RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Occurrence and Movement of Antibiotic Resistant Bacteria and Resistance Genes in Tile-Drained Agricultural Fields Receiving Swine Manure Application - **NPB #12-089**

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Industry Summary:

The use of antibiotics by the swine industry to increase production efficiency and treat disease is thought to contribute to antibiotic resistance in the environment. When manure from hog operations is applied to fields with subsurface drainage, it is possible that the antibiotics and bacteria with resistance will be transported through tile systems and discharged into surface waters. To investigate this, tylosin, enterococci (a pathogen indicator organism), and antibiotic resistance genes (ARGs) were assessed in manure, soil and tile water samples. The ARG examined in this study were the *erm* genes which confer resistance to macolide anibiotics, including tylosin and erythromycin. Manure from a swine facility which administers tylosin at sub-therapeutic levels was applied to chisel plow and no-till plots with separate tile drains. The use of tylosin in swine production caused an increase in *erm* genes in manure and in manured-treated soil above the background levels of *erm* genes in soils not receiving manure. This increase in soil is greatest immediately after manure application; and *erm*B and *erm*F persist in manure injection band in concentrations greater than in non-manured soils over winter. However, the manure band concentrations eventually decreased to levels equivalent to the non-manured control soils. This is potentially due to a reduction in *erm*-hosting bacteria in the soil following manure application. The same trend was seen in the decline of total enterococci populations over time potentially due to die off and other environmental factors, but enterococci were less persistent than *erm* genes after manure application. 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 were not different between tillage or manure treatments, suggesting that off-site transport of *erm* genes was not increased by the application of manure from antibiotic-treated swine.

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Scientific Abstract:

The use of tylosin at subtherapeutic levels by the swine industry provides selective pressure for antibiotic resistance in the animal gut and manure. Land application of manure from tylosin-treated swine introduces tylosin-resistant enterococci, *erm* genes, which confer resistance to tylosin, and tylosin. This study documents the occurrence and transport of tylosin-resistant enterococci, *erm* genes and tylosin in tile-drained chisel plow and no-till agricultural fields treated with liquid swine manure in alternating years. Nearly 75% of the enterococci in manure were resistant to tylosin and *erm*B concentrations exceeded 10^8 copies g^{-1} manure while the mean *erm*F concentrations exceeded 10^7 copies g^{-1} manure. e*rm*T was not detected. The mean concentration of tylosin was 73 ng g^{-1} manure. Soil collected from the manure injection band closely following application contained $>10^9$ copies g^{-1} soil of both *erm*B and *erm*F in 2010 and $> 10^8$ copies g^{-1} soil after the 2011 application compared to 3×10^3 to 3×10^5 copies g^{-1} soil in the no-manure control plots. Gene abundances declined over the subsequent two-year period to levels similar to those in the no-manure controls. Concentrations of enterococci in tile water were low while tylosin-resistant enterococci was rarely detected. E*rm*B was detected in approximately 75% of tile water samples and *erm*F was detected in 30% of tile water samples but levels of these genes were not elevated due to manure-application and no difference was found between both tillage practices. These results show that tylosin usage 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 in years of below average precipitation.

Introduction:

Antimicrobials are used in the swine industry at therapeutic levels for disease treatment and at sub-therapeutic levels to prevent the occurrence or spread of disease and promote growth. Tylosin is not completely metabolized in the gut and up to three-quarters of the mass of administered antibiotics to animals can be excreted in urine and feces (Mackie et al. 2006). Kumar et al. (2004) reported tylosin concentrations in swine manure ranging from 0 to nearly 4 mg L^{-1}. Antibiotic use results in resistant bacteria in the excreted feces. There is concern over the possible transport of antibiotic resistant bacteria into larger streams, or the possible transfer of antibiotic resistance genes to pathogenic microorganisms (Chee-Sanford et al., 2009: Heuer et al., 2011)

Erm (erythromycin resistance rRNA methylase) genes are responsible for resistance to macroloide-lincosamide-streptogramin (MLS) antibiotics, including tylosin. *Erm* genes have been reported in a varied assemblage of diverse bacteria which are principally, but not exclusively Firmicutes, Bacteriodes and Actinobacteria (Park et al. 2010). In *Enterococcus*, MLS resistance is most commonly mediated by the *erm*B gene (Portillo et al., 2000; Jackson et al., 2004). 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).

Land application of animal manure is a significant route by which fecal indicator organisms, antibiotics, ARB and antibiotic resistance genes (ARGs) enter the environment (Heuer et al. 2011). Between 25-35% of cropland in Iowa is artificially drained (Zucker and Brown, 1998) to enhance crop production, and much of this land is treated with swine manure. Transport of indicator bacteria (*E. coli* and *Enterococcus*) in tile drainage during high flows have also been reported previously (Dean and Foran, 1992; Joy et al., 1998; Hunter et al., 2000; Pappas et al., 2008). Tylosin and other antibiotics have also been detected agricultural streams, manure storage lagoons and in tile drainage water (Campagnolo et al., 2002; Kay et al., 2005; Dolliver and Gupta, 2008).

