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Fate and transport of tylosin-resistant bacteria and macrolide resistance genes in artificially drained agricultural fields receiving swine manure

Abstract

Application of manure from swine treated with antibiotics introduces antibiotics and antibiotic resistance genes to soil with the potential for further movement in drainage water, which may contribute to the increase in antibiotic resistance in non-agricultural settings. We compared losses of antibiotic-resistant *Enterococcus* and macrolide-resistance (*erm* and *msrA*) genes in water draining from plots with or without swine manure application under chisel plow and no till conditions. Concentrations of *ermB*,*ermC* and *ermF* were all > 10^9 copies g^{-1} in manure from tylosin-treated swine, and application of this manure resulted in short-term increases in the abundance of these genes in soil. Abundances of *ermB*, *ermC* and *ermF* in manured soil returned to levels identified in non-manured control plots by the spring following manure application. Tillage practices yielded no significant differences ($p > 0.10$) in enterococci or *erm* gene concentrations in drainage water and were therefore combined for further analysis. While enterococci and tylosin-resistant enterococci concentrations in drainage water showed no effects of manure application, *ermB* and *ermF* concentrations in drainage water from manured plots were significantly higher ($p < 0.01$) than concentrations coming from non-manured plots. *ErmB* and *ermF* were detected in 78% and 44%, respectively, of water samples draining from plots receiving manure. Although *ermC* had the highest concentrations of the three genes in drainage water, there was no effect of manure application on *ermC* abundance. *MsrA* was not detected in manure, soil or water. This study is the first to report significant increases in abundance of resistance genes in waters draining from agricultural land due to manure application.

Keywords

Antibiotic resistence, erm genes, Enterococcus, Tile drainage, Manure, Swine, Soil

Disciplines

Agriculture | Bioresource and Agricultural Engineering | Environmental Indicators and Impact Assessment | Water Resource Management

Comments

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Fate and transport of tylosin-resistant bacteria and macrolide resistance genes in artificially drained agricultural fields receiving swine manure

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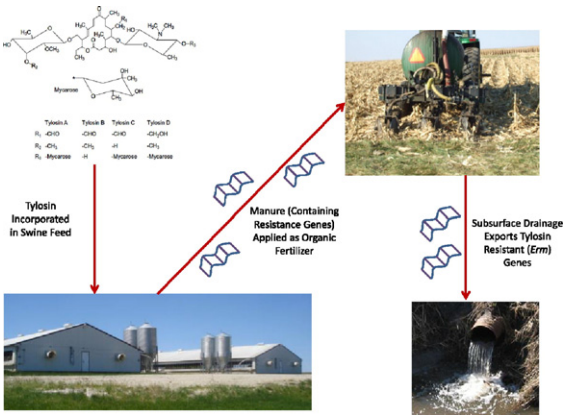
HIGHLIGHTS

- Higher levels of *ermB* and *ermF* in drainage are attributable to manure.
- Fall manure application resulted in transient increases in soil enterococci.
- Manure application did not affect enterococci levels in drainage water.

GRAPHICAL ABSTRACT



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ABSTRACT

Application of manure from swine treated with antibiotics introduces antibiotics and antibiotic resistance genes to soil with the potential for further movement in drainage water, which may contribute to the increase in antibiotic resistance in non-agricultural settings. We compared losses of antibiotic-resistant *Enterococcus* and macrolide-resistance (*erm* and *msrA*) genes in water draining from plots with or without swine manure application under chisel plow and no till conditions. Concentrations of *ermB*, *ermC* and *ermF* were all $>10^9$ copies g^{-1} in manure from tylosin-treated swine, and application of this manure resulted in short-term increases in the abundance of these genes in soil. Abundances of *ermB*, *ermC* and *ermF* in manured soil returned to levels identified in non-manured control plots by the spring following manure application. Tillage practices yielded no significant differences ($p > 0.10$) in enterococci or *erm* gene concentrations in drainage water and were therefore combined for further analysis. While enterococci and tylosin-resistant enterococci concentrations in drainage water showed no effects of manure application, *ermB* and *ermF* concentrations in drainage water from manured plots were significantly higher ($p < 0.01$) than concentrations coming from non-manured plots. *ErmB* and *ermF* were detected in 78% and 44%, respectively, of water samples draining from plots receiving manure. Although *ermC* had the highest concentrations of the three genes in drainage water, there was no effect of manure application on *ermC* abundance. *MsrA* was not detected in manure, soil or water. This study is the first to report significant increases in abundance of resistance genes in waters draining from agricultural land due to manure application.
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1. Introduction

Swine production is an economic cornerstone in the Midwestern United States and provides a substantial portion of the region's gross farm income. More than 66 million swine were produced in the United States in 2012, with over 67% grown in feeding operations containing over 5,000 pigs (USDA, 2014). Many farmers use a variety of antimicrobial additives in swine feed at sub-therapeutic levels as a prophylactic and growth promoter. Research has documented the positive effects of antibiotics in swine feed at subtherapeutic levels in a variety of contexts, including: improvement of growth rates, increased feeding efficiencies, reduced mortality rates and heightened reproductive rates (Hays, 1977; CAST, 1981; Cromwell, 1991). These improvements coupled with declining prices has led to approximately 90% of starter feeds, 75% of grower feeds and 50% of finisher feeds incorporating antibiotics (Cromwell, 2002). The most frequently used antimicrobials in the swine industry include: tetracyclines, tylosin, and sulfamethazine or other sulfonamides (McEwen and Fedorka-Cray, 2002). Apley et al. (2012) estimated an annual use of 533,973 kg of chlortetracycline, 165,803 kg of tylosin and 154,973 kg of oxytetracycline in swine feed in the United States using data from the National Animal Health Monitoring System (NAHMS) and a 2009 survey of swine-exclusive practitioners.

