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Seasonal variation of macrolide resistance gene abundances in the South Fork Iowa River Watershed

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HIGHLIGHTS

- Subsurface artificial drainage contained significantly higher concentrations of *ermB* and *ermF* than surface water.
- Differences in resistance gene concentrations significantly differed according to temporal variations.
- Resistance gene concentrations in surface water strongly correlated with average daily flowrates.

GRAPHICAL ABSTRACT

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ABSTRACT

The Midwestern United States is dominated by agricultural production with high concentrations of swine, leading to application of swine manure onto lands with artificial subsurface drainage. Previous reports have indicated elevated levels of antibiotic resistance genes (ARGs) in surface water and groundwater around confined animal feeding operations which administer antimicrobials. While previous studies have examined the occurrence of ARGs around confined swine feeding operations, little information is known how their transport from tile-drained fields receiving swine manure application impacts downstream environments. To further our knowledge in this area, water samples were collected from five locations in the agriculturally dominated South Fork Iowa River Watershed with approximately 840,000 swine present in the 76,000 ha basin. Samples were collected monthly from three stream sites and two main artificial subsurface drainage outlets. Samples were analyzed for macrolide resistance genes *ermB*, *ermF* and 16S rRNAgene abundance using qPCR. Abundance of *erm* genes ranged from below limits of quantification to $>10^7$ copies 100 mL^{-1} water. Eighty-nine percent of stream water samples contained one of these two ARGs. Results indicate significantly more *ermB* and *ermF* in main drainage outlets than stream samples when normalized by 16S rRNA abundance ($p < 0.0001$). Both artificial drainage locations revealed temporal trends for *ermB* and *ermF* abundance when normalized to 16S rRNA abundance. The higher resistance gene concentrations identified in artificial drainage samples occurring mid-Spring and late-Fall are likely due to manure application.

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1. Introduction

Over the past few decades technological advances in in genetics, nutrition, housing and veterinary services coupled with a shift to contract production farms has led to increases in hog production sizes, a reduction in farm numbers and an increase in antibiotic consumption. The number of hog farms in the United States decreased 70% from 1992 to 2009 from over 240,000 thousand farms to approximately 71,000, while the number of farms containing 2000 head or more increased from 30% to 86% (McBride and Key, 2013). Large contract farms concentrate substantial quantities of manure in pits housed below swine confinements, which is readily applied to agricultural land as organic fertilizer. While manure application provides beneficial nutrients for crops, application is capable of transporting excessive nutrients and pathogens off site into surrounding waterways (Randall and Mulla, 2001; Oliver et al., 2005; Nguyen et al., 2013; Given et al., 2016; Hruby et al., 2016). Of additional concern is the ability of swine manure to harbor antibiotic resistant bacteria.

Antibiotics are administered to swine for disease treatment, disease control, disease prevention and until recently for growth promotion (Veterinary Feed Directive, 2016). Over 5.07×10^5 kg of tetracycline and 1.65×10^5 kg of tylosin were estimated for incorporation into swine production in 2012 (Apley et al., 2012). A study performed by Looft et al. (2012) identified significantly higher concentrations of antibiotic resistance genes (ARGs) in swine intestinal tracts that were administered regimens of performance enhancing antibiotics when compared to an antibiotic free control group. Additionally, antibiotics, antibiotic resistant bacteria and ARGs have been identified in ground and surface water surrounding confined animal feeding operations in the USA and elsewhere (Bonelli et al., 2014; Campagnolo et al., 2002; Chee-Sanford et al., 2009; Heuer et al., 2011; Zhu et al., 2013). However, less is known regarding the export of ARGs off agricultural land into surrounding surface waters.

Waterways in agriculturally dominated watersheds in the Upper Midwestern United States are impacted by overland flow and artificial subsurface drainage. Subsurface artificial drainage consists of a network of corrugated piping approximately 1 m below hydric soils, hastening the movement of shallow groundwater to surface waters. Previous studies have identified elevated nutrient and pathogen concentrations in artificial subsurface drainage (David, 1997; Jaynes et al., 2001; Given et al., 2016). Additionally, Luby et al. (2016) identified significantly higher concentrations of *erm* (erythromycin ribosome methylation) genes in plot scale artificial subsurface drainage receiving manure application when compared to non-manured drainage. The *erm* family of genes encodes methyltransferase enzymes, which are responsible for reducing the binding ability of antibiotics within the macrolide, lincosamides and streptogramin B (MLS_B) family of antibiotics (Weisblum, 1998; Leclercq and Courvalin, 1991). Increasing levels of resistance to the MLS_B family is of great concern due to the group's inclusion of antibiotics which are critically important to agricultural and human health (Huerta et al., 2013).