Presently, there is limited information on antibiotic and resistance gene transport to tile waters under natural conditions. Previously, Hoang et al. (in press) quantified tylosin resistance in *Enterococcus* spp. from liquid swine manure, treated soil and tile drainage water. *Erm*B, *erm*F and *erm*T was detected in 69%, 78% and 9.5% of 200 *Enterococcus* isolates from manure, soil and water samples, indicating that these genes are likely to be found in quantifiable levels.

Objectives:

The overall goal of this three year research project is to further our understanding of the occurrence and transport of antibiotics, antibiotic-resistant bacteria (ARB), and antibiotic resistance genes (ARGs) in tile-drained agricultural fields that have received multi-year application of liquid swine manure through injection. Key project objectives include 1) determining the occurrence of tylosin, tylosin -resistant enterococci, and resistance genes in soil and drainage water from fields receiving manure and chemical fertilizer; 2) relating the transport of tylosin, tylosin-resistant enterococci, and resistance genes in tile drainage from fields receiving manure to concentrations in soil, time following manure application and patterns of rainfall/drainage; and 3) assessing the potential horizontal transfer of resistance genes in drainage water using a microbial bioassay. We will also compare the data obtained at the plot scale to drainage water samples obtained at selected other sites, which will allow us to extend our findings from the research site to more broad geographical areas. In this final report we present the results of our monitoring data collected in 2011 and 2012 (funded by two 1-year contracts: NPB #10-119 and NPB #12-089). The microbial bioassay and larger scale monitoring studies are ongoing and results will be included in future reports.

The key outcome of the project is an improved scientific understanding of ARB and ARG loading and distribution in soils and tile flow following land application of swine manure compared to background levels. This understanding will help the pork industry ensure that policy makers, media, individuals in agriculture, and the general public are appropriately educated about the occurrence and transport of ARB and ARGs from manure amended fields compared to natural background levels.

Materials & Methods:

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%. 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 prevented 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. Subsurface drainage flow is metered as a function of pumped volume and are recorded weekly while the tile lines are flowing. Precipitation data was obtained from the Iowa Environmental Mesonet.

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 no-manure control for assessing background levels. (Table 1). All corn plots receive swine manure or UAN fertilizer as a nitrogen source prior to each crop season. The plots are in a 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. The control plots have no manure applied since 1978, while the manured plots have been in various manure rotations since 1993. Specific plot locations at the project site are described by Kanwar et al. (1999).

Table 1: Northeast Research and Demonstration Farm plots and experimental treatments.

Plot	Tillage	Nitrogen Management
23†	Chisel plow	2010 Fall inject swine manure at 168 kg N ha^{-1}
24†	Chisel plow	Spring preplant spoke inject UAN at 168 kg N ha^{-1}
25†	No-till	2010 Fall inject swine manure at 168 kg N ha^{-1}
34†	No-till	Spring preplant spoke inject UAN at 168 kg N ha^{-1} with Cover Crop
29‡	Chisel plow	Spring preplant spoke inject UAN at 168 kg N ha^{-1}
30‡	Chisel plow	2011 Fall inject swine manure at 168 kg N ha^{-1}
19‡	No-till	Spring preplant spoke inject UAN at 168 kg N $ha^{-1}$with Cover Crop
20‡	No-till	2011 Fall inject swine manure at 168 kg N ha^{-1}

† Plots (PSA) with data for 2 full years after 2010 manure application.
‡ Plots (PSB) with data for 1 full year after 2011 manure application.

Manure was injected 10 to 15 cm below the soil surface with shanks (76 cm spacing) forming bands of treated soil, as described by Al-Kaisi and Kwaw-Mensah (2007), on October 28 in both 2010 and 2011 (Table 1). The manure was applied 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 plots were field cultivated (10 cm depth) prior to planting corn the next May (Al-Kaisi and Kwaw-Mensah, 2007). Manure samples were collected directly from the manure applicator.

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 sample and analy sis 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 and DNA extraction) or 48 hours (tylosin). Water samples were only collected from tile lines in the first year after manure application.

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 µm filter within 24 hours. Soil and manure samples were diluted (1 g/ 9 mL) with distilled water prior to filtration. Total and tylosin-resistant enterococci were enumerated on mEnterococcus (mE) agar (Difco, Detroit, MI) without antibiotics and mE agar infused with tylosin at 35 mg L^{-1} (Kaukas et al., 1988; FDA, 2009; CLSI, 2010). All samples were analyzed in triplicate. Results for manure or soil were expressed on a dry weight basis in terms of colony forming units (cfu)/g and results for water were expressed as cfu 100-mL^{-1}.