Macrolide antibiotics, such as erythromycin and tylosin, block protein synthesis (Weisblum, 1995; Weisblum, 1998). Antibiotic resistance genes are capable of reducing the effectiveness of antibiotics through a variety of mechanisms including: altered antibiotic target sites, decreased uptake or efflux, "bypass" pathways and enzymatic inactivation or modification (Hawkey, 1998). Erythromycin ribosome methylation (*erm*) genes code for methyltransferase enzymes, which add one or two methyl groups to a single adenine (A2058) (Weisblum, 1998). The methyl groups reduce the ability of erythromycin and tylosin to bind to the 50S ribosomal subunit, therefore hindering the effectiveness of the antibiotic. Furthermore, the binding site for erythromycin overlaps binding sites for other macrolides, lincosamides and streptogramin B antibiotics (MLS_B) (Leclercq and Courvalin, 1991). Therefore, resistance encoded by *erm* genes may cause cross-resistance in the MLS_B family of antibiotics. In addition to macrolide resistance being conferred by alteration of target sites, other classes of genes which code for antibiotic efflux systems have been identified. The *msr* gene family has been classified as a predecessor for proteins which are part of the ABC transporter superfamily (Roberts et al., 1999). ABC transporter proteins utilize energy from adenosine triphosphate binding and hydrolysis to translocate substances across membranes. Chouchani et al. (2012) identified *msrA* in clinical *Enterococcus faecalis* isolates. Antibiotic-resistance is a major threat to public health due to the growing demands for new antibiotics in order to keep up with the wide variety of resistance mechanism identified.

The growing number of animals receiving antibiotics have led to concerns over the increased abundance of antibiotic-resistant bacteria in commensal microbiota and excreted in their feces (Khachatourians, 1998). Koike et al. (2009) found *ermB*, *ermC and ermF* genes present in 100% of manure samples taken from confined animal feeding operations known to administer antibiotics. Additionally, Chen et al. (2007) identified genes conferring erythromycin (*erm*) and tetracycline (*tet*) resistance persisting in swine manure post biofilter treatment. Prior studies have identified elevated levels of antibiotics, antibiotic-resistant bacteria and antibiotic resistance genes in ground and surface water surrounding confined animal feeding operations (Campagnolo et al., 2002; Chee-Sanford et al., 2009; Heuer et al., 2011). The potential for antibiotics, antibiotic-resistant bacteria and antibiotic-resistance genes leaching into the environment is becoming of greater concern, with approximately 9.2 million hectares of farmland receiving manure annually (Dolliver and Gupta, 2007).

Approximately one third of Iowa cropland utilizes subsurface drainage systems (Zucker and Brown, 1998). Artificial drainage systems transport shallow groundwater from fields to surface waters which increases the losses of pesticides and nutrients from farmlands (Kanwar et al., 1999; Bakhsh et al., 2005; Kladivko et al., 1998). Additionally, various tillage practices affect macropore formation and therefore drainage and chemical transport. For example, Kanwar et al. (1997) identified higher average peak tile flow volumes and larger atrazine losses in cropping systems under no till management compared to chisel plow management.

While there is significant knowledge regarding the release of nutrients from agricultural fields, less is known regarding the export of bacteria. Rainfall simulations on no-till tile drained, swine manure treated plots found peak bacterial densities during periods of high drainage flows (Hoang et al., 2013). Previously, Garder et al. (2014) quantified antibiotic-resistant bacteria and resistance genes in tile drained agricultural fields receiving swine manure application. Elevated levels of antibiotic-resistant bacteria and resistance genes were found in manure injection bands in soil following swine manure application, but these genes returned to levels equivalent to the no-manure control plot concentrations one year after application. Tile drainage samples from the same plots maintained under no-till and chisel plow tillage and manure treatments did not show significant differences in antibiotic-resistant bacteria and resistance gene concentrations. The authors suggested that below average precipitation and cumulative tile drainage flow may have contributed to the lack of statistically significant differences. The objective of this study is to identify the effects of tillage and manure treatments on antibiotic-resistant bacteria and resistance gene levels in soil and tile drainage and determine tile flow impacts. This report describes the movement of antibiotic-resistance genes under more normal precipitation and drainage patterns.

2. Materials and methods

2.1. Study site

Four plots were used for this study at Iowa State's Northeast Research and Demonstration Farm, near Nashua, IA (43.0° N, 92.5° W). The soils at the site consist of moderately well to poorly drained Floyd loam, Kenyon silty–clay loam and Readlyn loam overtop of glacial till, with slopes ranging from 1 to 3% (Bakhsh et al., 2005). Plots are maintained in corn–soybean rotations, with nitrogen application in the form of swine manure (one chisel plow and one no-till plot each) or urea and ammonium nitrate (UAN) (one chisel plow and one no-till plot each) prior to the corn growing season (Table S1, supplemental material). Manure has not been applied to the control plots (plots receiving UAN application) since 1978, while the manure plots have been under various manure application rates since 1993. Manure was injected as parallel bands 10 to 15 cm below the soil surface by shanks spaced 76 cm apart on October 31, 2012. UAN was injected into the control plots in late April of 2013. No-till and chisel plow tillage were chosen for this study due to their prevalence in Midwestern agriculture. Tillage disrupts preferential flow paths, or macropores, formed between soil aggregates. Chisel plow tillage loosens and aerates soils while leaving crop residue on top of the soil, limiting erosion potential.

Each 4047 m^2 plot is individually drained by a 10 cm diameter subsurface drain located 1.2 m below the plot's surface. Border drains are located around the edge of each plot to prevent cross flow between plots. Connected to each plot's drain is a sump furnished with a metered effluent pump and a Neptune T-10 2.54 cm diameter flowmeter which allow quantification of drainage. Subsurface flow of individual plots has been monitored at the research site since 1988.

2.2. Sample collection

The manure used in this study was obtained from a commercial swine operation, which incorporates tylosin into feed at subtherapeutic rates (facility manager, personal communications, 2012).

Manure samples were collected directly from the injector on the day of application. Samples were stored in a 4 °C refrigerator overnight before being transported back to Iowa State in a cooler on ice. After subsamples were removed for enterococci analysis, the remaining samples were frozen at −20 °C for DNA extractions to be performed within three months.

Soil samples were collected the day after manure application (November 1, 2012) and the following spring prior to field seeding on May 7, 2013. The process was repeated in the second year of the rotation with samples collected on November 15, 2013 and April 17, 2014. Three composite samples were collected from both the band injection location and interband locations on the two plots which received manure during the first year of the crop rotation. Three composite samples were collected from the plots during the second year due to manure bands no longer being distinguishable. Additionally, three composite samples were also collected from each non-manured control plot. Each composite sample consisted of three 15 cm cores collected along parallel transects. Soil probes were cleaned with 70% ethanol between manure band, interband and control plot sample collections. Each composite sample was placed in a one gallon plastic bag and transported back to Iowa State University in coolers containing ice. Prior to removing subsamples for enterococci and tylosin-resistant enterococci analysis, composite samples were sieved through an 8 mm soil sieve to increase the homogeneity of the sample. Additional subsamples were removed within 24 h of collection for moisture content analysis. The remaining soil was frozen at −20 °C for DNA extraction within three months.

