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 #14-015**

revised

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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 macrolide antibiotics, 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, *erm*C, 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 observed 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 during the dry to average years of precipitation (2011 and 2012), but in 2013 and 2014 when above average precipitation occurred significantly higher concentrations of *erm*B genes were observed in tile drainage from the manure amended plots. However, concentrations of *erm* genes observed in 2013 and 2014 are similar to levels observed in our previous years of study. A significant reduction of *erm* genes in drainage water samples was observed in the second year of the corn-soybean two year rotation. However, the increasing prevalence of crop rotations with two or more years of corn and manure indicates the need to monitor corn-corn rotations that receive manure application yearly.

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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, macrolide resistant genes (*ermB*, *ermC*, *ermF*, *ermT*, and *msrA*), which confer resistance to tylosin, and other macrolide antibiotics. 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 from 2011-2014. In manure enterococci and tylosin resistant enterococci concentrations ranged from $>10^3$ to $>10^5$ CFUg^{-1} manure and tylosin resistant fractions of total enterococci ranged from 70%-100%. In drainage water enterococci levels were highly variable in all four plots and no significant differences ($p>0.10$) in enterococci concentrations were detected between tillage practices or manure treatment. Tylosin-resistant enterococci were rarely detected and concentrations were not significantly different ($p>0.10$) between manure or tillage treatments. High concentrations of *ermB*, *ermC*, and *ermF* were found in manure. Averaged over four years, *ermB* concentrations were greatest (1.51×10^{12} copies g^{-1} manure) followed by *ermF* (7.54×10^{11} copies g^{-1} manure) and *ermC (*4.54×10^8 copies g^{-1} manure), respectively. The highest soil concentrations for *ermB*, *ermC*, and *ermF* were detected in manure bands immediately following manure application; *msrA* and *ermT* were not found in quantities above the specified LOD. Gene concentrations in soils collected from the interband location of manured plots and control plots immediately after manure application were below detection limits for each *erm* gene. Gene concentrations in both the chisel plow and no-till soil bands the following spring were approximately an order of magnitude lower than the previous fall. *ErmB* was detected in 75% of soil samples from manure treated plots in the second year after manure application. *ErmF* was only detected in one soil sample in the second year of the crop rotation, while *ermC* was not detected. Similarly, *ermB*, *ermC*, and *ermF* were detected in tile drainage samples, while *msrA* and *ermT* were not above the limit of detection. Concentrations of *ermB* in positive samples were consistent over the four years of drainage sampling (6.36×10^2 to 5.17×10^4 copies 100 ml^{-1}), however, manured plot samples contained a significantly higher percentage of *ermB* positive samples ($p<0.10$) only in 2013 and 2014. While the first two years of study found no effect on tile drainage water quality in years of below average precipitation, higher levels of precipitation in 2013 and 2014 resulted in significant differences in resistance gene concentrations in agricultural drainage water from plots receiving manure application when compared to no-manure controls.

Introduction:

Antimicrobials are used in the swine industry at therapeutic levels for prevention, control, and treatment of disease and at sub-therapeutic levels to 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 in 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. (2013) 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 from 2011-2014.

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 in the fall 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 (2011), only 4 plots were sampled (hereafter referred to as plot system A, or PSA). In the second and third 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, 2012 Fall inject swine manure at 168 kg N ha^{-1}
24†	Chisel plow	Spring preplant spoke inject UAN at 168 kg N ha^{-1}
25†	No-till	2010, 2012 Fall inject swine manure at 168 kg N ha^{-1}
34†	No-till	Spring preplant spoke inject UAN at 168 kg N ha^{-1} with Cover Crop
29‡	Chisel plow	Spring preplant spoke inject UAN at 168 kg N ha^{-1}
30‡	Chisel plow	2011, 2013 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, 2013 Fall inject swine manure at 168 kg N ha^{-1}

† Plots (PSA) which received manure application in 2010 and 2012.
‡ Plots (PSB) which received manure application in 2011 and 2013.

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 each fall. 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 analysis protocol as in the initial sampling. The manure bands were flagged in the fall to allow accurate repeat sampling.

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.

Additional water samples were gathered from the South Fork of the Iowa River at sampling sites described by Tomer et al. (2008). The watershed is largely agricultural, tile-drained and with over 100 confined animal feeding operations in the 193,000 acre watershed. These samples were analyzed for tylosin-resistance genes and tylosin using the methods described subsequently.

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, *erm*C, and *msr*A. 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. Due 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).