DNA Extraction and qPCR

Quantitative PCR assays were performed to quantify *erm*B, *erm*F and *erm*T. DNA in tile water samples (250 mL) were extracted using the MoBio Power Water DNA kit within 48 hours of collection. Soil DNA extractions (10 g, wet weight) were performed using the MoBio UltraClean Soil DNA kit. ue to the complexity of the manure matrix, the repeated bead beating plus column extraction method 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).

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 subsamples of 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 2 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 2). The plasmids containing *erm*B and *erm*T fragments 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.

Table 2: qPCR primer sequences, annealing temperatures, and amplicon size for erm genes.

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

The effect of inhibitory substances co-extracted with the DNA 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. DNA extracts from soil and water matrices were selected 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 PCR products were sequenced at the the DNA Facility of the Iowa State University. The forward and reverse sequences were aligned and consensus sequences were developed using Vector NTI software. These sequences were matched to NCBI DNA sequences using mega BLAST.

Tylosin Extraction and Analysis

Analytical methods were developed and validated for tylosin A. 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 solvent 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) for verification. 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 (m/z 772.4) was present along with ions having m/z of 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^{-1}) and quantification (6.8 ng mL^{-1}) in the extracts from the first study year where concentrations of tylosin as low as 2 ng mL^{-1} were detected. In the second year, optimizing the procedure allowed for tylosin A to be detected at 0.3 ng mL^{-1} and quantified at 0.8 ng mL^{-1}.

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.

Results:

Enterococci in manure, soil, and tile drainage water

Entercocci were present in liquid swine manure with average concentrations of 5.7×10^5 cfu g^{-1} and 8.9×10^4 cfu g^{-1} for year 1 (PSA, 2010) and year 2 (PSB, 2011), respectively. Of those, 4.0×10^5cfu g^{-1} (70%) and 1.1×10^5 cfu g^{-1} (100%) were resistant to tylosin in PSA and PSB, respectively. The concentrations of enterococci and tylosin-resistant enterococci were significantly lower ($p < 0.1$) in year two than in the previous year. In 2011, the samples were analyzed 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. Previously, between 31% and 100% of enterococci from swine manure were tylosin-resistant (Jackson et al., 2004; Hoang et al., in press).

In soil, enterococci concentrations were the greatest in the manure injection band and the lowest in the no-manure (control) soils (Table 3). 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. ANOVA found that enterococci populations in soil after manure application in the 2010-2011 period (PSA) is significantly greater than in 2011-2012 period (PSB), which is expected due to the difference between the concentrations of enterococci in the applied manure. Additionally, there is a significant decrease in the manure band concentrations from the fall after manure application to the following spring in both years. 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 mean concentration across both years, are presented at the bottom of Table 3 and show that the enterococci concentration in the manure band is significantly greater than in the manure inter-band, but the mean inter-band enterococci concentration is not significantly greater than the no-manure control.

Table 3: Mean† enterococci (ENT, cfu/g) and tylosin-resistant enterococci (TYL, cfu/g) concentrations in soil in the first year after manure application. Standard deviations are 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 **y** (±58)	22 **y** (±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 comparing treatment (x, y, z) are not significantly different ($P \leq 0.1$).
‡ PSA plots, as shown in Table 1.
§ PSB plots, as shown in Table 1.
¶ Mean over both 2010 and 2011.

The long-term survival of enterococci in soil is shown in Table 4. Over the two years following manure application in 2010 (PSA), the enterococci concentration decreased in the manure band and reached concentrations equivalent to the no-manure and inter-band soils after two years. In the first year, enterococci concentrations in the band are statistically greater than the no-manure and the inter-band soils, but populations in the no-manure and inter-band soils were not statistically different. Enterococci in the manure band in 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 4. Mean† enterococci (ENT, cfu g^{-1}) and tylosin-resistant enterococci (TYL, cfu g^{-1}) concentrations in soil over 2-years after manure application in the fall of 2010. 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**
Treatment 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 comparing treatment (x, y, z) are not significantly different ($P \leq 0.1$).
‡ System A plots, as shown in Table 1.
NS-No Sample, this sampling time is not included in the calculation of the overall mean.