Tile water samples were collected directly from tile discharge in each plot's sump. Samples were collected on a weekly basis following the beginning of tile flow on April 15, 2013 until flow ceased July 15, 2013. Grab samples were also collected following rainstorms to ensure a range of flows were represented. Precipitation patterns were used to predict when increased flow would occur. Four samples were collected from each plot on the day following storms which produced more than 5.08 cm of rain, (typical amount necessary to identify change in drainage flow). Fourteen total grab samples were collected from chisel plow manured drainage, 16 from no-till manured drainage, and 15 from both control plots' drainage. Drainage water was collected in two 1-L plastic bottles and transported back to the Water Quality Research Lab at Iowa State University on ice. Flow meter readings were recorded at each sampling. Instantaneous flow rates were also measured by recording the time necessary to fill 1-L plastic bottles.

2.3. Total and tylosin-resistant enterococci enumeration

Manure, soil and tile water samples were analyzed for enterococci and tylosin-resistant enterococci through membrane filtration as described by APHA (APHA, 1998) with 0.45 μm filters (Millipore, Billerica, MA). Samples were analyzed in triplicate within 24 h of collection. Soil and manure samples were diluted prior to filtration. After filtration the membranes were placed on mEnterococcus agar (Difco, Detroit Michigan) or mEnterococcus agar infused with 35 mg L^{-1} tylosin (Sigma-Aldrich, St. Louis, MO). Tylosin concentrations in mEnterococcus agar were slightly higher than the tylosin resistance breakpoint for enterococci established by the Clinical and Laboratory Standards Institute (CLSI, 2010). Colonies were enumerated after incubation at 35 ± 0.5 °C for 48 h. Results for water samples were reported as colony forming units (cfu) per 100 mL and per gram of manure and soil on a dry weight basis.

2.4. DNA extraction

Tile water samples (250 mL) were filtered through 0.22 μm sterile filters. Mo Bio Power Water DNA kits were used to extract DNA from the filters. Filters were extracted within 24 h of tile water collection or frozen in bead tubes for extraction on a later date. Soil samples were frozen after collection and subsamples (10 g) were later thawed for DNA extraction. DNA was extracted using MoBio Power Soil DNA kits. In order to maximize the yield and purity of manure DNA extracts, the repeated bead beading plus column extraction method (RBBC) was used (Yu and Morrison, 2004). The RBBC method combines bead beating with a lysis buffer containing sodium dodecyl sulfate and EDTA.

2.5. Quantitative PCR analyses

Quantitative PCR was performed on a MJ Research Opticon2 qPCR instrument operated in the 96-well format. Each gene was analyzed separately. Each individual reaction had cumulative volume of 25 μL, consisting of: 2.5 μL of DNA, 5 μL each of forward and reverse primer and 12.5 μL of Qiagen SYBR Green Master Mix. Conditions and primer sequences defined by Garder et al. (2014) were used for *ermB* and *ermF*. *ErmC* qPCR protocols and primer sequences were adapted from Koike et al. (2009). Temperature gradients resulted in an optimal annealing temperature of 51.4 °C for *ermC*. PCR primers and protocols for *msrA* were adapted for this study (Sutcliffe et al., 1996). The optimal annealing temperature for *msrA* was 54 °C. The 16S-rRNA gene in *Bacteria* was also enumerated with Eub338/Eub518 primers and protocols adapted from Fierer et al. (2005). 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 *Escherichia coli* using TOPO TA cloning kits (Invitrogen Corp., Carlsbad, CA). DNA from transformed *E. coli* was extracted using a 5 Prime FastPlasmid Mini Kit. *ErmB* and *ermC* PCR products were amplified from *Enterococcus* isolate Man T1-C, described by Hoang et al. (2013). *ErmF* product originated from a reference *E. coli* strain purchased from M. C. Roberts (University of Washington). *MsrA* product originated from plasmid pAT10 inside *Staphlococcus aureus* strain RN4220, which was also purchased from M. C. Roberts. Product from PCR with Eub primers originated from *Pseudomonas stutzeri* genomic DNA (ATCC 14405). Blanks and negative controls were included in each qPCR assay. Negative controls consisted of PCR grade water and *P. stutzeri* genomic DNA (ATCC 14405). All samples were run in triplicate wells within a single 96-well plate. 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.

Multiple 96-well qPCR plate runs were necessary due to the number of samples analyzed in this study. A single limit of quantification (LOQ) and limit of detection (LOD) was used for each gene for multiple plates. The LOQ copy number per reaction well for each 96-well plate was calculated from the most dilute DNA standard before Ct values deviated from the linear range of the standard curve or from the average Ct of a false positive (amplification above Ct in wells with water as template or *P. stutzeri* genomic DNA) noted in a single run. Once all qPCR runs for a specific gene were complete, the LOQ was set as the highest copies per reaction identified from standard curve analysis or false positive copies per well from the set of plates (Table 1). The LOD was set as smallest copies per reaction identified from standard curve analysis or false positive copies per well from the set of plates. Only values above the LOQ were enumerated. Values between the LOQ and LOD were reported as detected, but unquantifiable. Additionally drainage samples containing concentrations of *ermB*, *ermC* and *ermF* above limits of quantification were normalized to 16S-rRNA gene abundances.

2.6. Statistical analysis

Statistical analysis was performed with JMP®, Version 10.0.2. (SAS Institute Inc., Cary, NC, 1989–2007). The non-parametric Wilcoxon ranked sum test was used to determine if resistance gene concentrations in tile drainage from different plots were

Table 1
Limits of quantification (LOQ) and detection (LOD) for qPCR amplification of macrolide resistance genes extracted from manure, soil and water.