Quantitative PCR was performed on a MJ Research Opticon2 qPCR instrument operated in the 96-well format. Each gene was analyzed separately. Each individual reaction had cumulative volume of 25 μL, consisting of: 2.5 μL of DNA, 5 μL each of forward and reverse primer and 12.5 μL of Qiagen SYBR Green Master Mix. Conditions and primer sequencesare summarized in Table 2. Additionally, the molarities of each primer used in reactions were optimized by combining forward and reverse primers at various concentrations. Quantitative PCR standards were created by inserting amplified qPCR product into pCR-4TOPO in *E coli* using TOPO TA cloning kits (Invitrogen Corp., Carlsbad, CA). DNA from transformed *E coli* was extracted using a 5 Prime FastPasmid Mini Kit. *ErmB* and *ermC* product were derived from *Enterococcus* isolate Man T1-C, described by Hoang et al. (2010). *ErmF* product originated from a reference *E coli* strain purchased from M. C. Roberts's lab (University of Washington). *MsrA* product originated from plasmid pAT10 inside *S. aureus* strain RN4220, which was also purchased from M. C. Roberts's lab. Blanks and negative controls were included in each qPCR assay. Negative controls consisted of PCR grade water and *Pseudomonas stutzeri* genomic DNA (ATCC 14405).

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

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

ErmC-FW *ErmC*-RV	*erm*C	AATCGTGGAATACGGGTTTGC CGTCAATTCCTGCATGTTTTAAGG	293	51.4	Koike et al. 2010
MsrA-F *MsrA-rev*	*msrA*	GCAAATGGTGTAGGTAAGACAACT ATCATGTGATGTAAACAAAAT	399	54	Sutcliffe et al. 1996

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}.

Horizontal Resistance Gene Transfer

Water samples were collected from directly from tile discharge or streams in watersheds containing large numbers of swine confinements. The 250 mL water sample was filtered using a 0.45 μm filter within 24 hours. Following water filtration, 0.5 mL of recipient *Enterococcus faecalis* strain JH2-2 (resistant to rifampicin and fusidic acid) grown to log phase was passed through the same filter. The filter was placed on BHI agar and incubated for 24 hours. Following incubation, filters were placed in 15 mL centrifuge tube with 10 mL of PBS and vortexed to suspend cells. Next, 50 μL aliquots were spread on mE agar containing 25 μg mL^{-1} of fusidic acid, 50 μg mL^{-1} of rifampicin and either 16 μg mL^{-1} of tetracycline or 35 μg mL^{-1} of tylosin and incubated for 48 hours. JH2-2 colonies containing newly acquired resistance genes were isolated and streaked for purity. DNA from the isolates will be extracted and sent for sequencing analysis with the aim of identifying the resistance gene transferred and the mechanism of transfer.

Statistical Tests and Analysis

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

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

Statistical analysis was performed with JMP®, Version 10.0.2. (SAS Institute Inc., Cary, NC, 1989-2007). Water samples analyzed for resistance genes below the specified LOQ and above the LOD were assigned the average of the LOQ and LOD for statistical analysis. Additionally, samples below the LOD were assigned a value of zero for analysis. The non-parametric Wilcoxon ranked sum test was used to determine if resistance gene concentrations in tile drainage from different plots were significantly different. Wilcoxon ranked sum test was also performed on enterococci concentrations present in tile drainage

Results:

Enterococci in manure, soil, and tile drainage water

Enterococci and tylosin resistant enterococci concentrations in manure ranged from $>10^3$ to $>10^5$ $CFUg^{-1}$ manure (Table 3). Tylosin resistant fractions of total enterococci ranged from 70%-100%.

Table 3: Enterococci and tylosin resistant enterococci concentrations in swine manure

Indicator Organism (CFU/g)	Year			
	2010	2011	2012	2013
Enterococci	5.66×10^4	8.64×10^3	1.76×10^5	7.61×10^4
Tylosin Resistant Enterococci	3.97×10^4	7.22×10^3	1.46×10^5	7.84×10^4

Enterococci concentrations were greatest in soil samples collected from the band location immediately following manure application. The average enterococci concentrations in the band locations for manured plots decreased to background concentrations as defined by the concentrations in the control plots by the time samples were collected the following spring (Table 4). Band locations were unidentifiable during the second year of the crop rotation. Concentrations of enterococci in soil in the second year after manure application were similar to levels in the control plots. Tylosin-resistant enterococci in the manure band immediately following application were detected at similar concentrations to total enterococci concentrations. The resistant enterococci levels dropped two orders of magnitude in band samples collected the following spring. No tylosin resistant enterococci were detected in interband or control plot samples in the year following manure application or in any of the soil samples collected during the second year of the crop rotation.

Table 4: Enterococci and tylosin resistant enterococci concentrations in soil manure band and interband locations and no-manure control plots under no-till and chisel plow tillage for PSA over a two year period.