Tylosin-resistant enterococci concentrations in soil are also shown in Tables 3 and 4. Resistant enterococci were most frequently detected in the manure band soils, and rarely detected in the inter-band or control soils. 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 differ slightly from studies by Onan and LaPara (2003) and Halling-Sorenson et al. (2005) where 16% or less of culturable bacteria from soil with a manure history were macrolide-resistant. Hoang et al. (in press) reported total and tylosin-resistant enterococci in manured soil averaged 9.8×10^3 cfu g-^{1} soil and 7.5×10^3 cfu g^{-1} soil, respectively. The enterococci concentrations immediately after manure application in this study were slightly less, and two orders of magnitude less for tylosin-resistant enterococci.

There was no correlation ($r < 0.5$) between enterococci concentrations and drainage flow (data not shown) or time after manure application (Figure 1); therefore data were analyzed by analysis of variance for the effects of tillage, manure treatment, and year. There was no statistical difference in the concentration of enterococci in tile water due to manure application or study year (2010-11 compared to 2011-12) as shown in Figure 1. The second year of monitoring from PSA (data not shown) supported our first year findings that there

was no statistical difference due to tillage or manure treatment. Pappas et al. (2008) measured the concentration of enterococci, *E. coli* and fecal coliform in drainage water over 3 years under various swine manure treatments and manure-free control plots located in central Iowa. Concentrations of enterococci in tile water were similar from both manure-free and manure-amended soils.

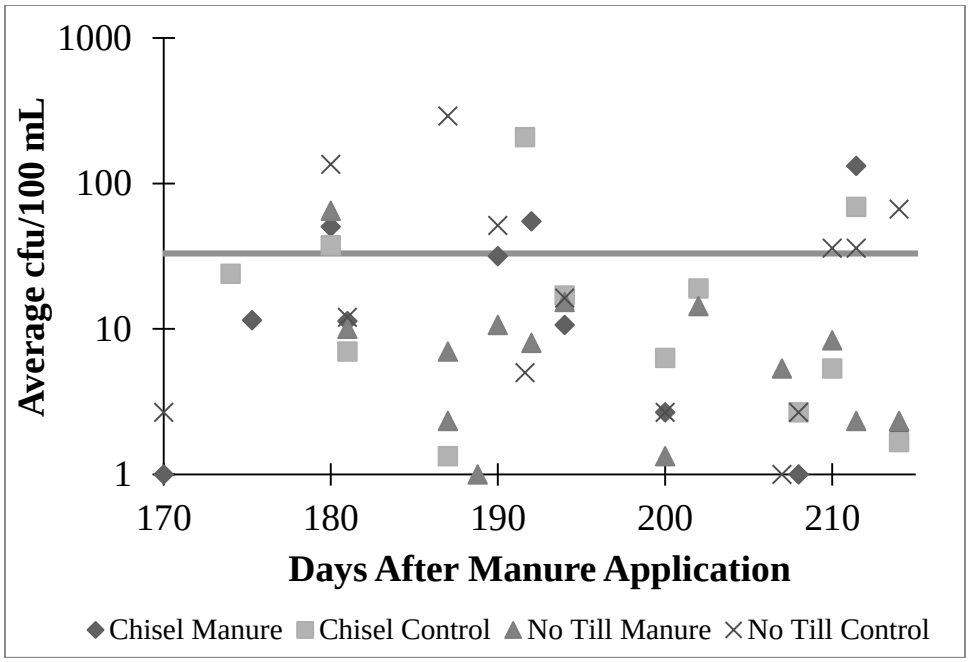

Figure 1: Enterococci in individual tile water samples in the first growing season after manure application in 2010 (PSA) and 2011 (PSB). The recreational water quality limit for *Enterococcus* (33 cfu 100-mL^{-1}) is shown for reference.

Tylosin-resistant enterococci 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 detected in 16% and 2% of the 86 tile water samples collected in 2011 and 2012 respectively. Only 5% of 46 samples collected from PSB in 2012 had detectible levels of tylosin-resistant enterococci.

erm genes in manure, soil, and tile drainage water

Quantitative PCR analysis was conducted on DNA extracted from manure, soil, and water samples for *erm*B, *erm*F and *erm*T. Average detection limits for *erm*B and *erm*F were 1.1 x 10^5 and 2.5 x 10^5 copies g^{-1} soil, respectively. For water samples, the average detection limits were 2.5 x 10^2 and 3.6 x 10^5 copies 100-mL^{-1} for *erm*B and *erm*F respectively. Detection limits are based on observations of non-specific amplification in control wells and were determined separately for each PCR run (Osbourne and Smith, 2009). *Erm*B and *erm*F were found in all three matrices, while *erm*T was not detected. 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 standard DNA spiked into in manure, soil and water ranged from 73 to 251%. There appeared to be no inhibition of PCR due to the sample matrix, as recovery of *erm* genes was greater than 100%.

