TECHNICAL REPORTS

SURFACE WATER QUALITY

Transport and Persistence of Tylosin-Resistant Enterococci, *erm* Genes, and Tylosin in Soil and Drainage Water from Fields Receiving Swine Manure

Jason L. Garder, Thomas B. Moorman,* and Michelle L. Soupir

Abstract

Land application of manure from tylosin-treated swine introduces tylosin, tylosin-resistant enterococci, and erythromycin resistant rRNA methylase (*erm*) genes, which confer resistance to tylosin. This study documents the persistence 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. Between 70 and 100% 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 (*erm*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 2-yr period to levels similar to those in the no-manure controls. Concentrations of enterococci in tile water were low, while tylosin-resistant enterococci were rarely detected. In approximately 75% of tile water samples, *erm*B was detected, 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 tillage practices. These results show that tylosin usage increased the short-term occurrence of tylosin-resistant enterococci, *erm* genes, and tylosin in soils but had minimal effect on tile drainage water quality in years of average to below average precipitation.

A NTIMICROBIALS 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 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 antibiotic-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).

Erythromycin resistance rRNA methylase (*erm*) genes are responsible for resistance to macroloide-lincosamide-streptogramin (MLS) antibiotics, including tylosin. Various *erm* genes have been reported in a varied assemblage of diverse bacteria that 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 *erm* genes have been found in swine waste lagoons including *erm*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, antibiotic-resistant bacteria (ARB), and antibiotic resistance genes (ARGs) enter the environment (Heuer et al., 2011). Between 25 and 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 (*Escherichia*

J.L. Garder, Dep. of Civil, Construction, and Environmental Engineering, Iowa State Univ., Ames, IA; T.B. Moorman, National Laboratory for Agriculture and the Environment, USDA–ARS, Ames, IA; M.L. Soupir, Dep. of Agricultural and Biosystems Engineering, Iowa State Univ., Ames, IA; J.L. Garder, current address: CDM Smith, Inc. Kansas City, MO. Assigned to Associate Editor Ed Topp.

coli and *Enterococcus* spp.) in tile drainage during high flows have been previously reported (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. The objectives of this study were to quantify total and tylosin-resistant enterococci, *erm*B, *erm*F, *erm*T, and tylosin in liquid swine manure, manure-treated soil, and subsurface tile drainage. No-till (NT) and chisel plow (CP) field plots receiving multiyear applications of liquid swine manure were studied and compared to nonmanured control plots.

Materials and 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, (43.0° N, 92.5° W) from 2010 to 2012. The soils are moderately well to poorly drained Floyd loam (fine-loamy, mixed, superactive, mesic aquic Pachic Hapludolls), Kenyon loam (fine-loamy, mixed, superactive, mesic Typic Hapludolls), and Readlyn loam (fine-loamy, mixed, superactive, mesic Aquic Hapludolls), which overlie loamy glacial till, as described previously by Fathelrahman et al. (2011). Soil slopes vary from 1 to 3%. Each 0.404-ha (1-acre) plot was 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). Crossflow between plots was prevented by border drains. Central drainage lines from each plot were connected to individual sumps equipped with an effluent pump and a 2.54-cm-diameter Neptune T-10 flow meter (Neptune Technology Group). Subsurface drainage flow was metered as a function of pumped volume and was recorded weekly while the tile lines were flowing. Precipitation data were obtained from the Iowa Environmental Mesonet (Iowa State University, 2012).

The selected plots encompass two tillage practices, CP and 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 (*Zea mays* L.) plots receive swine manure or UAN fertilizer as a nitrogen source before each crop season. The plots are in a corn–soybean (*Glycine max* [L.] Merr.) rotation; therefore, a total of eight plots were selected to obtain 2 yr of data. In the first year of the study, only four plots were sampled (hereafter referred to as plot system A, or PSA). In the second year of the study, four additional plots were added (hereafter referred to as plot system B, or PSB) along with PSA. The control plots had no manure applied since 1978, while the manured plots have been in various rotations with swine manure applications since 1993. Specific plot locations at the project site are described by Kanwar et al. (1999).

Manure slurry 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 28 October 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 subtherapeutic levels of 44.1 g Mg^{-1} feed (~1.85 mg tylosin kg^{-1} body weight) for growth promotion for 16 out of 20 wk of each animal rotation or 2.5 turns per year (facility manager, personal communication, 2012). Urea and ammonium nitrate (168 kg N ha^{-1}) was knifed into the control plots in late April of 2011 and 2012. The CP plots were field cultivated (10-cm depth) before planting corn in May of 2011 and 2012 (Al-Kaisi and Kwaw-Mensah, 2007). Three manure samples were collected directly from the manure applicator and were kept refrigerated until analyzed. In 2010, the manure samples were analyzed for enterococci (described subsequently) 1 d after application, while in 2011 the samples were held for 3 d before being analyzed. After analyzed for enterococci, the manure samples were frozen until extraction of DNA and tylosin.

Soil samples were collected following manure application in the fall of 2010 and 2011. In 2010 the post-manure-application sampling was done 1 d after application, but in 2011 sampling was done 3 d after application. Six composite soil samples were prepared 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 disinfected with 75% ethanol between sampling in the manure injection band, inter-band, and nonmanured soils.

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 (plot system A) with data for 2 full years after 2010 manure application.

‡ Plots (plot system B) with data for 1 full year after 2011 manure application.