While there is significant knowledge regarding increases in ARG concentrations in agricultural settings due to manure application, less is known regarding their transport into larger stream networks where human exposure may occur (Pei et al., 2006). The presence of *erm* genes in artificial drainage suggests there is opportunity for horizontal transfer to pathogenic species in connecting waterways (West et al., 2011). In order to characterize potential risks associated with antibiotic resistance in recreational waterbodies, the impact of agriculturally derived inputs must first be identified. The objective of the study is to quantify *erm* genes in main artificial drainage and surface waters in an agriculturally dominated watershed under varying spatial and temporal conditions. This information is needed to identify impacts of swine manure additions on ARG environmental reservoirs.

2. Methods

2.1. Study site

Five locations were sampled in the South Fork Iowa Watershed. The Iowa River's South Fork Watershed, resides mainly in Hamilton and Hardin, IA on the Des Moines Lobe in the United States' Midwestern Corn Belt. The 76,000 ha watershed is dominated by row crop agriculture, primarily in corn and soybean rotation and contains 169 confined animal feeding operations (CAFOs) (Tomer et al., 2008). Approximately 80% of the watershed contains artificial subsurface drainage, commonly referred to as tile drainage (Green et al., 2006a, 2006b). Two of the five sampling locations were main artificial subsurface drainage outlets which drain directly into Tipton Creek (TC241 and TC242). No CAFOs are located in the drainage districts associated with either drainage outlet; however, swine manure is readily applied to cropland within the boundaries (Personal communication, Kevin Cole, USDA-ARS). The remaining three sites were located on Beaver Creek (BC350), South Fork main branch (SF450) and Tipton Creek (TC323) (Fig. 1). Tipton Creek drains into the South Fork main branch prior to reaching sampling site SF450. Downstream of the watershed the Iowa River continues to flow southeast until reaching the Mississippi River. Given et al. (2016) estimated that between 30 and 60% of the watershed receives 93–186 $m^3\ ha^{-1}$ of swine manure annually and the majority of this manure is injected as bands in late fall. Additional potential sources of fecal pollution in surface waters within the watershed include human and naturally occurring wildlife additions.

2.2. Sample collection

Grab samples were collected monthly or bimonthly from August 2011 to December 2014 from three stream sites and two main artificial subsurface drainage outlets in the South Fork Iowa Watershed. Between 35 and 43 samples were collected from each location. Samples for the two drainage outlets, which feed into Tipton Creek, were collected directly from the outlets. TC241 and TC242 are responsible for draining approximately 150 and 1040 ha, respectively. Surface water samples were collected directly from the three streams. Samples were transported on ice to the USDA National Laboratory for Agriculture and the Environment. Daily stream flow measurements were derived from methods described by Tomer et al. (2008).

2.3. DNA extraction and quantitative PCR

Water samples (250–500 mL) were filtered on 0.22 um filters (Millipore, Billerica, MA) within 24 h of sample collections. Filters were then frozen at −20 °C for DNA extraction at a later date. DNA was extracted using Mo Bio Power Water DNA kits. Conditions and primer sequences defined by Luby et al. (2016) were used for *ermB*, *ermF* and Eub338/Eub518 for 16S rRNA bacterial gene concentrations. *Erm* resistance genes were chosen based on the frequent detection of tylosin by Washington et al. (2017) in surface waters and tile drainage within South Fork Iowa River Watershed. Quantitative standards for qPCR were created through insertion of amplified product into pCR-4TOPO in *Escherichia coli* using TOPO TA cloning kits (Invitrogen Corp., Carlsbad, CA). Transformed *E. coli* plasmid DNA was extracted using 5 Prime FastPlasmid Mini Kit (5 Prime, Gaithersburg, MD). PCR product was amplified from *Pseudomonas stutzeri* genomic DNA (ATCC 14405) using Eub338/Eub518 primers. *ErmB* product was amplified from *Enterococcus* isolate Man T1-C described by Hoang et al. (2013). *ErmF* product originated from plasmid pVA831 in *E. coli* strain HB101 bought from M.C. Roberts (University of Washington). All samples were run in triplicate wells in the same 96-well plate. *P. stutzeri* DNA and PCR grade water were used as template for negative controls. *P. stutzeri* (ATCC 14405) DNA does not contain *ermB* or *ermF* and was used as a negative control to identify if resistance gene primers were amplifying sequences