In manure, the mean *erm*B concentrations were 8 x 10^8 copies g^{-1} in 2010 and 6 x 10^{12} copies g^{-1} in 2011 respectively. The mean *erm*F concentrations were 4 x 10^7 copies g^{-1} and 3 x 10^{12} copies g^{-1} in the first and second study years, respectively. *Erm*T was not detected in manure in either year. Previously reported concentrations of *erm*B, *erm*F, and *erm*T exceed 1 x 10^9 copies/g in liquid swine manure (Chen et al., 2007, Koike et al., 2010). Differences in the abundance of these genes among these studies may be due to the manure handling and storage or differences in farm tylosin administration practices.

In soil, the mean *erm*B concentrations were the greatest in the manure injection band followed by the inter-band and no-manure soil, in the first year after manure application (Table 5). Statistical analysis found that the effects of tillage on *erm*B abundance were not significant. The *erm*B levels in the 2011 manure band were slightly less than in the band in 2010. In the over-winter time period *erm*B abundances declined following both the 2010 and 2011 manure applications (Table 5). The decline in abundance of *erm*B in the manure band continued in the year after manure application and reached concentrations equivalent to concentrations in the inter-band and no-manure control soils by one year after manure application (Figure 2).

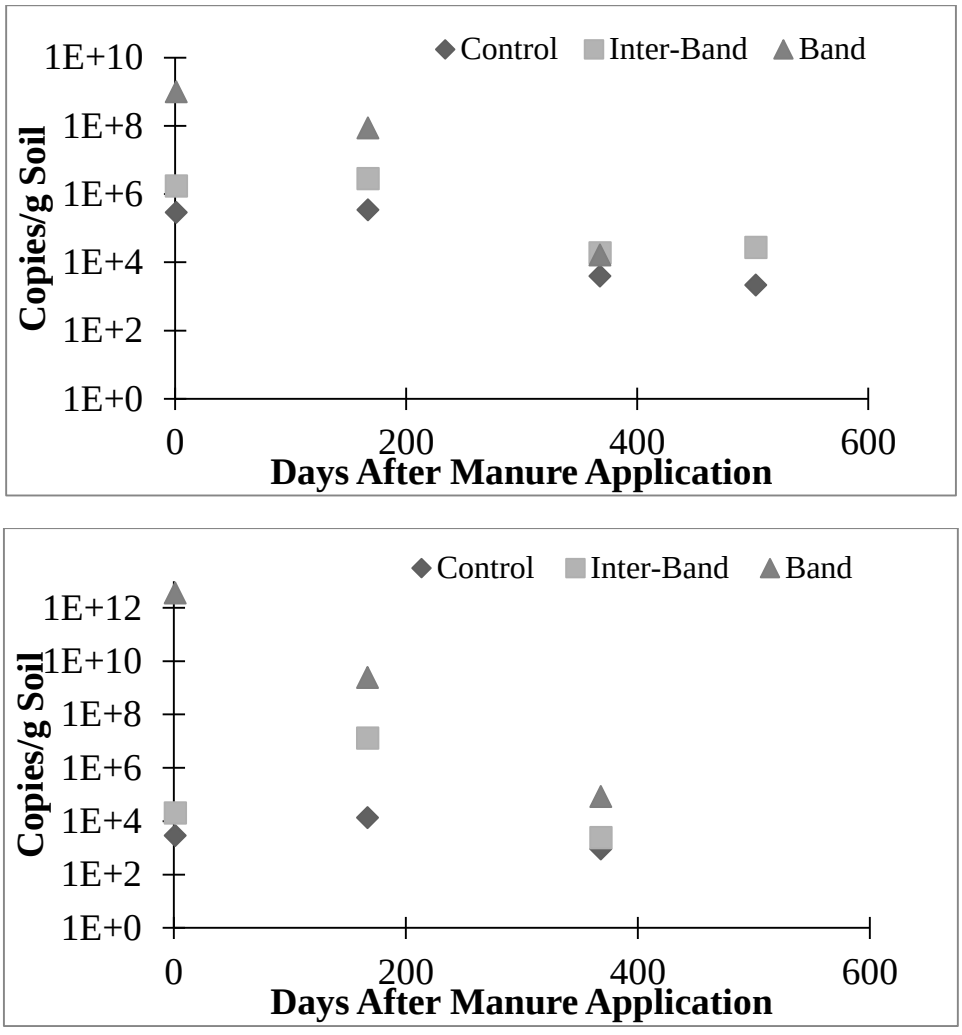

Figure 2: Persistence of *erm*B (top) and *erm*F (bottom) in soil over 2 years (PSA) after receiving swine manure in 2010. No manure band samples were collected in spring of 2012 (day 503) as the bands were no longer visible.

Table 5. Mean† *erm*B (copies g^{-1}) 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 1.
§ System B plots, as shown in Table 1.
¶ Mean over both 2010 and 2011.