Samples were placed in plastic bags and transported back to Iowa State University on ice in a cooler. Samples were mixed using surface spatulas disinfected with 75% ethanol. A subsample was removed for analysis of total enterococci and tylosin-resistant enterococci and processed within 24 h. Another subsample was removed for moisture analysis and the remaining sample was frozen for DNA and tylosin extraction. A second set of soil samples was collected in mid-April (2011 and 2012) 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 the following spring. Mean soil moisture content from all samples was 0.17, 0.16, 0.19, and 0.24 kg water kg^{-1} soil 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 (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 2500 mL was collected: 250 mL for analysis of tylosin, 250 mL for DNA extraction, and 2000 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, IA, on ice and analyzed within 24 h (enterococci and DNA extraction) or 48 h (tylosin). Water samples were only collected from tile lines in the first year after manure application. Instantaneous flow was measured volumetrically at each sampling.

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 h. Soil and manure samples were diluted (1 g 9 mL^{-1}) with sterile, distilled water before filtration. Total and tylosin-resistant enterococci were enumerated on mEnterococcus (mE) agar (Difco) 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^{-1}) and results for water were expressed as cfu 100 mL^{-1}.

DNA Extraction and qPCR

Quantitative PCR assays were performed to quantify ermB, ermF, and ermT. These genes were chosen based on their abundance in fresh swine manure and swine waste lagoons (Chen et al., 2007; Chen et al., 2010). DNA in tile water samples

(250 mL) were extracted using the MoBio Power Water DNA kit within 48 h of collection. Soil DNA extractions (10 g, wet weight) were performed using the UltraClean Mega Soil DNA Isolation Kit (Mo Bio Laboratories, Inc). 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 of manure slurry was combined with QIAamp DNA Stool protocol (Qiagen). This method uses bead beating in the presence of a lysis buffer with sodium dodecyl sulfate, salt, and EDTA. The concentration of extracted DNA was determined with an Eppendorf biophotometer. Afterward, the DNA was frozen until qPCR analysis.

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 ermB, ermF, and ermT. Each qPCR reaction was performed on a Opticon2 qPCR instrument (MJ Research) with total reaction volume of 25 μL containing 2.5 μL of DNA, 12.5 μL of Qiagen SYBR Green Master Mix, and 5 μL of each primer (forward and reverse at 2.5 μM). The qPCR conditions for all genes consisted of an initial denaturation of 95°C for 15 min followed by 40 cycles of 30 s of denaturation at 95°C, 1 min of annealing at the temperature specified in Table 2 and 1 min of extension at 70°C. This was followed by a final extension at 70°C for 10 min. Melt curves were run afterward to confirm PCR product identity. The annealing temperatures for ermB, ermF, and ermT 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 from 1 g dry weight (manure or soil adjusted to a dry weight basis after extraction) or 100 mL (water). Standards of DNA were prepared from E. coli strains carrying plasmids with erm gene fragment inserts (Table 2). The plasmids containing ermB and ermT fragments were constructed from Enterococcus isolates Man T1-C and Soil T3-R, respectively, previously characterized by (Hoang et al., 2013). PCR products from these isolates were purified and cloned into pCR-4TOPO using the TOPO TA cloning kit (Invitrogen Corp.). A reference E. coli strain with a plasmid carrying ermF was provided by M. C. Roberts' laboratory (University of Washington). Both negative controls (three) and blanks (six) were run with each assay. Negative controls for PCR consisted of Pseudomonas stutzeri genomic DNA (ATCC 14405) and PCR-grade water, and all negative controls included SYBR Green mastermix. Calibrations (log of standard DNA plotted against threshold cycle) were linear from 10^8 copies per well to approximately 1000 copies per well. The lower limits of quantitation are based on observations of

Table 2. Quantitative polymerase chain reaction primer sequences, annealing temperatures, and amplicon size for erm genes.

Primer	Gene targeted	Primer sequence (5′→3′)	Amplicon size	Primer annealing temp.	Reference
			bp	°C	
ermB-FW	ermB	GGTTGCTCTTGCACACTCAAG	191	58.4	Koike et al. (2010)
ermB-RV		CAGTTGACGATATTCTCGATTG			
ermF-189f	ermF	CGACACAGCTTTGGTTGAAC	309	54.3	Chen et al. (2007)
ermF-497r		GGACCTACCTCATAGACAAG			
ermT-52f	ermT	CATATAAATGAAATTTTGAG	369	51.0	Chen et al. (2007)
ermT-420r		ACGATTTGTATTTAGCAACC			

nonspecific amplification in control wells and were determined separately for each PCR run (Smith and Osborn, 2009). For all assays, the average lower limit of quantitation for *erm*B and *erm*F were 1.1×10^5 ($\pm 1.4 \times 10^5$) and 2.5×10^5 ($\pm 3.5 \times 10^5$) copies g^{-1} soil, respectively. For water samples, the average limits of quantitation were 2.5×10^2 ($\pm 3.2 \times 10^2$) and 3.6×10^5 ($\pm 5.1 \times 10^5$) copies 100 mL^{-1} for *erm*B and *erm*F, respectively. We performed far fewer analysis of *erm*T, but similar limits of detection were obtained in these limited assays.

The effect of inhibitory substances coextracted with the DNA were characterized by spiking soil, water, and manure samples with known amounts of plasmid DNA containing the target genes at concentrations equivalent to 10^6 to 10^8 gene copies and comparing actual and theoretical recoveries for each *erm* gene. Mean recoveries of *erm*B, *erm*F, and *erm*T extracted 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%.

Amplified DNA from SYBR Green PCR 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 was 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). The purified PCR products were sequenced at the DNA Facility at Iowa State University. The forward and reverse sequences were aligned and consensus sequences were developed using Vector NTI v11.1 software (Life Technologies). These sequences were matched to NCBI DNA sequences using mega BLAST (http://blast.ncbi.nlm.nih.gov/mmtrace.shtml).