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

*Manure bands were no longer visible one year after manure application and could not be re-sampled

Enterococci levels in drainage water were highly variable in all four plots (Table 5). No significant differences ($p>0.10$) in enterococci concentrations were detected between tillage practices or manure application using the Wilcoxon Ranked Sum Test. Enterococci were frequently detected in drainage samples from all four plots. There was no correlation between time after application or instantaneous flow rate (data not shown) and enterococci concentrations ($r<0.50$). Tylosin-resistant enterococci were rarely detected and concentrations were not significantly different ($p>0.10$) between manure or tillage treatments using Wilcoxon Ranked Sum Test (Table 3). Average tylosin resistant concentrations in drainage water rarely exceeded >1 CFU/100 mL (Table 6).

Table 5: Detection frequency (%) of enterococci and tylosin resistant enterococci in drainage water during the first year after manure application.

Indicator	Plot Type	Year			
		2011	2012	2013	2014
		——— (%) ———			
Enterococci	No Till Control	62	78	73	100
	No Till Manure	93	88	63	100
	Chisel Plow Control	93	80	60	100
	Chisel Plow Manure	93	91	93	93
Tylosin Resistant Enterococci	No Till Control	8	9	7	10
	No Till Manure	0	13	13	15
	Chisel Plow Control	21	0	0	11
	Chisel Plow Manure	29	0	7	40

Table 6: Average concentrations of enterococci and tylosin resistant enterococci in drainage water during the first year after manure application.

Indicator	Plot Type	Year			
		2011	2012	2013	2014
		—— (CFU/100 mL) ——			
Enterococci	No Till Control	45	52	12	40
	No Till Manure	36	5	13	25
	Chisel Plow Control	99	7	8	9
	Chisel Plow Manure	385	6	15	54
Tylosin Resistant Enterococci	No Till Control	<1	<1	<1	8
	No Till Manure	ND[a]	<1	<1	<1
	Chisel Plow Control	28	ND	ND	<1
	Chisel Plow Manure	<1	ND	16	<1

[a]No enterococci were detected.

Antibiotic resistance genes in manure, soil, and tile drainage water

High concentrations of *ermB*, *ermC*, and *ermF* were found in manure (Table 7). Averaged over four years, *ermB* concentrations were greatest, followed by *ermF* and *ermC,* respectively. *ErmF* results contained the greatest amount of variability, spanning five orders of magnitude over the four years. *MsrA* and *ermT* were not detected in manure samples. Concentrations of antibiotic resistance genes are significantly greater than the population of antibiotic-resistant enterococci in manure. The concentrations of *ermB* and *erm F* are similar to those found in liquid swine manure in North Carolina (Chen et al., 2010).

Table 7: *Erm* gene concentrations in swine manure.

Resistance Gene	Year			
	2010	2011	2012	2013
	gene copies g^{-1} manure			
ermB	8.00×10^8	6.0×10^{12}	7.29×10^9	1.52×10^{10}
ermC	*	*	2.44×10^7	8.83×10^8
ermF	4.0×10^7	3.0×10^{12}	1.26×10^8	1.53×10^{10}

**ErmC* was first analyzed in 2012

Table 8: *Erm* gene concentrations in soil following manure application in plots under no-till and chisel plow management for PSA over a two year period.

Gene	Treatment	Location	Fall 2012	Spring 2013	Fall 2013	Spring 2014
			(gene copies g-1 soil)			
ermB	No Till Manure	Band	5.46×10^7	2.66×10^5	<LOD*	1.59×10^5*
		Interband	<LOQ[a]	<LOD[b]		
	No Till Control	Composite	<LOD	6.61×10^4	<LOD	<LOD
	Chisel Plow Manure	Band	1.73×10^6	5.77×10^5	2.45×10^4	4.18×10^4
		Interband	<LOD	<LOD		
	Chisel Plow Control	Composite	<LOD	<LOD	2.42×10^4	<LOD
ermC	No Till Manure	Band	1.53×10^6	3.24×10^5	<LOD	<LOD
		Interband	<LOD	<LOD		
	No Till Control	Composite	<LOD	<LOD	<LOD	<LOD
	Chisel Plow Manure	Band	<LOD	5.77E+05	<LOD	<LOD
		Interband	<LOD	<LOD		
	Chisel Plow Control	Composite	<LOD	2.88E+05	<LOD	<LOD
ermF	No Till Manure	Band	2.58×10^6	2.28×10^5	<LOD	5.16×10^4
		Interband	<LOD	<LOD		
	No Till Control	Composite	<LOD	6.14×10^4	<LOD	<LOD
	Chisel Plow Manure	Band	1.29×10^7	8.75×10^4	<LOD	<LOD
		Interband	<LOD	<LOD		
	Chisel Plow Control	Composite	<LOD	<LOD	<LOD	<LOD

