErmF-	>ErmFP25519
Water	tcgacacagctttggttgaacatttacgaaaattattttctgatgcccgaaatgttcaagttgtcggttgtgattttaggaattttgcagttccgaaatt
Plot 25	tcctttcaaagtggtgtcaaatattccttatggcattacttccgatattttcaaaatcctgatgtttgagagtcttggaaattttctgggaggttccatt
	gtccttcaattagaacctacacaaaagttattttcgaggaagctttacaatccatataccgttttctatcatacttttttgatttgaaacttgtctatga
	ggtaggtcc

APPENDIX H: REFERENCES

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