Tylosin Extraction and Analysis

Analytical methods were developed and validated for detection of 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). 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 (mass-to-charge ratio (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 (m/z 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 four replicate soil samples spiked with tylosin averaged 88%.

Tylosin was extracted from the tile water samples by filtering 250 mL through an Oasis HLB 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 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. Nondetects were taken as half of the limit of detection (Croghan and Egeghy, 2003) for the *erm* gene and tylosin data. For the soil data (concentrations of *erm* genes and enterococci) analysis of variance was performed using the effects of tillage (CP or NT), treatment location (manure band, inter-band, or no-manure), season (time, fall, or spring), and year (2010 or 2011), which provides replication in time. Interaction effects were examined between tillage and treatment location and between season and treatment. For most sampling times, each manured plot had three replicate samples in the band and inter-band positions, and each control plot had three replicate samples. 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. Nonsignificant effects or interactions were removed from the model. Mean separation was determined from pairwise differences of least-squares means. Effects were considered significant at $p \leq 0.1$. Data are reported as back-transformed means.

For the water data, correlation analysis was used to determine the relationships between concentrations of enterococci or *erm* genes in tile water and time after manure application or tile flow rate. Effects were considered significant if the correlation coefficient (r) exceeded 0.9. Analysis of variance was also performed to test the effects of tillage and treatment (manured or control) using both years of data to achieve replication in time.

Results and Discussion

Enterococci in Manure, Soil, and Tile Drainage Water

Enterococci 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, 4.0×10^5 cfu 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 2011 manure than in the 2010 manure. In 2011, the manure samples were analyzed 3 d after application,

while in 2010, sampling and analysis took place the day after application; therefore, bacterial die-off during sample storage may account for some of the differences between the 2010 and 2011 enterococci populations. Previously, between 31 and 100% of enterococci from swine manure were tylosin-resistant (Jackson et al., 2004; Hoang et al., 2013).

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. Using ANOVA, we found that enterococci populations in soil after manure application in the 2010–2011 period (PSA) was significantly greater than in the 2011–2012 period (PSB), which may be due to the difference between the concentrations of enterococci in the applied manure. Cools et al. (2001) reported that populations of *Enterococcus* in soil at 5°C did not decline for 80 d, which suggests that the difference in timing of sampling after manure application in 2010 and 2011 may have had a minimal effect. There were significant ($p < 0.1$) decreases in the manure band concentrations from the fall after manure application to the following spring in both years. The enterococci population

in manure bands declined from 826 cfu g^{-1} soil in the fall of 2010 to 246 cfu g^{-1} soil in the spring of 2011. In the spring of 2011, the manure band enterococci population was greater ($p < 0.1$) than the populations in the inter-band and control soil. Similarly, enterococci populations declined over the winter following the 2011 fall manure application. The spring 2012 populations in the manure band (6 cfu g^{-1} soil) were equivalent to the enterococci populations in the inter-band and no-manure control soils at that time. Using mean concentration across both years, the enterococci concentration in the manure band was significantly greater than in the inter-band, but the mean inter-band enterococci concentration was not significantly greater than the no-manure control (Table 3, bottom row). There was no significant interaction between treatment (band, inter-band, or no-manure control) and season (fall or spring).

The long-term survival of enterococci in soil is shown in Table 4. Over the 2 yr 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 1 yr. 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

Table 3. Mean† enterococci (ENT) and tylosin-resistant enterococci (TYL) concentrations in soil in the first year after manure application. Standard deviations are in parentheses.

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

† Treatment means are averaged across tillage treatments. 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 ($p \leq 0.1$).

‡ Plot system A plots as shown in Table 1.

§ Plot system B plots as shown in Table 1.

¶ Mean over both 2010 and 2011.

Table 4. Mean† enterococci (ENT) and tylosin-resistant enterococci (TYL) concentrations in soil over 2 yr after manure application in the fall of 2010. Standard deviations are presented in parentheses.

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

† Treatment means are averaged across tillage treatments. Means in columns followed by the same letter (a, b, c) or rows comparing treatment (x, y, z) are not significantly different ($p \leq 0.1$).

‡ Plot system A, as shown in Table 1.

§ NS, no sample; this sampling time is not included in the calculation of the overall mean.

no-manure or inter-band samples from 2010, indicating that the manured plots return to the background levels measured in the manure-free plots.

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-Sorensen et al. (2005) where 16% or less of culturable bacteria from soil with a manure history were macrolide-resistant. Hoang et al. (2013) reported total and tylosin-resistant enterococci in manured soil averaged 9.8 × 10³ cfu g⁻¹ soil and 7.5 × 10³ cfu g⁻¹ soil, respectively. The enterococci concentrations immediately after manure application in this study were slightly less than those concentrations 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 (Fig. 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 (2010–2011 vs. 2011–2012) as shown in Fig. 1. The second year of monitoring from PSA (data not shown) supported the 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 yr under various swine manure treatments and manure-free control plots located in central Iowa. Mean concentrations of enterococci in tile water from both manure-free and manure-amended soils were similar, ranging from 27 to 206 cfu 100 mL⁻¹, which were slightly greater than those found in this study.