*Manure band were no longer present one year after application
[a] below limit of quantification
[b] below limit of detection

The highest soil concentrations for *ermB*, *ermC*, and *ermF* were detected in manure bands immediately following manure application; *msrA* and *ermT* were not found in quantities above the specified LOD (Table 8). Each gene exceeded 10^6 copies g^{-1} soil in manure bands (Table 8), except for *ermC* in the chisel plowed plot. Gene concentrations in soils collected from the interband location of manured plots and control plots immediately after manure application were below detection limits for each *erm* gene. Gene concentrations in both the chisel plow and no-till soil bands the following spring were approximately an order of magnitude lower than the previous fall. *ErmB* was detected in 75% of soil samples from manure treated plots in the second year after manure application. *ErmF* was only detected in one soil sample in the second year of the crop rotation, while *ermC* was not detected.

Quantitative PCR detected *ermB*, *ermC*, and *ermF* in tile drainage grab samples, while levels of *msrA* and *ermT* were not above the limit of detection (Table 9). Nine to 17 drainage samples were collected from each plot each year. Samples were collected during or immediately following rainfall events and weekly until drainage ceased. The quantities of samples from each plot depended on the length of the drainage season. *ErmB* was most frequently detected in drainage samples in 2011 and 2012 (Table 9). Concentrations of *ermB* in positive samples were consistent over the four years of drainage sampling (Table 10), however, manured plot samples contained a significantly higher percentage of *ermB* positive samples ($p<0.10$) only in 2013 and 2014. Mean concentrations in drainage samples above the limit quantification were of greatest in *ermF*, followed by *ermC* and *ermB*,

respectively (Table 10). *ErmF* was detected at significantly higher concentrations in manured plot samples than non manured control plot samples in 2013. *ErmC* concentrations in drainage grab samples were not significantly higher in manured plot samples in 2013 or 2014 (Table 9). *ErmC* was only detected in one sample from each manured plot in 2014 drainage. No significant differences in the three genes were observed between chisel plow and no-till regimes.

Table 9: Frequency of *erm* gene detection (%) in drainage water during the first year after manure application.

Gene	Treatment	Year			
		2011	2012	2013	2014
		——— (%) ———			
ermB	No Till Control	100	100	13	0
	No Till Manure	91	100	58	25
	Chisel Plow Control	91	100	6	10
	Chisel Plow Manure	100	100	27	31
ermC	No Till Control	*	*	31	0
	No Till Manure	*	*	29	8
	Chisel Plow Control	*	*	25	0
	Chisel Plow Manure	*	*	27	8
ermF	No Till Control	27	47	0	11
	No Till Manure	45	54	38	25
	Chisel Plow Control	27	47	0	10
	Chisel Plow Manure	36	50	6	23

**ErmC* was first analyzed in 2012

Table 10: Average *erm* gene concentrations (excluding non-detects) in drainage water during the first year after manure application.

Gene	Treatment	Year			
		2011	2012	2013	2104
		—— (gene copies/100 mL) ——			
ermB	No Till Control	1.57×10^4	1.08×10^4	3.17×10^3	ND[a]
	No Till Manure	1.58×10^3	1.26×10^3	4.67×10^3	1.45×10^4
	Chisel Plow Control	2.28×10^3	1.62×10^3	6.36×10^2	8.32×10^3
	Chisel Plow Manure	3.94×10^3	2.10×10^4	3.94×10^3	5.17×10^4
ermC	No Till Control	*	*	6.35×10^4	ND
	No Till Manure	*	*	1.79×10^4	5.21×10^4
	Chisel Plow Control	*	*	1.36×10^4	ND
	Chisel Plow Manure	*	*	9.71×10^4	1.86×10^5
ermF	No Till Control	3.35×10^5	1.46×10^5	ND	1.68×10^4
	No Till Manure	2.28×10^5	1.65×10^5	1.81×10^3	9.67×10^4
	Chisel Plow Control	3.75×10^5	1.63×10^5	ND	1.12×10^4
	Chisel Plow Manure	1.75×10^6	1.17×10^6	1.23×10^3	1.94×10^5

*_ErmC_ was first analyzed in 2012
[a]Not detected

The number of drainage water samples containing *ermB, ermC and ermF* resistance gene concentrations above limits of quantification greatly decreased during the second year of drainage after manure application (Figure 1a,1b,1c). *ErmB* and *ermC* were not detected in drainage water during the second year after manure . *ErmF* was detected infrequently in the second year after application. Manured plot *ermF* concentrations in the second year of drainage were not significantly different from control plot concentrations ($p>0.10$).