Gene	Manure		Soil		Water	
	LOQ	LOD	LOQ	LOD	LOQ	LOD
	Copies g^{-1} manure		Copies g^{-1} soil		Copies 100 mL^{-1}	
ermB	4,800	480	6,400	640	480	48
ermC	7.52×10^4	ND[a]	1.00×10^5	ND	7,520	ND
ermF	6,880	2240	9,170	2,990	688	224
msrA	7.92×10^4	ND	1.06×10^5	ND	7,920	ND

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

significantly different. This test was chosen because it does not require normally-distributed data and can accommodate missing data. Water samples with resistance genes below the specified LOQ and above the LOD were assigned the average of the LOQ and LOD for analysis. Additionally, sample concentrations below the LOD were assigned a value of one for analysis. Wilcoxon ranked sum test was also performed on enterococci present in tile drainage. Resistant enterococci concentrations were not analyzed due to a lack of positive samples. The Wilcoxon rank sum test is a one-way test; therefore initial analysis compared the effect of tillage using water samples within a growing season as replicates. We concluded that tillage was non-significant and further analysis used no-till and chisel plow plots as additional replication to assess the effect of manure application (results described subsequently).

3. Results

3.1. Enterococci and tylosin-resistant enterococci

Total enterococci followed the expected trends in relative concentrations with the greatest levels found in manure followed by soil and water (Tables 2, 3). Enterococci concentrations were greatest in soil samples collected from the manure band location immediately following manure application. Average enterococci concentrations in the manure band for both no-till and chisel plow plots were equivalent to control plots the following spring (Table 2).

The average enterococci concentration in manure was 1.76×10^3 CFU g^{-1}, with 83% resistant to tylosin. Band locations were unidentifiable during soil sample collection in the second year following manure application. Concentrations of enterococci in the manured plot soils were similar in the second year to levels in the control plot soils. Tylosin-resistant enterococci concentrations were the same order of magnitude as total enterococci in the band location immediately following manure application. The resistant enterococci levels dropped two orders of magnitude in band samples collected the following spring. No tylosin-resistant enterococci were detected in interband or control plot samples in the spring of 2013 following manure application or any of the soil samples collected during the second year of the crop rotation.

Enterococci levels in drainage water from manured plots ranged from 0 to 110 CFU 100 ml^{-1} and 0 to 47 CFU 100 ml^{-1} in control plot drainage (Table 3). No significant differences ($p > 0.10$) in enterococci concentrations were detected between tillage practices or manure application using the Wilcoxon Ranked Sum Test. Enterococci were frequently detected in drainage samples from all four plots. The geometric mean for enterococci in recreational waterbodies of 33 CFU 100 mL^{-1} (USEPA, 1986) was exceeded in 8 of 64 samples. There was not a significant relationship between time after application or instantaneous flow rate (data not shown) and enterococci concentrations ($p > 0.10$). Cumulative tile drainage for the 4 plots in 2013 ranged from 370 to 465 m^3, compared to the 10-year averages which ranged from 161 to 337 m^3 (Table S2, supplemental material).

Tylosin-resistant enterococci were rarely detected in drainage water (Table 3) and concentrations were not significantly different ($p > 0.10$) between manure or tillage treatments using Wilcoxon Ranked Sum Test. Mean tylosin-resistant enterococci concentrations in drainage water were less than 1 CFU 100 mL^{-1} in two plots and not detected in a third. Tylosin-resistant enterococci were only detected in three samples from plots with histories of manure application.

3.2. Antibiotic resistance genes

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

The highest soil concentrations for all *erm* genes were detected in soil manure bands immediately following manure application, and only *msrA* was not found in quantities above the specified LOD (Table 4). Each gene exceeded 10^6 copies g^{-1} soil in manure bands (Table 4), 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 manure band soils 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

Table 2
Mean soil concentrations of total and tylosin-resistant enterococci in manure band and interband sampling locations and in no-manure control plots under no-till and chisel plow tillage.

Enterococci	Treatment	Sample location	Fall 2012	Spring 2013	Fall 2013	Spring 2014
			Mean CFU g^{-1} soil ± std. dev.			
Total	No-till manure	Band	314 ± 154	8 ± 10	1 ± 4[a]	1 ± 4
		Interband	3 ± 4	3 ± 5		
	No-till control	Composite	26 ± 26[b]	13 ± 38	7 ± 12	7 ± 12
	Chisel–plow manure	Band	254 ± 24	7 ± 6	0	4 ± 6
		Interband	1 ± 2	0		
	Chisel plow control	Composite	13 ± 16	5 ± 8	4 ± 6	6 ± 6
Tylosin-resistant	No-till manure	Band	322 ± 200	10 ± 10	0	0
		Interband	0	0		
	No-till control	Composite	0	0	0	0
	Chisel–plow manure	Band	226 ± 32	7 ± 10	0	0
		Interband	0	0		
	Chisel plow control	Composite	0	0	0	0

[a] Composite samples were collected in the second year after manure bands were no longer visible.
[b] Control plots had no manure application and the samples were composites.

Table 3
Total and tylosin-resistant enterococci in drainage water during April through July 2013.

Treatment	Total enterococci			Tylosin resistant		
	Mean[a] (CFU 100 mL^{-1}) ± std. dev.	n[c]	Non-detects (%)	Mean (CFU 100 mL^{-1}) ± std. dev.	n	Non-detects (%)
No-till manure	16 ± 20	12	25	<1	2	88
No-till control	19 ± 16	12	20	ND[b]	0	100
Chisel plow manure	22 ± 31	13	7	16 ± 0	1	94
Chisel plow control	9 ± 11	12	20	<1	1	93

[a] Means were calculated excluding the samples where enterococci were not detected.
[b] Tylosin-resistant enterococci were not detected in drainage samples from the no-till control plot.
[c] Number of samples above LOQ included in means.

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 water grab samples, while levels of *msrA* were not above the limit of detection (Table 5). *ErmB* was detected in 82% of the water samples collected from the no-till, manure treated plot, with 59% above the LOQ (Table 5). This was followed by the manure treated, chisel plow plot in which *ermB* was detected in 73% of all samples, with 33% of samples above the LOQ. Only one drainage sample in each control plot was above the limit of quantification for *ermB*. However, similar percentages of samples from all plots were above the limit of detection and below quantification (24–44%). Mean concentrations of *ermB* in samples above the limit of quantification were similar in chisel plow and no till plots receiving manure application. The Wilcoxon Ranked Sum Test did not identify significant differences ($p > 0.10$) in *ermB* concentrations between the no-till and chisel plow treatments for both the manured and control plots and therefore were combined for further analysis. After data for the two tillage regimes were combined, concentrations of *ermB* in water from the manure treated plots were significantly greater ($p < 0.01$) (4258 copies 100 mL^{-1}) than in water from the control plots (3170 copies 100 mL^{-1}) using the Wilcoxon Ranked Sum Test. *ErmB* was detected in all five water samples from no-till, manure treated plot and in all but one sample from the chisel plow, manure treated plot collected during rainfall events. Additionally, the 61% of quantifiable *ermB* samples were from the first half of the sampling period (Fig. 1).