Tylosin-resistant enterococci in the tile water were rarely detected, and when present, the maximum concentration was 1 cfu 100 mL⁻¹ (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.
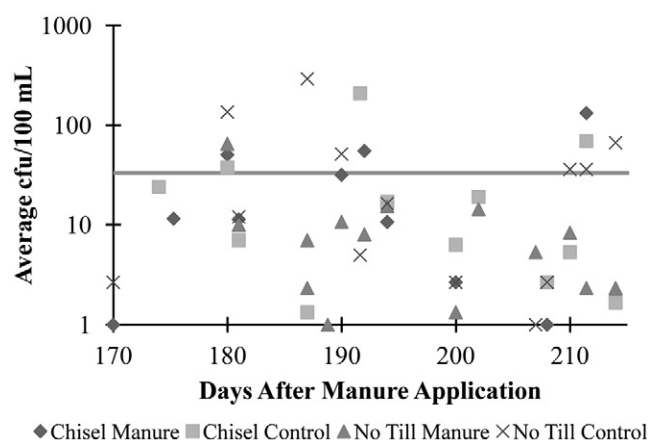

Fig. 1. Enterococci in individual tile water samples in the first growing season after manure application in 2010 (plot system A) and 2011 (plot system B). The recreational water quality limit for *Enterococcus* (33 cfu 100 mL⁻¹) is shown for reference.

erm Genes in Manure, Soil, and Tile Drainage Water

Quantitative PCR analysis conducted on DNA extracted from manure, soil, and water detected *erm*B and *erm*F, while *erm*T was not detected. In manure, the mean *erm*B concentrations were 8 × 10⁸ copies g⁻¹ in 2010 and 6 × 10¹² copies g⁻¹ in 2011. The mean *erm*F concentrations were 4 × 10⁷ copies g⁻¹ and 3 × 10¹² copies g⁻¹ in 2010 and 2011, respectively. *erm*T was not detected in manure in either year. Previously reported concentrations of *erm*B, *erm*F, and *erm*T exceed 1 × 10⁹ copies g⁻¹ 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 significantly ($p < 0.1$) 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

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
		——————————— copies g⁻¹ ———————————		
2010‡	Fall 2010	1 × 10⁹a (±2 × 10⁸)	2 × 10⁶a (±3 × 10⁵)	3 × 10⁵a (±2 × 10⁴)
	Spring 2011	9 × 10⁷b (±1 × 10⁷)	3 × 10⁶a (±8 × 10⁵)	3 × 10⁵a (±4 × 10⁴)
	Annual mean‡	5 × 10⁸x (±5 × 10⁸)	2 × 10⁶y (±6 × 10⁵)	3 × 10⁵y (±3 × 10⁴)
2011§	Fall 2011	5 × 10⁸a (±2 × 10⁵)	1 × 10⁶a (±1 × 10⁶)	<6.3 × 10³a
	Spring 2012	2 × 10⁶b (±1 × 10⁶)	1 × 10⁵a (±1 × 10⁵)	2 × 10⁴b (±0 × 10⁰)
	Annual mean§	2 × 10⁸x (±2 × 10⁸)	6 × 10⁵x (±4 × 10⁵)	2 × 10⁴x (±0 × 10⁰)
Treatment mean¶		4 × 10⁸x (±4 × 10⁸)	1 × 10⁶y (±1 × 10⁶)	3 × 10⁵z (±1 × 10⁵)

† Means are averaged across tillage treatments. Means in columns followed by the same letter (a, b, c, d) or rows (x, y, z) are not significantly different ($p \leq 0.1$).

‡ Plot system A, as shown in Table 1.

§ Plot system B, as shown in Table 1.

¶ Mean over both 2010 and 2011.

concentrations equivalent to concentrations in the inter-band and no-manure control soils by 1 yr after manure application (Fig. 2).

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 nonmanured 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 2 yr, after manure application in PSA in 2010, and reached concentrations equivalent to concentrations in the inter-band and no-manure control soils by 1 yr after manure application (Fig. 2). In the spring of 2012, *erm*F concentrations were below the detection limit in all soils.

Tylosin-resistant enterococci, *erm*B, and *erm*F decreased to levels that were comparable to those observed in the control plots after a complete year following manure application, suggesting that corn–soybean rotations with alternating years of swine manure application will not have increasing levels of antibiotic-resistant bacteria in soil. The elevated levels of *erm*B and *erm*F genes in the inter-band samples compared to the *erm* abundance in soils without manure (Tables 4 and 5) 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; but, the magnitude of any differences between inter-band and control soil gene abundances is relatively small. However, a continuous-corn rotation receiving annual manure application might maintain higher levels of resistance genes without the biennial decrease reported here. Zhou et al. (2010) reported transient (20–40 d) 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 1940s, suggesting an increase in antibiotic resistance. The long-
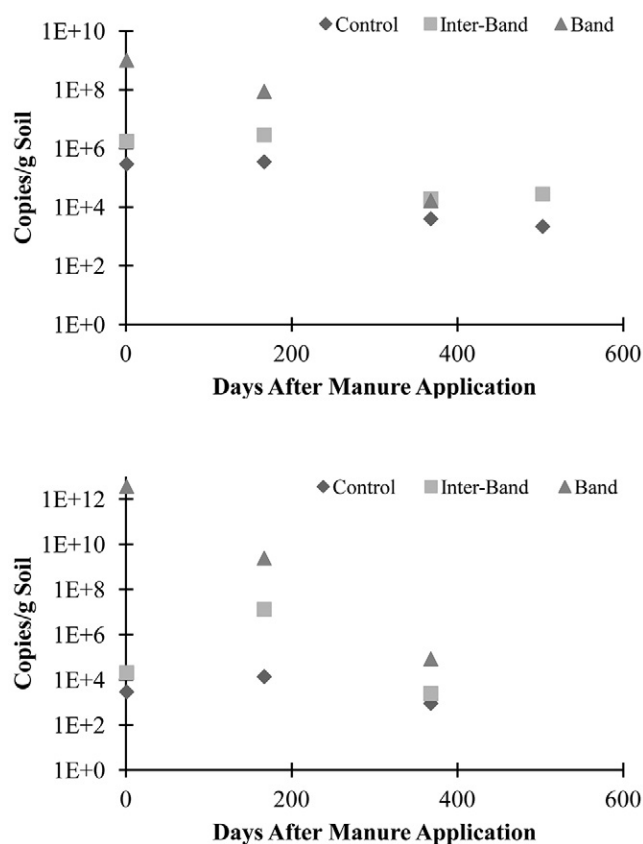