Mean *erm*F concentrations in soil were also greatest in the manure injection band, with lower concentrations detected in the inter-bands, and the lowest concentrations in the non-manured soils (Table 6). Similar to *erm*B, statistical analysis found that the effects of tillage were not significant. The concentrations in the manure band in 2010 were significantly greater than the concentrations in the band in 2011. There were significantly ($p < 0.1$) lower *erm*F concentrations in the no-manure soil sampled in the fall and spring of both 2010 and 2011 than the inter-band soil (Table 6). However, 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 (Figure 2). In the spring of 2012, *erm*F concentrations were below the detection limit in all soils.

Table 6. Mean† *erm*F concentrations (copies g^{-1}) 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	4×10^{12} **a** ($\pm 2 \times 10^{12}$)	2×10^{4} **a** ($\pm 6 \times 10^{3}$)	3×10^{3} **a** ($\pm 3 \times 10^{1}$)
	Spring 2011	2×10^{9} **b** ($\pm 1 \times 10^{9}$)	1×10^{7} **b** ($\pm 9 \times 10^{6}$)	1×10^{4} **a** ($\pm 2 \times 10^{3}$)
	Annual Mean ‡	2×10^{12} **x** ($\pm 2 \times 10^{12}$)	7×10^{6} **y** ($\pm 1 \times 10^{7}$)	8×10^{3} **z** ($\pm 6 \times 10^{3}$)
2011§	Fall 2011	5×10^{8} **a** ($\pm 3 \times 10^{8}$)	5×10^{6} **a** ($\pm 58 \times 10^{6}$)	$< 7.0 \times 10^{3}$ **a**
	Spring 2012	4×10^{6} **a** ($\pm 3 \times 10^{6}$)	1×10^{5} **a** ($\pm 1 \times 10^{5}$)	$< 7.0 \times 10^{3}$ **a**
	Annual Mean §	2×10^{8} **x** ($\pm 3 \times 10^{8}$)	3×10^{6} **y** ($\pm 5 \times 10^{6}$)	$< 7.0 \times 10^{3}$ **z**
Treatment Means¶		9×10^{11} **x** ($\pm 1 \times 10^{12}$)	7×10^{6} **y** ($\pm 7 \times 10^{6}$)	6×10^{3} **z** ($\pm 6 \times 10^{3}$)

† 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.

The relative abundance of both *erm*B and *erm*F (Figure 3) in tile water was lower than in soil or manure. *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 9.0×10^{3} copies 100-mL^{-1}. *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 2.4×10^{5} copies 100-mL^{-1}. 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 due to tillage or manure treatment for each year. Figure 3 also shows that both *erm*B and *erm*F were found in drainage water from the control plots, which is consistrent with the detection of both *erm* genes in the non-manured soil. To date, no other published study has quantified *erm* genes in tile water. However, Bockelmann (2009) detected *erm*B in groundwater receiving artifial recharge and 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^{-1}, whereas nine samples of *erm*B were within the detection range of 40-4×10^{8} copies 100 mL^{-1} (Koike et al., 2010).