ErmC was detected in drainage water from all four plots. Although *ermC* had the greatest average concentration in samples above the limit of quantification between the three genes detected (Table 5), levels in water from the manure treated plots were not significantly different ($p > 0.10$) from water draining from the control plots using the Wilcoxon Ranked Sum Test. The frequencies of detection for the four plots were quite similar, ranging from 25 to 33%. The majority of *ermC* detections were from samples collected during the second half of the sampling season (Fig. 2).

ErmF was detected in 44% of tile drainage samples from the manure applied, no-till plot and 33% of samples from the manure applied, chisel plow plot. However, the majority of samples collected from the manure applied no-till plot were above the specified LOQ, while bulk of detects in the manure applied chisel plow plot were below the LOQ (Table 5). *ErmF* was not detected in any water samples from the chisel plow, control plot, and only one sample collected from the no-till, control plot. The Wilcoxon Ranked Sum Test did not identify significant differences ($p > 0.10$) in *ermF* concentrations between the no-till and chisel plow treatments for both the manure applied and control plots. Therefore the data for the different tillage treatments were combined for further analysis. Average concentrations of *ermF* in the drainage from manure treated plots (1679 copies 100 mL^{-1}) were significantly greater ($p < 0.01$) than those in drainage from the control plots (no detects over the LOQ) using the Wilcoxon Ranked Sum Test. The majority of water samples containing *ermF* concentrations above the LOQ were collected during the first half of the sampling season (Fig. 3), similar to *ermB*.

The 16S rRNA concentrations in drainage water samples ranged from 3.27×10^5 to 7.17×10^7 copies 100 ml^{-1} and 16S-rRNA abundance for all treatments decreased over the sampling period. For drainage samples with *ermB*, *ermC* and *ermF* over the limits of quantification,

Table 4
Erm gene concentrations in soil under no till or chisel plow management after swine manure application or without manure application.

Gene	Treatment	Sample location	Fall 2012	Spring 2013	Fall 2013[a]	Spring 2014[a]
			Average gene copies g^{-1} soil			
ermB	No-till manure	Band	5.46×10^7	2.66×10^5	<LOD[b]	1.59×10^5
		Interband	<LOQ[c]	<LOD		
	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	5.77×10^5	<LOD	<LOD
		Interband	<LOD	<LOD		
	Chisel plow control	Composite	<LOD	2.88×10^5	<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

[a] Composite sample: manure bands could not be identified.
[b] Less than limit of detection (LOD) for specified gene (Table 1).
[c] Less than limit of quantification (LOQ), greater than limit of detection for specified gene (Table 1).

Table 5
Detection frequency and concentrations of *erm* genes in tile drainage following manure application in plots under no-till and chisel plow tillage regimes from April through July 2013.

Gene	Treatment	Mean (>LOQ)		Frequency (% of samples)	
		(gene copy 100 mL^{-1})	n[b]	<LOQ and >LOD	Non-detects
ermB	No-till manure	4,670 ± 7,530	10	24	18
	No-till control	3,170 ± 2,330	2	31	56
	Chisel plow manure	3,940 ± 4,090	5	40	33
	Chisel plow control	636 ± 0	1	44	50
ermC	No-till manure	$1.79 \times 10^4 \pm 1.06 \times 10^4$	5	0	71
	No-till control	$6.35 \times 10^4 \pm 6.59 \times 10^4$	5	0	69
	Chisel plow manure	$9.71 \times 10^4 \pm 1.55 \times 10^5$	5	0	73
	Chisel plow control	$1.36 \times 10^4 \pm 5{,}140$	4	0	75
ermF	No-till manure	1,810 ± 756	7	6	56
	No-till control	NQ[a]	0	6	94
	Chisel plow manure	1,230 ± 204	2	27	67
	Chisel plow control	NQ[a]	0	0	100

[a] Not quantifiable (NQ): no drainage water samples contained concentrations of *ermF* above the specified LOQ.
[b] Number of samples above LOQ.

the ratio of *erm* genes to 16S-rRNA genes ranged from 0.001% to 3.1% (Supplemental Figs. S1, S2, S3).

4. Discussion

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

Concentrations of total and tylosin-resistant enterococci in manure bands following application were approximately an order of magnitude lower than reported by Hoang et al. (2013) and Garder et al. (2014). Additionally, enterococci concentrations in band locations dropped to background concentrations within six months, while Garder et al. (2014) reported concentrations above those in non-manured control plots after overwintering. Prior studies have reported percentages of tylosin-resistant bacteria in manure treated soils ranging from 1 to 100% (Hoang et al., 2013; Garder et al., 2014; Onan and LaPara, 2003; Halling-Sorensen et al., 2005). The large range of tylosin resistance in soils noted in previous studies likely stems from variable initial concentrations in manure. Levels of antibiotics administered in feed vary depending on the growth cycle of the swine, which affect concentrations of resistant bacteria excreted in manure.