Fig. 2. Persistence of *erm*B (top) and *erm*F (bottom) in soil over 2 yr after receiving swine manure in 2010 (plot system A). No manure band samples were collected in spring of 2012 (day 503), as the bands were no longer visible.

term history of antibiotic use in the swine production facility that supplied the manure for our study is not available presently.

The abundance of both *erm*B and *erm*F (Fig. 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 2-yr 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 2-yr mean concentration of 2.4×10^5 copies 100 mL^{-1}. There was no correlation ($r < 0.5$) between *erm*B and *erm*F concentrations relative to flow (data not shown) or time after manure application.

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
		——————————— copies g^{-1} ———————————		
2010‡	Fall 2010	4×10^{12}a ($\pm 2 \times 10^{12}$)	2×10^4a ($\pm 6 \times 10^3$)	3×10^3a ($\pm 3 \times 10^1$)
	Spring 2011	2×10^9b ($\pm 1 \times 10^9$)	1×10^7b ($\pm 9 \times 10^6$)	1×10^4a ($\pm 2 \times 10^3$)
	Annual mean‡	2×10^{12}x ($\pm 2 \times 10^{12}$)	7×10^6y ($\pm 1 \times 10^7$)	8×10^3z ($\pm 6 \times 10^3$)
2011§	Fall 2011	5×10^8a ($\pm 3 \times 10^8$)	5×10^6a ($\pm 58 \times 10^6$)	$<7.0 \times 10^3$a
	Spring 2012	4×10^6a ($\pm 3 \times 10^6$)	1×10^5a ($\pm 1 \times 10^5$)	$<7.0 \times 10^3$a
	Annual mean§	2×10^8x ($\pm 3 \times 10^8$)	3×10^6y ($\pm 5 \times 10^6$)	$<7.0 \times 10^3$z
Treatment means¶		9×10^{11}x ($\pm 1 \times 10^{12}$)	7×10^6y ($\pm 7 \times 10^6$)	6×10^3z ($\pm 6 \times 10^3$)

† Means are averaged across tillage treatments. Means in columns followed by the same letter (a, b) or rows (x, y, z) are not significantly different ($p \leq 0.1$).

‡ Plot system A, as shown in Table 3.

§ Plot system B, as shown in Table 3.
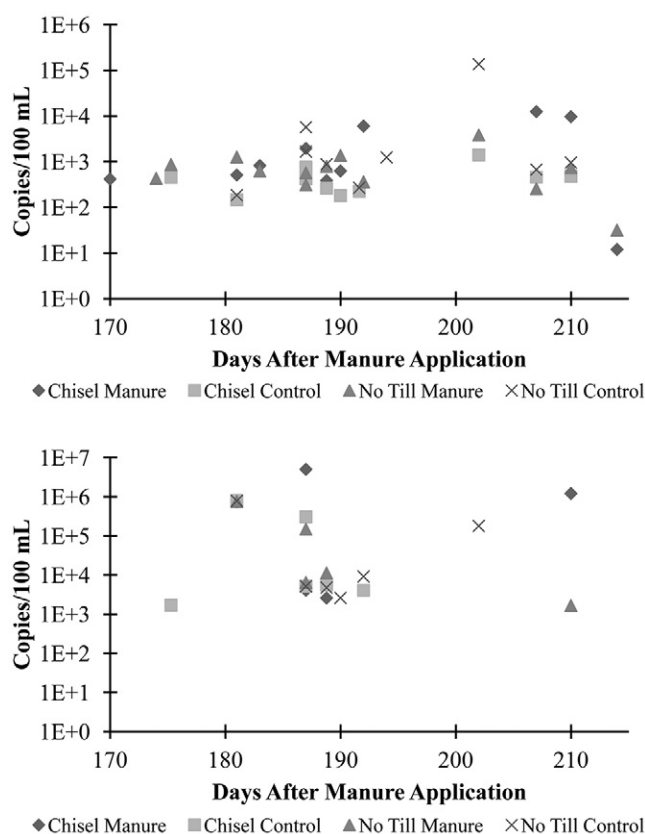
¶ Mean over both 2010 and 2011.

Fig. 3. Abundance of *erm*B (top) and *erm*F (bottom) in tile water in first year after manure application for plot system A and plot system B.

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 consistent with the detection of both *erm* genes in the nonmanured soil. To date, no other published study has quantified *erm* genes in tile water. However, Bockelmann et al. (2009) detected *erm*B in groundwater receiving artificial recharge and Koike et al. (2010) detected both *erm*B and *erm*F in shallow groundwater wells near swine lagoons. The *erm*F in the groundwater 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 to 4×10^8 copies 100 mL^{-1} (Koike et al., 2010).