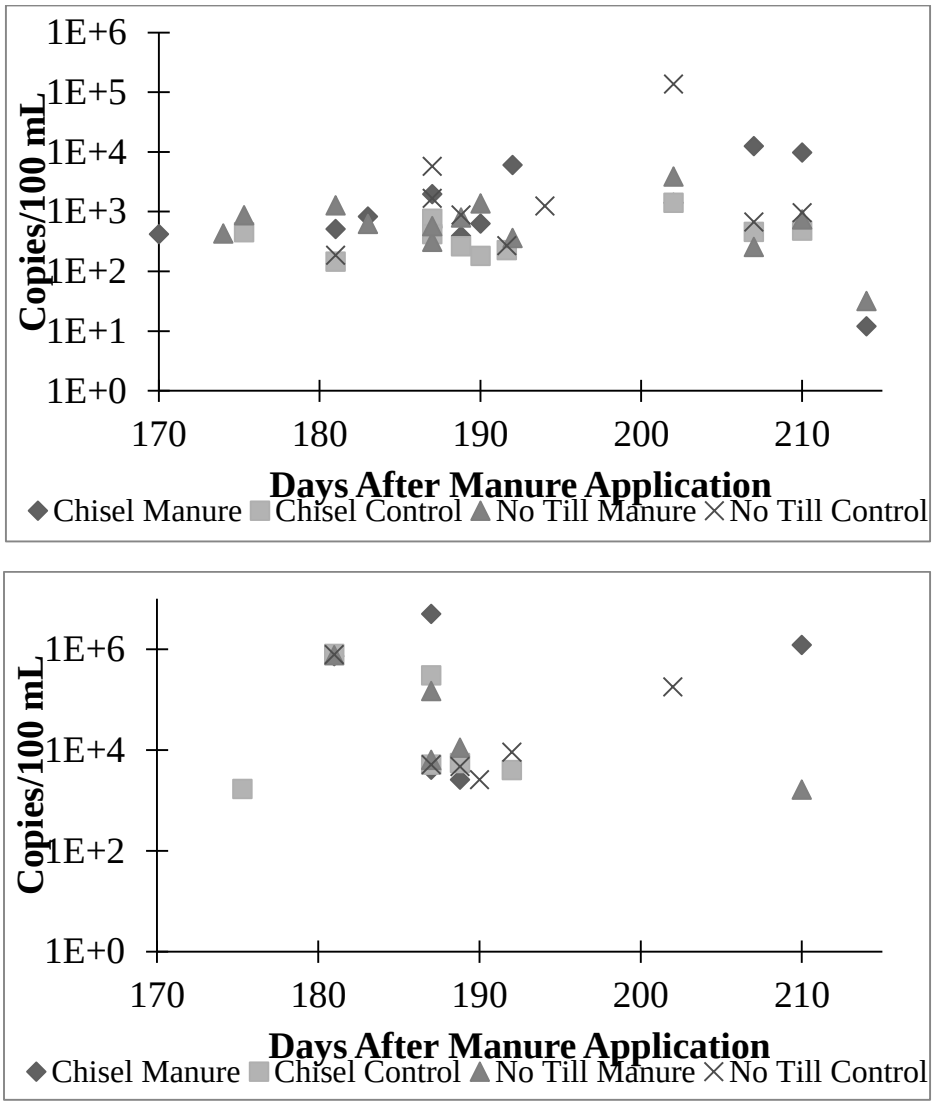

Figure 3. Abundance of *erm*B (top) and *erm*F (bottom) in tile water in 1st year after manure application for PSA and PSB.

DNA sequencing of qPCR products confirmed that *erm* genes were selectively amplified. The *erm*B product was 182-185 bp and the *erm*F fragment was 310 bp which correspond to the 191 bp *erm*B and and 309 bp *erm*F amplicons described previously (Chen, et al., 2007; Koike et al., 2007). All of the PCR products derived from standards and samples for *erm*B were successfully sequenced. However, only the PCR products from standards and the water samples produced consensus sequences for *erm*F. Matches to the consensus sequences were identified using Mega BLAST searches of the NCBI nucleotide database. Matches to *erm*B and *erm*F 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 non-specific matches were obtained.

Tylosin in manure, soil, and tile drainage water

Tylosin A was detected in manure applied to plots in both 2010 and 2011with an average tylosin concentration of 17±1.5 ng g^{-1} in 2010 and 128±19 ng g^{-1} in 2011. 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

each year with tylosin being administered 16 out of 20 weeks per turn; manure applied in 2010 might be from the beginning of a new cycle, which would have lower amounts of tylosin in the excreted manure. Dolliver and Gupta (2008) quantified tylosin at levels ranging from 0.4 to 4.9 μg g^{-1} in swine manure while Kolz et al. (2005) reported concentrations of tylosin B and D ranging from 50 to 1700 μg L^{-1} and 15 to 270 μg L^{-1}, respectively in swine lagoons. The concentrations found in the present study are significantly lower than in these previous studies. This might be due to the amounts of tylosin fed to swine or the length of manure storage. The rapid loss of tylosin in swine manure has been demonstrated (Teeter and Myerhoff, 2003; Kolz et al., 2005), which might also explain some of the differences in reported values.

In soil, tylosin concentrations were affected by the manure treatment and year. Mean concentrations (including the non-detects) for the manure band, inter-band and control soils for 2010 were 1.33, 0.22, and 0.09 ng g^{-1} respectively. There were no statistical differences 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, 0.34, and 0.37 ng g^{-1} 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, 0.79, and 0.57 ng g^{-1} 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 (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 x 10^3 to 50 x 10^3 μg g^{-1} (Halling-Sorensen et al., 2005).

The concentration of tylosin in tile water was less than 1 ng mL^{-1} (Table 7). In 2010, tylosin was detected frequently, but in 2011 tylosin was only detected once. The limit of detection in 2010 ranged from 0.016 ng mL^{-1} for the first 7 sampling times to 0.0096 ng mL^{-1} for the last 8. Except for one sample, only tylosin A was detected. For 2011, we chose to quantitate only tylosin A resulting in an improved detection limit of 0.0024 ng mL^{-1}. Concentrations of tylosin up to 1.2 ng mL^{-1} have been detected in tile flow (Dolliver and Gupta, 2008). Kay et al. (2004), however, were unable to detect tylosin in tile-drained clay soil at a quantification limit of 0.35 ng mL^{1}.