Erm genes concentrations in soil followed a similar pattern to enterococci concentrations during the first year after manure application. *Erm* genes were greatest in the manure-band soil immediately after manure application. However, concentrations of *ermB* and *ermF* were both at least two orders of magnitude lower than concentrations previously reported by Garder et al. (2014). Additionally, *ermB* was only detected

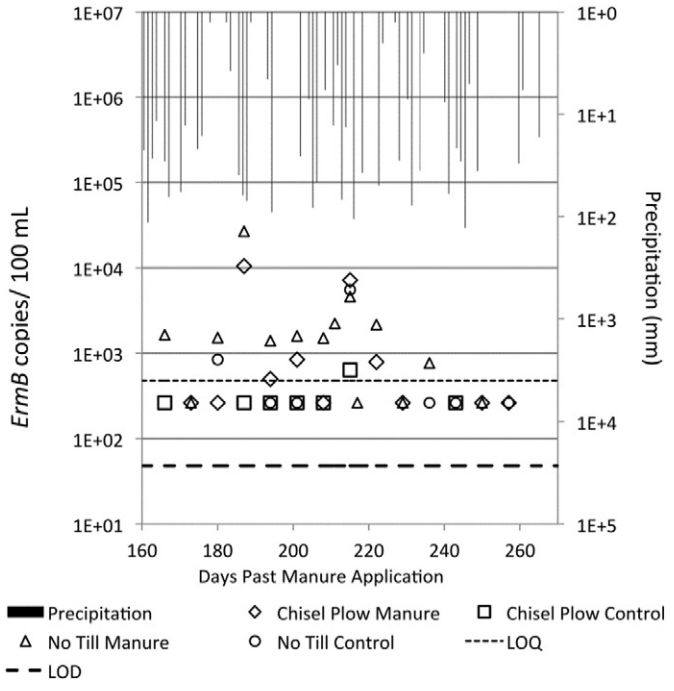

Fig. 1. *ErmB* concentrations in tile drainage water following manure application in plots under no-till and chisel plow regimes with LOQ and LOD. Concentrations less than the LOQ and greater than LOD were assigned the average value of the LOQ and LOD for visualization.

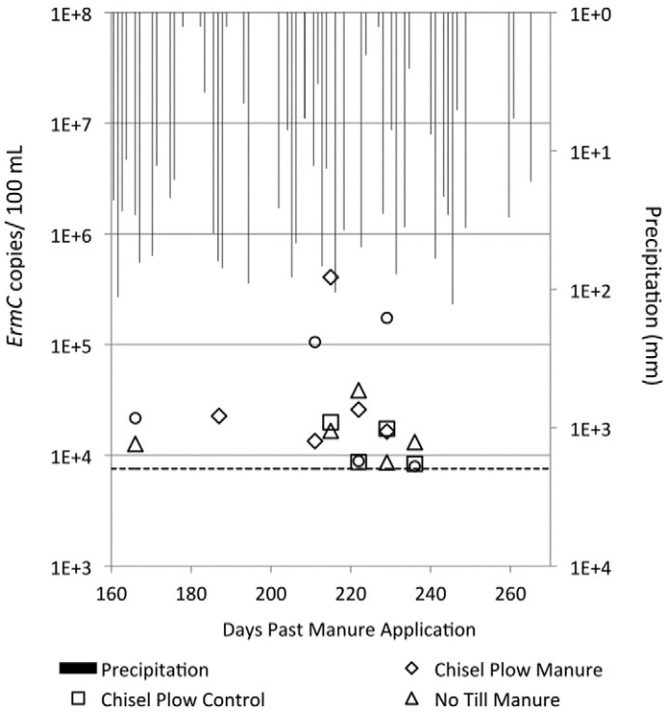

Fig. 2. *ErmC* concentrations in tile drainage water following manure application in plots under no-till and chisel plow regimes with the LOQ (dotted line).

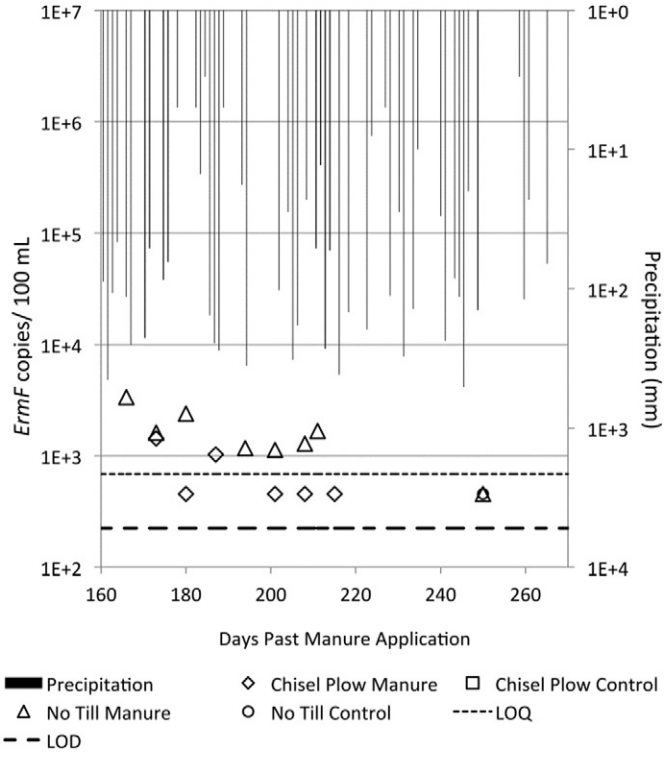

Fig. 3. *ErmF* concentrations in tile drainage water following manure application in plots under no-till and chisel plow regimes with LOQ and LOD. Concentrations less than the LOQ and greater than LOD were assigned the average value of the LOQ and LOD for visualization.

in one interband sample in the first year of the crop rotation, while Garder et al. (2014) detected *ermB* and *ermF* at quantifiable levels in every interband sample in the two previous years (fall 2010–spring 2012). The drought conditions in the summer of 2012 in Northeast Iowa may have caused additional stress to bacteria hosting the resistance genes and therefore hastened the return of overall concentrations to those found in no-manure control plot soils (Cools et al., 2001; Byappanahalli et al., 2012). The reduction in *ermB* and *ermF* genes in soil over winter are similar to those reported previously (Garder et al., 2014; Fahrenfeld et al., 2014).

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

Precipitation totals for April through June in 2011 and 2012 at the study site were 31.8 cm and 26.5 cm, respectively. These totals were nearly doubled in 2013, with 62.4 cm of precipitation from April through June. Additionally, total drainage from plots in 2011 and 2012 were below the ten year average, while flows from the same plots in 2013 exceeded the average. Therefore, above average precipitation witnessed in spring of 2013 may have created additional opportunities for bacteria harboring resistance genes to be transported from soil to drainage water.

When tillage treatments were combined, both *ermB* and *ermF* concentrations in tile drainage were significantly greater ($p < 0.01$) in plots with manure application than their control plot counterparts. Garder et al. (2014) did not detect any significant differences in gene concentrations in drainage water due to tillage or manure treatments during 2011 and 2012 at the same study site. Additionally, Garder et al. (2014) used a unique LOQ for each 96-well qPCR run. Therefore, samples in this study lower than the specified LOQ, but greater than the LOD would have been classified as quantifiable by Garder et al. (2014). Setting a conservative LOQ in this study allowed for greater confidence by eliminating false positives. However, the sensitivity of the Wilcoxon Ranked Sum analysis was likely reduced by assigning a uniform rank for samples below the LOQ and LOD.