The DNA sequencing of qPCR products confirmed that *erm* genes were selectively amplified. The *erm*B product was 182–185 nucleotide base pairs (bp) and the *erm*F fragment was 310 bp, which correspond to the 191 bp *erm*B and 309 bp *erm*F amplicons described previously (Chen et al., 2007; Koike et al., 2010). 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. However, non-consensus partial sequences were obtained from *erm*F PCR products amplified from two soil samples. Matches to the consensus sequences were identified using Mega BLAST searches of the NCBI nucleotide database. Matches to *erm*B and *erm*F (>99% similarity) indicated that gram-positive gut bacteria, including various species of *Enterococcus*, *Streptococcus*, *Clostridium*, *Bacteriodes*, and *Capnocytophaga*, are the likely host of the *erm* genes found in this study. No nonspecific matches

were obtained. The bacterial genera matching our PCR product sequencing agree with previous studies that identify the known hosts of *erm* genes (Zhou et al., 2010; 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.

Tylosin in Manure, Soil, and Tile Drainage Water

Tylosin A was detected in manure applied to plots in both 2010 and 2011 with 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 2 yr 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 wk 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 Meyerhoff, 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 nondetects) 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 ($p < 0.1$) in concentrations between the inter-band and band or 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 ($p < 0.1$) between the three means. The mean concentrations of tylosin in soils across the 2-yr 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 ($p < 0.1$) between the three means over the 2 yr. 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 25×10^3 to 50×10^3 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 seven sampling times to 0.0096 ng mL^{-1} for the last eight. Except for one sample, only tylosin A was detected. In 2011 only tylosin A was quantified, 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}.

Our results are 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,

Table 7. Mean concentrations (ng mL⁻¹) of tylosin in tile water in the first year after fall manure applications in 2010 and 2011.

Year		Chisel with manure	Chisel control	No till with manure	No till control
			———————— ng mL⁻¹ ————————		
2010–2011	Mean of detects†	0.20 ± 0.38	0.24 ± 0.28	0.03 ± 0.02	0.04 ± 0.03
	Mean of all data‡	0.15 ± 0.24	0.21 ± 0.21	0.01 ± 0.01	0.02 ± 0.02
2011–2012	Mean of detects†	–	–	–	0.004
	Mean of all data‡	–	–	–	0.0002 ± 0.001

† Mean concentration for samples above the detection limit, non-detects are indicated with a dash. In 2010–2011, 14 samples were collected from each plot. In 2011–2012, 10 to 15 samples were collected.

‡ Mean concentration for all samples using half of the detection limit for those falling below the detection limit. For treatments without detectable tylosin in any sample, the mean concentrations were not estimated, as indicated with a dash.

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 likely facilitates a gross underestimation of the actual concentrations in soil due to limitations of the antibiotic extraction procedure. 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 to128 μg mL⁻¹ for 78 *Enterococcus* isolates, which are well above tylosin concentrations observed in soil or tile water during this study. 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).

Precipitation and Drainage

Precipitation during the 2-yr study was below normal. The 10-yr rainfall average during the first 6 mo of the year is 37.4 cm: in 2011 it was 30.8 cm, and in 2012 it was 21.2 cm. However, average drainage for these plots was near normal in 2011: 6.5 ± 3.8 cm drainage compared to the 10-yr average of 6.0 ± 2.0 cm. In 2012, the average drainage was only 3.8 ± 1.9 cm, reflecting the lower precipitation at the end of 2011 and in 2012. The transport of bacteria (and potentially *erm* genes and tylosin) in tile drainage water might be less than that expected in a normal flow year. Furthermore, the grab sampling scheme may have underestimated 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 NT plots over CP 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 concentrations of ARB and ARG in tile water with respect to manure or tillage treatments.

Conclusions

Swine manure application increased the abundance of *erm* genes in soil above the background levels in soils not receiving manure. This increase in soil was greatest immediately after manure application, and *erm*B and *erm*F persist in manure injection band in concentrations greater than in nonmanured soils over winter. However, the manure band concentrations eventually decreased to levels equivalent to the nonmanured 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, but initial concentrations of enterococci after manure application and subsequent persistence were substantially less than than concentrations of *erm* genes. 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. These results suggest that injection of swine manure into soil does not result in substantial effects on water quality, although further research on the bacteria hosting resistance genes is needed.

References

Al-Kaisi, M., and D. Kwaw-Mensah. 2007. Effect of tillage and nitrogen rate on corn yield and nitrogen and phosphorus uptake in a corn–soybean rotation. Agron. J. 99:1548–1558. doi:10.2134/agronj2007.0012

Allaire, S.E., J. Del Castillo, and V. Juneau. 2006. Sorption kinetics of chlortetracyline and tylosin on sandy loam and heavy clay soils. J. Environ. Qual. 35:969–972. doi:10.2134/jeq2005.0355

Allen, H.K., J. Donato, H.H. Wang, K.A. Cloud-Hansen, J. Davies, and J. Handelsman. 2010. Call of the wild: Antibiotic resistance genes in natural environments. Nat. Rev. Microbiol. 8:251–259. doi:10.1038/nrmicro2312

Andersson, D.I., and D. Hughes. 2012. Evolution of antibiotic resistance at non-lethal drug concentrations. Drug Resist. Updat. 15:162–172. doi:10.1016/j.drup.2012.03.005

APHA. 1998. Standard methods for the examination of water and wastewater 9230C. American Public Health Association, Washington, DC.