Table 7: Mean concentrations (ng mL^{-1}) of tylosin in tile water in the first year after manure application in 2010 and 2011.

		Chisel w/ Manure	Chisel Control	No Till w/ Manure	No Till Control
2010	Mean of detects†	0.20	0.24	0.03	0.04
	Mean of all data‡	0.15	0.21	0.01	0.02
2011	Mean of detects†	-	-	-	0.004
	Mean of all data‡	-	-	-	0.0004

† Mean concentration for samples above the detection limit
‡ Mean concentration for all samples, using ½ of the detection limit for those falling below the detection limit

Discussion:

Manure from tylosin-treated swine introduced environmentally significant levels of tylosin-resistant enterococci and *erm* genes into soil, while a smaller fraction of MLS-resistant bacteria were naturally present in soils that had not received manure in many years. Concentrations of tylosin-resistant enterococci, *erm*B and *erm*F concentrations in the manure band were significantly higher ($p < 0.1$) then the no-manure controls in the fall and spring samplings. The levels of tylosin-resistant enterococci, *erm*B and *erm*F decreased to those observed in the control plots after a complete year, suggesting that corn-soybean rotations with alternating years of swine manure application will not have increasing levels of anitibiotic-resistant bacteria. However, a continuous corn rotation receiving annual manure application might maintain high levels of resistance genes without the biennial decrease reported here. Zhou et al. (2010) reported transient (20 to 40 days) increases in MLS-resistant bacteria after swine manure application, but no increase in MLS resistance in field soils receiving

antibiotic-treated manure over controls (both no manure application or manure with no antimicrobial use). In contrast, Knapp et al. (2010) observed an increase in the ratio of *erm*/16S-rRNA genes over time in soils sampled over multiple decades since the 1940's. The elevated levels of *erm*F genes in the inter-band samples compared to the *erm*F in soils without manure may result from redistribution of resistant microorganisms in soil after manure application or longer-term changes in the microbial community due to repeated manure application. The long-term history of antibiotic use in the facility that supplies the manure is not available presently.

The *erm* gene abundance in manure-treated soil declines over time, suggesting that some of the microorganisms carrying the *erm* genes are not adapted to long-term survival in the soil environment. Our post-PCR product sequencing and the findings of other researchers suggest that gram-positive bacteria including, *Enterococcus*, *Staphylococcus*, *Streptococcus*, *Lactobacillus*, and *Clostridium* species are the principal hosts of *erm* genes (Zhou et al., 2009; Koike et al., 2010; Park et al, 2010). These bacteria are found in both soil and manure, but individual species and strains indigenous to manure may not be adapted to soil. *Enterococcus* is considered a fecal indicator bacterium and populations in soil also declined in a pattern similar to the *erm* genes, but the abundance of tylosin-resistant *Enterococcus* spp. never exceeded 10^3 cells g^{-1} soil.

Precipitation during the two year study was below normal. The 10-year rainfall average during the first 6 months of the year is 37.4 cm; in 2011 it was 30.8 cm and in 2012 it was 21.2 cm (Iowa State University Department of Agronomy, 2012). The transport of bacteria (and potentially *erm* genes and tylosin) in tile drainage water might be less than that expected during a year under normal flow. Furthermore, the grab sampling scheme may have under estimated transport during storm events since the greatest concentrations are often observed in the rising limb or the peak of the hydrograph (Cullum, 2009), which were likely missed. Therefore, the concentrations reported in his study potentially underestimated 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 more bacteria would have been transported to the tile lines by macropore flow in the no-till plots over chisel plow plots (Cullum, 2009; Ramirez et al., 2009). However, macropore flow would require nearly saturated soil water content. Reduced macropore flow may have contributed to the lack of differences in concentations of ARB and ARG in tile water with respect to manure or tillage treatments.

Although tylosin was detected in manure, soil and tile drainage water, the concentrations are small. This is in general agreement with previous research indicating that tylosin has little risk of accumulation in soil or groundwater after manure application (Kay et al. 2005, Blackwell et al., 2007; Blackwell et al., 2009). 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. Tylosin concentrations are very low in the soil and water, and do not likely impact the selective pressures on the microbial community. For instance, Portillo et al. (2000) reported tylosin minimum inhibitory concentrations (MIC) from 0.125-128 µg ml^{-1} for 78 *Enterococcus* isolates, which are well above tylosin concentrations in soil or tile water. However, the diversity of the soil microbial community and the potential for selection for resistance genes at sub-MIC concentrations suggest that caution is needed in assessing the possible impacts of tylosin residues in soil on abundance of resistance genes (Heuer et al., 2011; Andersson and Hughes, 2012).

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