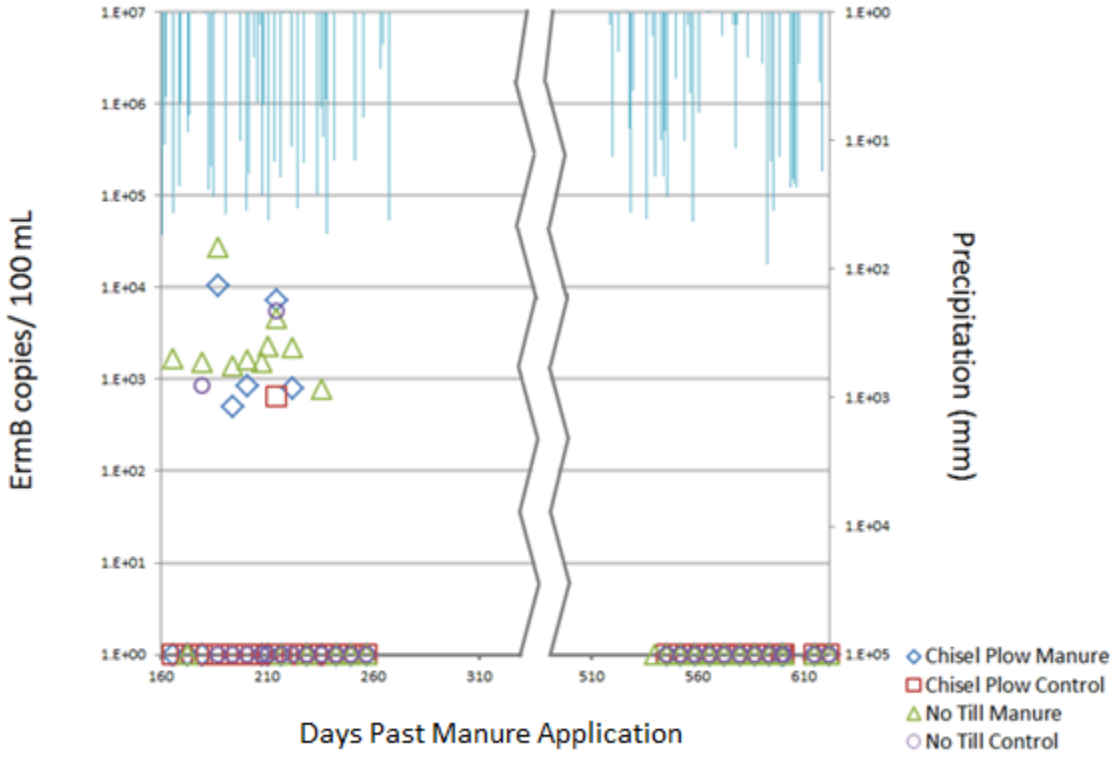

Figure 1a: *ErmB* concentrations in tile drainage following manure application in plots under no-till and chisel plow regimes