Blackwell, P.A., P. Kay, R. Ashauer, and A.B.A. Boxall. 2009. Effects of agricultural conditions on the leaching behaviour of veterinary antibiotics in soils. Chemosphere 75:13–19. doi:10.1016/j.chemosphere.2008.11.070

Blackwell, P.A., P. Kay, and A.B. Boxall. 2007. The dissipation and transport of veterinary antibiotics in a sandy loam soil. Chemosphere 67:292–299. doi:10.1016/j.chemosphere.2006.09.095

Bockelmann, U., H.H. Dorries, M.N. Ayuso-Gabella, M. Salgot de Marcay, V. Tandoi, C. Levantesi, C. Masciopinto, E. Van Houtte, U. Szewzyk, T. Wintgens, and E. Grohmann. 2009. Quantitative PCR monitoring of antibiotic resistance genes and bacterial pathogens in three European artificial groundwater recharge systems. Appl. Environ. Microbiol. 75:154–163. doi:10.1128/AEM.01649-08

Campagnolo, E.R., K.R. Johnson, A. Karpati, C.S. Rubin, D.W. Kolpin, M.T. Meyer, J.E. Esteban, R.W. Currier, K. Smith, K.M. Thu, and M. McGeehin. 2002. Antimicrobial residues in animal waste and water resources proximal to large-scale swine and poultry feeding operations. Sci. Total Environ. 299:89–95. doi:10.1016/S0048-9697(02)00233-4

Chee-Sanford, J.C., R.I. Mackie, S. Koike, I.G. Krapac, Y.F. Lin, A.C. Yannarell, S. Maxwell, and R.I. Aminov. 2009. Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. J. Environ. Qual. 38:1086–1108. doi:10.2134/jeq2008.0128

Chen, J., F.C. Michel, Jr., S. Sreevatsan, M. Morrison, and Z. Yu. 2010. Occurrence and persistence of erythromycin resistance genes (erm) and tetracycline resistance genes (tet) in waste treatment systems on swine farms. Microb. Ecol. 60:479–486. doi:10.1007/s00248-010-9634-5

Chen, J., Z. Yu, F.C. Michel, Jr., T. Wittum, and M. Morrison. 2007. Development and application of real-time PCR assays for quantification of erm genes conferring resistance to macrolides-lincosamides-streptogramin B in livestock manure and manure management systems. Appl. Environ. Microbiol. 73:4407–4416. doi:10.1128/AEM.02799-06

Clinical and Laboratory Standards Institute. 2010. Performance standards for antimicrobial susceptability testing. Twentieth Informational Supplement. M100-S20. CLSI, Wayne, PA.

Cools, D., R. Merckyx, K. Vlassak, and J. Verhaegen. 2001. Survival of E. coli and Enterococcus spp. derived from pig slurry in soils of different texture. Appl. Soil Ecol. 17:53–62. doi:10.1016/S0929-1393(00)00133-5

Croghan, C., and P.P. Egeghy. 2003. Methods of dealing with values below the limit of detection using SAS. Presented at Southeastern SAS User Group, St. Petersburg, FL. 22–24 September.

Cullum, R.F. 2009. Macropore flow estimations under no-till and till systems. Catena 78:87–91. doi:10.1016/j.catena.2009.03.004

Dean, D.M., and M.E. Foran. 1992. The effect of farm liquid waste application on tile drainage. J. Soil Water Conserv. 47:368–369.

Dolliver, H., and S. Gupta. 2008. Antibiotic losses in leaching and surface runoff from manure-amended agricultural land. J. Environ. Qual. 37:1227–1237. doi:10.2134/jeq2007.0392

Fathelrahman, E.M., J.C. Ascough, D.L. Hoag, R.W. Malone, P. Heilman, L.J. Wiles, and R.S. Kanwar. 2011. Economic and stochastic efficiency comparison of experimental tillage systems in corn and soybean under risk. Exp. Agric. 47:111–136. doi:10.1017/S0014479710000979

FDA. 2009. NARMS Retail Meat Annual Report, 2009. FDA, Silver Spring, MD.

Halling-Sorensen, B., A.M. Jacobsen, J. Jensen, G. Sengelov, E. Vaclavik, and F. Ingerslev. 2005. Dissipation and effects of chlortetracycline and tylosin in two agricultural soils: A field-scale study in southern Denmark. Environ. Toxicol. Chem. 24:802–810. doi:10.1897/03-576.1

Heuer, H., H. Schmitt, and K. Smalla. 2011. Antibiotic resistance gene spread due to manure application on agricultural fields. Curr. Opin. Microbiol. 14:236–243. doi:10.1016/j.mib.2011.04.009

Hoang, T.T.T., M.L. Soupir, , P. Liu., and A. Bhandari. 2013. Occurrence of tylosin-resistant enterococci in swine manure and tile drainage systems under no-till management. Water Air Soil Pollut. 224:1754. doi:10.1007/s11270-013-1754-3

Hu, D., and J.R. Coats. 2009. Laboratory evaluation of mobility and sorption for the veterinary antibiotic, tylosin, in agricultural soils. J. Environ. Monit. 11:1634–1638. doi:10.1039/b900973f

Hunter, C., J. Perkins, J. Tranter, and P. Hardwick. 2000. Fecal bacteria in the waters of an upland area in Derbyshire, England: The influence of agricultural land use. J. Environ. Qual. 29:1253–1261. doi:10.2134/jeq2000.00472425002900040032x

Iowa State University. 2012. Daily data request form. Iowa Environmental Mesonet. Iowa State University of Science and Technology, Department of Agronomy, Ames. http://mesonet.agron.iastate.edu/agclimate/hist/dailyRequest.php (accessed 21 Sept. 2012).

Jackson, C.R., P.J. Fedorka-Cray, J.B. Barrett, and S.R. Ladely. 2004. Effects of tylosin use on erythromycin resistance in enterococci isolated from swine. Appl. Environ. Microbiol. 70:4205–4210. doi:10.1128/AEM.70.7.4205-4210.2004

Joy, D.M., H. Lee, C.M. Reaume, H.R. Whiteley, and S. Zelin. 1998. Microbial contamination of subsurface tile drainage water from field applications of liquid manure. Can. Agric. Eng. 40:153–160.