ErmB was the most frequently detected gene in water, with the majority of drainage samples from manure treated plots containing concentrations above the limit of detection. *ErmF* was the next most prevalent gene in manure treated plots with 44% of samples above the limit of detection. The detection frequency and magnitude for *ermB* and *ermF* were consistent with results obtained by Garder et al. (2014); however, their detection frequencies were similar with respect to the manure treatment and no-manure control. Koike et al. (2009) detected *ermB* in 87% of samples and *ermF* in 40% of samples taken from wells near swine lagoons, which were previously identified as being contaminated by swine lagoon leachate. The concentrations and frequencies of antibiotic resistance genes in groundwater beneath the lagoons were greater than those seen in results from this study.

ErmC was detected at the highest concentrations of the three genes which were identified, but detection frequency and concentrations were comparable across all treatments. Additionally, the majority of the positive samples were from later in the sampling season, as opposed to *ermB* and *ermF*, which were mainly detected during the first portion. Hoang et al. (2013), using PCR, only detected *ermC* in 9% of enterococci isolates which were phenotypically resistant to tylosin. Phylogenic analysis performed on resistance genes by Koike et al. (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. Isolates containing *ermC* were in the low-G + C content group. *ErmC* was not detected in water or soil collected from wastewater trenches exporting waste from a swine farm (Li et al., 2013). *ErmC* was detected in hog house effluent by Chen et al. (2007), but less frequently than the five other *erm* genes screened for in the study. *ErmC* concentrations in water samples from this study are likely from naturally occurring bacterial communities in soil, due to similar concentrations in manured and control plot drainage and the majority of quantifiable concentrations occurring towards the end of the tile drainage period.

MsrA was not detected in any samples, including manure; however, Hoang et al. (2013) detected *msrA* in 97% of tylosin-resistant enterococci isolated from manure, soil and water samples. While *erm* genes confer resistance by target site modification, *msrA* is responsible for encoding a transport protein containing two ATP-binding domains. The ATP-binding domains are part of an efflux system which works to translocate macrolides across cell membranes (Ross et al., 1995). Although Hoang et al. (2013) identified *msrA* in nearly 100% of enterococci isolates resistant to tylosin, the proportion of tylosin-resistant enterococci to total *erm* genes in soil or water samples is quite small.

Increased levels of antibiotic-resistant bacteria in the environment are of great concern due to their associated public health risks. The export of *ermB* and *ermF* in drainage water at elevated levels due manure application may be of concern because of the large land areas that receive swine manure and have subsurface drainage. Currently, water quality standards do not exist for antibiotic-resistant bacteria or resistance genes. However, relative concentrations of resistant bacteria and genes may be monitored in order to identify the effects of anthropogenic activities on microbial communities in soil and water. This study utilized both phenotypic resistance to tylosin in enterococci and enumeration of macrolide antibiotic resistance genes through qPCR. Although enterococci are used commonly as an indicator organism for

fecal contamination in surface waters (USEPA, 1986), results from this study indicate that concentrations of antibiotic-resistant enterococci do not accurately portray total concentrations of resistance genes found in soil and water microbial communities. The *erm* genes are found in a wide array of bacteria (Koike et al., 2009; Park et al., 2010), but we did not identify which species carry the *erm* genes in drainage water. In order to more accurately represent antibiotic resistance in environmental samples, additional research is needed to help identify the bacteria harboring the majority of resistance genes. Regardless, the transport of antibiotic resistance genes among different environmental compartments (e.g. soil, drainage water) might facilitate their spread among indigenous bacteria through horizontal gene transfer (West et al., 2010).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.01.132.

References

APHA, 1998. Standard Methods for the Examination of Water and Wastewater 9230C. American Public Health Association, Washington, D.C.

Apley, M.D., Bush, E.J., Morrison, R.B., Singer, R.S., Snelson, H., 2012. Use estimates of in-feed antimicrobials in swine production in the United States. Foodborne Path. Dis. 9 (3), 1–8.

Bakhsh, A., Kanwar, R.S., Karlen, D.L., 2005. Effects of liquid swine manure applications on NO_3–N leaching losses to subsurface drainage water from loamy soils in Iowa. Agric. Ecosyst. Environ. 109 (1–2), 118–128.

Byappanahalli, M.N., Nevers, M.B., Korajkic, A., Staley, Z.R., Harwood, V.J., 2012. Enterococci in the environment. Microbiol. Mol. Biol. Rev. 76 (4), 685–706.

Campagnolo, E.R., Johnson, K.R., Karpati, A., Rubin, C.S., Kolpin, D.W., Meyer, M.T., Esteban, J.E., Currier, R.W., Smith, K., Thu, K.M., McGeehin, M., 2002. Antimicrobial residues in animal waste and water resources proximal to large-scale swine and poultry feeding operations. Sci. Total Environ. 299, 1–3.

CAST, 1981. Antibiotics in Animal Feeds; Report No. 88. Council for Agricultural Science and Technology, Ames, IA.

Chee-Sanford, J.C., Mackie, R.I., Koike, S., Krapac, I.G., Lin, Y.F., Yannarell, A.C., Maxwell, S., Aminov, R.I., 2009. Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. J. Environ. Qual. 38 (3), 1086–1108.

Chen, J., Yu, Z., Michel Jr., F.C., Wittum, T., Morrison, M., 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 (14), 4407–4416.

Chouchani, C., El Salabi, A., Marrakchi, R., Ferchichi, L., Walsh, T.R., 2012. First report of *mefA* and *msrA*/*msrB* multidrug efflux pumps associated with bla_{TEM-1} β-lactamase in *Enterococcus faecalis*. Int. J. Infect. Dis. 16, e104–e109.

CLSI, 2010. Performance standards for antimicrobial susceptibility testing; twentieth informational supplement. M100-S20. Clinical and Laboratory Standards Institute, Wayne, PA.

Cools, D., Merckx, R., Vlassak, K., Verhaegen, 2001. Survival of *E. coli* and *Enterococcus* spp. derived from pig slurry in soils of different texture. Appl. Soil Ecol. 17, 53–62.