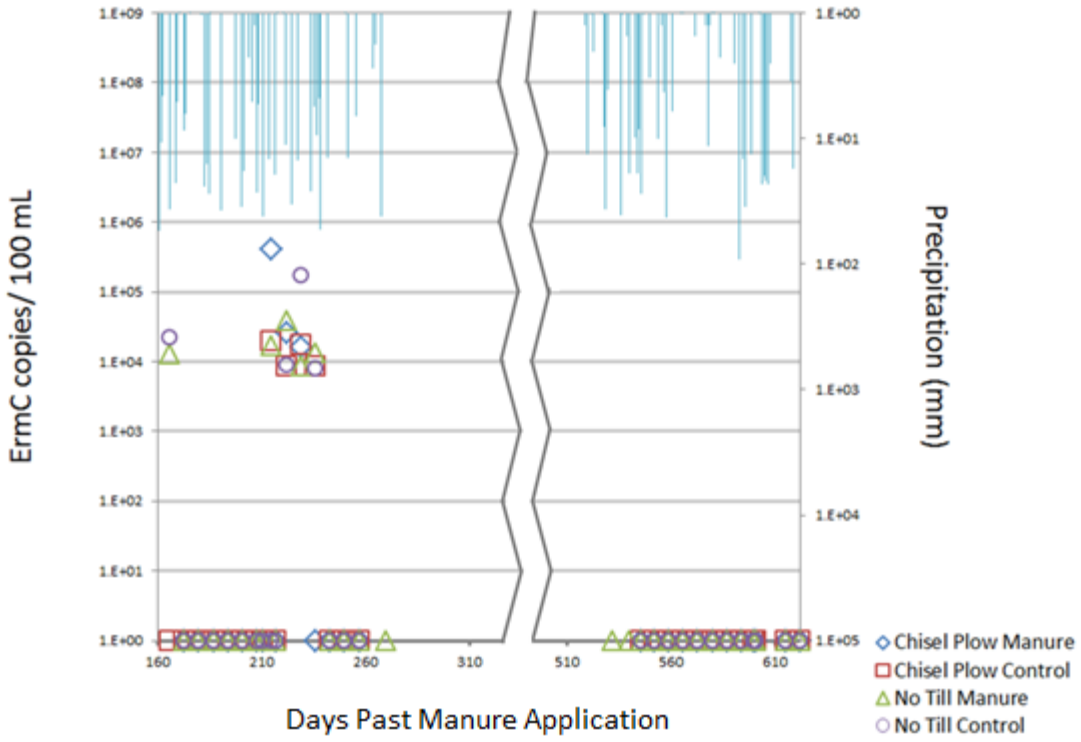

Figure 1b: *ErmC* concentrations in tile drainage following manure application in plots under no-till and chisel plow regimes.

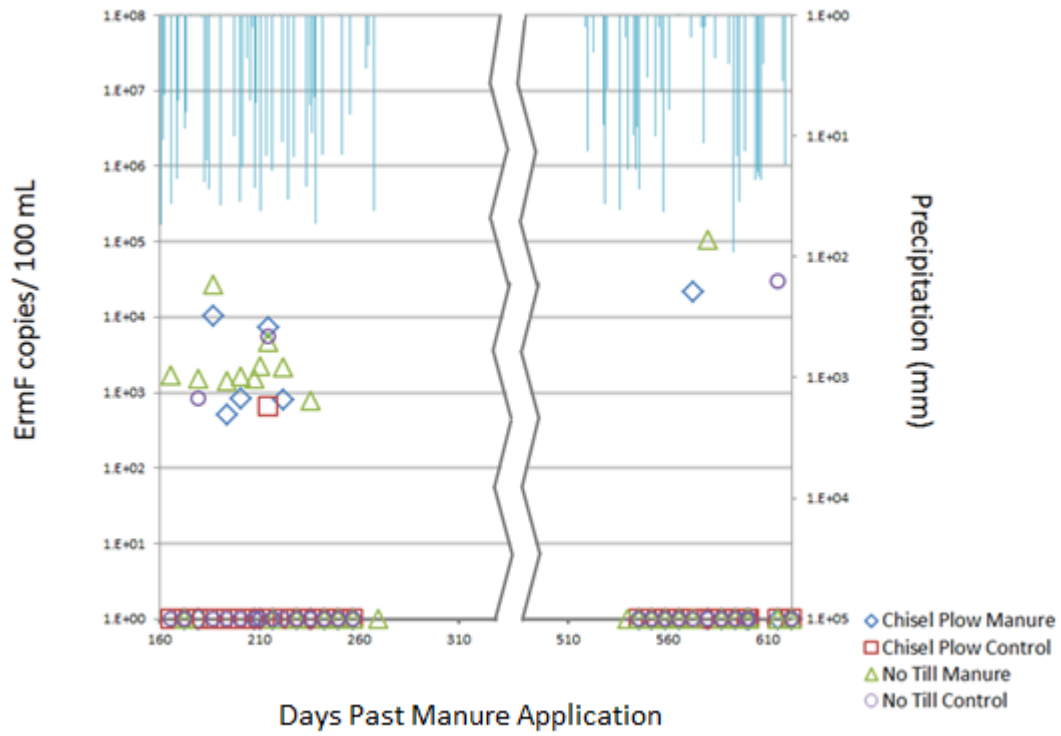

Figure 1c: *ErmF* concentrations in tile drainage following manure application in plots under no-till and chisel plow regimes

Horizontal Gene Transfer

Preliminary results from conjugation assays phenotypically confirm the transfer of tylosin and tetracycline resistance genes from environmental water samples to JH2-2. Transconjugant isolates have shown the ability to retain resistance genes through regrowth in mediums containing the aforementioned concentrations of antibiotics. Upon completion of sequencing analysis, the specific genes responsible for the newly found resistance will be identified. Additionally gene cassettes responsible for the transfer of resistance will be identified.