Kanwar, R.S., D. Bjorneberg, and D. Baker. 1999. An automated system for monitoring the quality and quantity of subsurface drain flow. J. Agric. Eng. Res. 73:123–129. doi:10.1006/jaer.1998.0398

Kaukas, A., M. Hinton, and A.H. Linton. 1988. The effect of growth-promoting antibiotics on the fecal enterococci of healthy young chickens. J. Appl. Bacteriol. 64:57–64. doi:10.1111/j.1365-2672.1988.tb02429.x

Kay, P., P.A. Blackwell, and A.B.A. Boxall. 2004. Fate of veterinary antibiotics in a macroporous tile drained clay soil. Environ. Toxicol. Chem. 23:1136–1144. doi:10.1897/03-374

Kay, P., P.A. Blackwell, and A.B.A. Boxall. 2005. A lysimeter experiment to investigate the leaching of veterinary antibiotics through a clay soil and comparison with field data. Environ. Pollut. 134:333–341. doi:10.1016/j.envpol.2004.07.021

Knapp, C.W., J. Dolfing, P.A.I. Ehlert, and D.W. Graham. 2010. Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. Environ. Sci. Technol. 44:580–587. doi:10.1021/es901221x

Koike, S., R.I. Aminov, A.C. Yannarell, H.D. Gans, I.G. Krapac, J.C. Chee-Sanford, and R.I. Mackie. 2010. Molecular ecology of macrolide–lincosamide–streptogramin B methylases in waste lagoons and subsurface waters associated with swine production. Microb. Ecol. 59:487–498. doi:10.1007/s00248-009-9610-0

Kolz, A.C., T.B. Moorman, S.K. Ong, K.D. Scoggin, and E.A. Douglass. 2005. Degradation and metabolite production of tylosin in anaerobic and aerobic swine-manure lagoons. Water Environ. Res. 77:49–56. doi:10.2175/106143005X41618

Kumar, K., A. Thompson, A.K. Singh, Y. Chander, and S.C. Gupta. 2004. Enzyme-linked immunosorbent assay for ultratrace determination of antibiotics in aqueous samples. J. Environ. Qual. 33:250–256. doi:10.2134/jeq2004.2500

Mackie, R.I., S. Koike, I. Krapac, J. Chee-Sanford, S. Maxwell, and R.I. Aminov. 2006. Tetracycline residues and tetracycline resistance genes in groundwater impacted by swine production facilities. Anim. Biotechnol. 17:157–176. doi:10.1080/10495390600956953

Martinez-Carballo, E., C. Gonzalez-Barreiro, S. Scharf, and O. Gans. 2007. Environmental monitoring study of selected veterinary antibiotics in animal manure and soils in Austria. Environ. Pollut. 148:570–579. doi:10.1016/j.envpol.2006.11.035

Onan, L.J., and T.M. LaPara. 2003. Tylosin-resistant bacteria cultivated from agricultural soil. FEMS Microbiol. Lett. 220:15–20. doi:10.1016/S0378-1097(03)00045-4

Pappas, E.A., R.S. Kanwar, J.L. Baker, J.C. Lorimor, and S. Mickelson. 2008. Fecal indicator bacterial in subsurface drain water following swine manure application. Trans. ASABE 51:1567–1573. doi:10.13031/2013.25313

Park, A.K., H. Kim, and H.J. Jin. 2010. Phylogenetic analysis of rRNA methyltransferases, Erm and KsgA, as related to antibiotic resistance. FEMS Microbiol. Lett. 309:151–162.

Portillo, A., F. Ruiz-Larrea, M. Zarazaga, A. Alonso, J.L. Martinez, and C. Torres. 2000. Macrolide resistance genes in Enterococcus spp. Antimicrob. Agents Chemother. 44:967–971. doi:10.1128/AAC.44.4.967-971.2000

Ramirez, N.E., P. Wang, J. Lejeune, M.J. Shipitalo, L.A. Ward, S. Sreevatsan, and W.A. Dick. 2009. Effect of tillage and rainfall on transport of manure-applied Cryptosporidium parvum oocysts through soil. J. Environ. Qual. 38:2394–2401. doi:10.2134/jeq2008.0432

R Development Core Team. 2011. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.r-project.org/ (accessed 22 Dec. 2011).

Schmitt, H., K. Stoob, G. Hamscher, E. Smit, and W. Seinen. 2006. Tetracyclines and tetracycline resistance in agricultural soils: Microcosm and field studies. Microb. Ecol. 51:267–276. doi:10.1007/s00248-006-9035-y

Smith, C.J. and A.M. Osborn. 2009. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. FEMS Microbiol. Ecol. 67:6–20. doi:10.1111/j.1574-6941.2008.00629.x

Teeter, J.S., and R.D. Meyerhoff. 2003. Aerobic degradation of tylosin in cattle, chicken, and swine excreta. Environ. Res. 93:45–51. doi:10.1016/S0013-9351(02)00086-5

Yu, Z., and M. Morrison. 2004. The improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques 36:808–812.

Zhou, Z., L. Raskin, and J.L. Zilles. 2010. Effects of swine manure on macrolide, lincosamide, and streptogramin B antimicrobial resistance in soils. Appl. Environ. Microbiol. 76:2218–2224. doi:10.1128/AEM.02183-09

Zucker, L.A., and L.C. Brown, editors. 1998. Agricultural drainage: Water quality impacts and subsurface drainage studies in the Midwest. The Ohio State University Extension Bulletin 871-98. OSU Extension. http://ohioline.osu.edu/b871/ (accessed 21 Sept. 2012).