Cromwell, G.L., 1991. Antimicrobial agents. In: Miller, E.R., Ullrey, D.E., Lewis, A.J. (Eds.), Swine Nutrition. Butterworth-Heinemann, Boston, MA, pp. 297–314.

Cromwell, G.L., 2002. Why and how antibiotics are used in swine production. Anim. Biotechnol. 13 (1), 7–27.

Dolliver, H., Gupta, S., 2007. Antibiotic losses in leaching and surface runoff from manure-amended agricultural land. J. Environ. Qual. 37, 1227–1237.

Fahrenfeld, N., Knowlton, K., Krometis, L.A., Hession, W.C., Xia, K., Lipscomb, E., Libuit, K., Green, B.L., Pruden, A., 2014. Effect of manure application on abundance of antibiotic resistance genes and their attenuation rates in soil: field-scale mass balance approach. Environ. Sci. Technol. 48, 2643–2650.

Fierer, N., Jackson, J.A., Vilgalys, R., Jackson, R.B., 2005. Assesment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Appl. Environ. Microbiol. 71, 4117–4120.

Garder, J.L., Moorman, T.B., Soupir, M.L., 2014. Transport and persistance of tylosin-resistant enterococci, *erm* genes, and tylosin in soil and drainage water from fields receiving swine manure. J. Environ. Qual. 43, 1484–1493.

Halling-Sorensen, B., Jacobsen, A.M., Jenson, J., Sengelov, G., Vaclavix, E., Ingerslev, F., 2005. Dissipation and effects of chlortetracycline and tylosin in two agricultural soils: a field-scale study in Southern Denmark. Environ. Toxicol. Chem. 24 (4), 802–805.

Hawkey, P.M., 1998. The origins and molecular basis of antibiotic resistance. Br. Med. J. 317, 657–660.

Hays, V.W., 1977. Effectiveness of Feed Additive Usage of Antibacterial Agents in Swine and Poultry Production. Office of Technology Assessment, U.S. Congress, Washington, DC. (Edited Version: Hays, V.W. The Hays Report; Rachelle Laboratories, Inc.: Long Beach, CA, 1981).

Heuer, H., Schmitt, H., Smalla, K., 2011. Antibiotic resistance gene spread due to manure application on agricultural fields. Curr. Opin. Microbiol. 14 (3), 236–243.

Hoang, T.T.T., Soupir, M.L., Liu, P., Bhandari, A., 2013. Occurrence of tylosin-resistant enterococci in swine manure and tile drainage systems under no-till management. Water Air Soil Pollut. 224 (11), 1–15.

Kanwar, R.S., Colvin, T.S., Karlen, D.L., 1997. Ridge, moldboard, chisel, and no-till effects on tile water quality beneath two cropping systems. J. Prod. Agric. 10 (2), 227–234.

Kanwar, R.S., Bjorneberg, D., Baker, D., 1999. An automated system for monitoring the quality and quantity of subsurface drain flow. J. Agric. Eng. Res. 73 (2), 123–129.

Khachatourians, G.G., 1998. Agricultural use of antibiotics and the evolution and transfer of antibiotic-resistant bacteria. CMAJ 159 (9), 1129–1136.

Kladivko, E.J., Grochulska, J., Turco, R.F., Van Scoyoc, G.E., Eigel, J.D., 1998. Pesticide and nitrate transport into subsurface tile drains of different spacings. J. Environ. Qual. 28, 997–1004.

Koike, S., Aminov, R.I., Yannarell, A.C., Gans, H.D., Krapac, I.G., Chee-Sanford, J.C., Mackie, R.I., 2009. Molecular ecology of macrolide–lincosamide–streptogramin B methylases in waste lagoons and subsurface waters associated with swine production. Microb. Ecol. 59 (3), 487–498.

Leclercq, R., Courvalin, P., 1991. Bacterial resistance to macrolide, lincosamide, and streptogramin antibiotics by target modification. Antimicrob. Agents Chemother. 35, 1267–1272.

Li, L., Sun, J., Liu, B., Zhao, D., Ma, J., Deng, H., Li, X., Hu, F., 2013. Quantification of lincomycin resistance genes associated with lincomycin residues in waters and soils adjacent to representative swine farms in China. Front. Microbiol. 4, 364.

McEwen, S.A., Fedorka-Cray, P.J., 2002. Antimicrobial use and resistance in animals. Clin. Infect. Dis. 34 (Suppl. 3), S93–S106.

Onan, L.J., LaPara, T.M., 2003. Tylosin-resistant bacteria cultivated from agricultural soil. FEMS Microbiol. Lett. 220 (1), 15–20.

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

Roberts, M.C., Sutcliffe, J., Courvalin, P., Jensen, L.B., Rood, J., Seppala, H., 1999. Nomenclacture for macrolide and macrolide–lincosamide–streptogramin B resistance determinants. Antimicrob. Agents Chemother. 43 (12), 2823–2830.

Ross, J.I., Eady, E.A., Cove, J.H., Baumberg, S., 1995. Identification of a chromosomally encoded ABC-transport system with which the staphylococcal erythromycin exporter MsrA may interact. Gene 153, 93–98.

Sutcliffe, J., Grebe, T., Tait-Kamradt, A., Wondrack, L., 1996. Detection of erythromycin-resistant determinants by PCR. Antimicrob. Agents Chemother. 40 (11), 2562–2566.

USDA, 2014. 2012 Census of Agriculture. United States Department of Agriculture. Washington, D.C.

USEPA, 1986. Ambient water quality criteria for bacteria — 1986. EPA440/5-84-002.

Weisblum, B., 1995. Erythromycin resistance by ribosome modification. Antimicrob. Agents Chemother. 39 (3), 577–585.

Weisblum, B., 1998. Macrolide Resistance. Drug Resist. Updat. 1, 29–41.

West, B.M., Liggit, P., Clemans, D., Francoeur, S.N., 2010. Antibiotic resistance, gene transfer, and water quality patterns observed in waterways near CAFO farms and wastewater treatment facilities. Water Air Soil Pollut. 217, 473–489.

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

Zucker, L.A., Brown, L.C., 1998. Agricultural drainage. Water Quality Impacts and Subsurface Drainage Studies in the Midwest. The Ohio State University Extension Bulletin, pp. 871–898.