South Fork Watershed Monitoring

Tylosin concentrations (Table 11) and the resistance genes *ermB* and *ermF* were also detected in tile drain and surface water samples collected from the South Fork of the Iowa River. For the IASF-400 and IASF-450 sites, *ermF* was detected in 59% of the 66 samples (2011-2014) and the detection frequency was 83% for *ermB*. The median gene copy concentrations (excluding non-detects) were 1.15×10^4 100 mL^{-1} water (*ermF*) and 2.12×10^3 100 mL^{-1} (*ermB*). The maximum concentrations were 4.67×10^5 (*ermF*) and 1.83×10^5 100 mL^{-1} (*ermB*). The samples at these two sites were surface water, but the watershed discharge is dominated by tile drainage (Tomer et al., 2008). For IATC-241 and IATC-242, which are large tile drains, similar results were obtained with respect to detection frequency and median *erm* gene concentrations, but the maximum gene concentrations were larger: 7.06×10^6 (*ermF*) and 2.89×10^7 gene copies 100 mL^{-1} (*ermB*). These results indicate that our plot-scale information obtained under controlled conditions at Nashua are relevant and similar to results obtained in a watershed with extensive swine production and tile drainage. Tylosin concentrations are lower than those reported from the manure amended plots at Nashua reported by Garder et al. (2014) which ranged from 0.15 to 0.24 ng mL^{-1}. These concentrations are similar to the concentrations found in drainage from the tile-drained plots at Nashua. Although, we have previously estimated that 66% of the land within the watershed receives manure (primarily swine manure) the potential contributions of overland flow and wildlife sources to the *erm* gene concentrations in the stream complicate the interpretation of these data.

Table 11. Tylosin A concentrations at two tile drain and two surface water monitoring locations in the South Fork Watershed.

Site	**IATC-241[a]**	**IATC-242[a]**	**IATC-323[b]**	**IASF-450[b]**	**IABC-350[b]**
		——————— (ng mL^{-1}) ———————			
2013	0.0089	0.0057	0.0155	0.0129	0.0135
2014	0.0004	0.0007	0.0006	0.0023	0.0026

[a]Tile drain
[b]Surface water

Discussion

We monitored *Enterocooccus* sp (enterococci) and several genes that confer resistance to tylosin in manure, in soil treated with swine manure and in drainage water leaving the plots treated with swine manure. Additionally, these constituents were monitored on parallel production systems without swine manure (controls).

Enterococci concentrations present in liquid swine manure were similar to levels reported previously as were enterococci resistant to tylosin in swine manure (65-100% of total enterococci) (Trang et al. 2013, Onan et al. 2003). The fractions of tylosin resistant enterococci from the soil manure bands immediately following application (93%-100%) were also comparable to prior studies which have reported tylosin resistant enterococci ranging from 5%-100% (Halling-Sorensen et al. 2005, Onan et al. 2003). Concentrations of total and tylosin

resistant enterococci in manure bands following application were comparable during the first two years of the study to those reported by Hoang et al. (2013), but were an order of magnitude lower during the final two years of sampling. Additionally, enterococci concentrations in the manure application bands dropped to levels similar to background concentrations either by the following spring or next fall. Enterococci concentrations in tile drainage samples were not significantly different ($p>0.10$) across tillage or manure treatments. Furthermore, enterococci concentrations were not correlated with time after application or instantaneous flow rates.

Soil concentrations of *ermB*, *ermC* and *ermF* were greatest in the manure application bands immediately after manure application. The elevated concentrations of resistance genes was sometimes present in the spring of the following year but elevated concentrations (relative to the controls) of resistance genes did not persist into the second year after manure application. Concentrations of *ermB* and *ermF* were both at least two orders of magnitude lower in 2013 and 2014 than concentrations reported previously in 2011 and 2012. Additionally, *ermB* was only identified in one interband sample in the first year of the crop rotation in spring of 2013, while *ermB* and *ermF* were detected at quantifiable levels in every interband sample collected at the study site in the two previous years (fall 2010 – spring 2012). Factors that may contribute to this variability include the length of manure storage prior to application, and environmental conditions at the time of manure application and subsequent to application.

ErmB was detected in the majority of the drainage samples in 2011 and 2012, with no significant differences ($p>0.10$) between manured and control plots (Garder et al. 2014). However, when data for both tillage treatments were combined for statistical analysis, *ermB* concentrations in tile drainage were significantly greater ($P<0.10$) in plots with manure application than their control plot counterparts in 2013 and 2014. Mean yearly concentrations for *ermB* positive samples were consistent over the four year sampling period.

ErmF was most frequently detected in drainage samples in 2011 and 2012. No significant differences between tillage practices or manure application were observed during these years ($p>0.10$). Concentrations of *ermF* in drainage samples from 2013 and 2014 were on average one to two orders of magnitude lower than the two previous years. After combining data for chisel plow and no-till treatments, *ermF* concentrations were found to be significantly greater in manured plot drainage water in 2013 compared to the no-manure control.

Precipitation totals for April through June in 2011 and 2012 at the study site were 31.6 cm and 26.4 cm, respectively. These totals were nearly doubled in 2013 and 2014, with 51.9 cm and 62.4 cm, respectively. Above average precipitation in spring of 2013 may have created additional opportunities for bacteria harboring resistance genes to be transported from soil to drainage water. The majority of drainage samples from plots receiving manure application which contained concentrations of *ermB* and *ermF* above detection limits in 2013 were collected in the first half of the sampling season or during rainfall events. The elevated concentrations of *ermB* and *ermF* in those water samples support the notion that the resistance genes found in tile drainage stemmed from bacteria in the manure application.

ErmC had the highest concentrations of the three genes in 2013, but detection frequency and concentrations of *ermC* were comparable across all treatments. Additionally, the majority of the positive samples were from later in the sampling season, as opposed to *ermB* and *ermF*, which were mainly detected during the first portion. Hoang et al. (2013), using PCR, only detected *ermC* in 9% of enterococci isolates which were phenotypically resistant to tylosin. Phylogenic analysis performed on resistance genes by Koike et al. (2009) concluded that RNA methylases can be organized into two major clusters: bacteria containing high-G + C contents, such as streptomyces, and bacteria containing low-G + C contents, which include commensal, pathogenic and environmental bacteria and *ermC* was present in the low G+C cluster. *ErmC* was not detected by Li et al. (2013) in water or soil samples collected from wastewater trenches exporting waste from a swine farm. *ErmC* concentrations in 2013 water samples from this study are likely from naturally occurring bacterial communities in soil, due to similar concentrations in manured and control plot drainage and the majority of quantifiable concentrations occurring towards the end of the tile drainage period. *ErmC* was only detected in one sample from each manured plot in 2014.

MsrA was not detected in any samples, including manure; however, Hoang et al. (2013) detected *msrA* in 97% of tylosin resistant enterococci isolated from manure, soil and water samples. While *erm* genes confer resistance by target site modification, *msrA* is responsible for encoding a transport protein containing two ATP-

binding domains. The ATP-binding domains are part of an efflux system which works to translocate macrolides across cell membranes (Ross et al. 1995). This mode of resistance may be less prevalent in the environment due to the transport systems having to utilize energy to export the antibiotic across the membrane. Although Hoang et al. (2013) identified *msrA* in nearly 100% of enterococci isolates phenotypically resistant to tylosin, the proportion of extracted enterococci DNA to total DNA extracted in an environmental sample may be quite small.

Previous studies have reported elevated levels of fecal indicator bacteria and antibiotic resistance genes in groundwater adjacent to swine production houses and lagoons. Koike et al. (2010) detected *ermB* in 87% of samples and *ermF* in 40% of samples collected from wells near swine lagoons, which were previously identified as being contaminated by swine lagoon leachate. Likewise in a series of studies Chee-Sanford and colleagues (Chee-Sanford et al, 2009) detected a number of tetracycline resistance genes in groundwater underlying swine production facilities. Previous studies have also demonstrated that fecal indicator bacteria can leach to shallow groundwater (Sapkota et al. 2007). This study is among the first to examine the presence of antibiotic resistance in tile drainage water. In our 4 years of water quality monitoring, we twice observed statistically significant differences in *ermB* concentrations drainage water from manured and no-manure plots in the first year after manure application. Additionally, statistically significant differences in *ermF* concentrations in drainage water from manured and no-manure plots in 2013. Clearly the above average precipitation levels in the spring of 2013 and 2014 impacted the export of resistance genes to tile drainage waters. The elevated export of antibiotic resistance genes after manure application in wetter years may be significant because of the potential for exchange and storage of these genes in river waters and sediments (Pei et al., 2006).

The results obtained with qPCR differed from those obtained with the fecal indicator *Enterococcus*. The concentration of resistance genes was far greater than concentrations of enterococci. The effect of manure application on resistance genes concentrations persisted longer after manure application than the enterococci. *Erm* genes are present in a wide variety of bacteria (Park et al., 2010), but we do not know the identity of bacteria carrying *erm* genes in tile drainage or in the soil.

Effects of manure application were not observed beyond the first year after application. We found no evidence that the long-term effects of manure application were increasing the soil-borne *erm* gene concentrations. The significant reduction *erm* genes in drainage water samples in the second year of the corn-soybean two year rotations supports this conclusion. However, the increasing prevalence of crop rotations with two or more years of corn and manure indicates the need to monitor corn-corn rotations that receive manure application yearly. Furthermore, the data from this study cannot preclude qualitative shifts in the microbial community due to repeated applications of manure in alternate years.

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