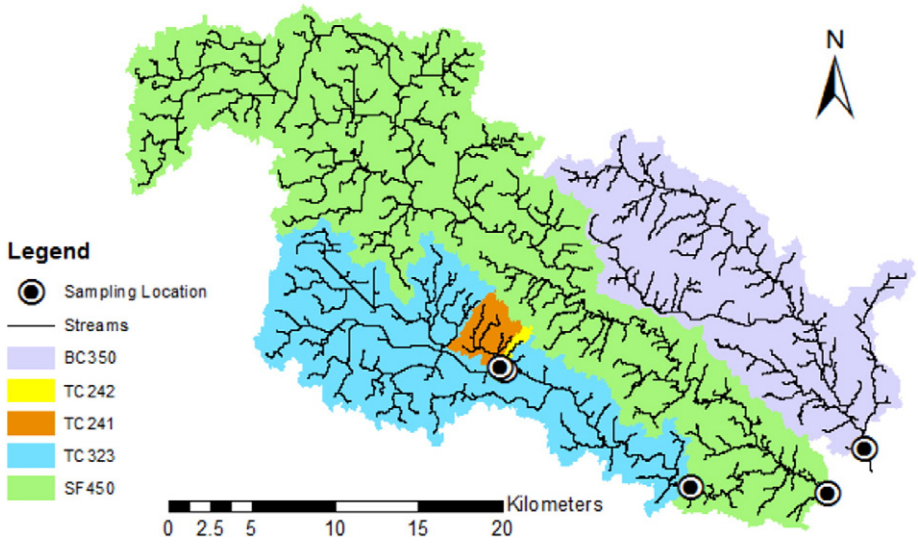

Fig. 1. Surface and drainage water sampling locations and drainage areas in the South Fork of the Iowa River. Subsurface drainage sampling locations TC241 and T42 drain directly into Tipton Creek. Tipton Creek drains into the South Forth Iowa River main branch before reaching sampling location SF450.

other than the targeted genes. Means and standard deviations for the two PCR wells with the smallest difference in copy numbers were calculated. If the third well copy number was not within three standard deviations of the mean, the value was labeled as an outlier and discarded; otherwise the mean copy number of the three reaction wells was used for each water sample. Multiple 96-well plates were necessary for analysis of each gene. Limits of quantification (LOQ) were unique for each plate. LOQ was set as the lowest copies per reaction identified from standard curve analysis or false positive copies from negative controls on each plate. *ErmB* LOQs ranged from 22 to 159 copies 100 mL^{-1}. *ErmF* LOQs ranged from 7 to 174 copies 100 mL^{-1}. 16S rRNA LOQs ranged from 5.66×10^4 to 4.14×10^5 copies 100 mL^{-1}. Additionally, samples with *ermB* and *ermF* concentrations above limits of quantification were normalized to 16S rRNA gene abundance by dividing *erm* gene copies 100 mL^{-1} by 16S rRNA copies 100 mL^{-1}. The 16S rRNA gene is conserved in all bacteria and archaea, allowing for estimation of percent of bacteria containing *ermB* and *ermF*.

2.4. Statistical analysis

All statistical analyses were performed using R version 3.3.1. Wilcoxon Ranked Sum tests were used to determine if there were statistically significant differences between resistance gene concentrations and concentrations normalized to 16S rRNA between sampling locations. Wilcoxon Ranked Sum tests were also performed to determine significant differences ($p < 0.01$) in resistance gene concentrations between samples based on temporal groupings. Using a more stringent criterion for determining significant differences between sites allowed for grouping of tile and surface samples for further analysis. Samples were divided into four categories based on temporal differences: frozen soil, drainage period, pre-fall manure application and post-fall manure application. Frozen soil samples were classified as samples collected when soil temperatures were below 0 °C, which generally occurs from early December to late March. Drainage period samples were classified as samples collected once soil temperatures rose above 0 °C in the spring until artificial drainage rates dropped to 0.01 of peak summer flows. Pre-fall manure application samples were classified as collected after artificial drainage rates were reduced to 0.01 of peak summer flows until soil temperatures dropped below 10 °C. Post-fall manure application samples were classified as samples collected after soil temperatures dropped below 10 °C soil temperatures reached 0 °C. Simple linear regressions were run to identify relationships between gene concentrations and average daily flowrates. Samples containing resistance gene concentrations below LOQs were excluded from the linear regression analyses. Additionally, resistance gene concentrations were log transformed prior to running the daily flow linear regression.

3. Results

Abundance of *ermB* and *ermF* ranged from below limits of quantification to $>10^7$ copies 100 mL^{-1} water. The Wilcoxon Ranked Sum Test did not identify significant differences ($p > 0.01$) in *erm* gene abundance between the two subsurface drainage sites and data for these sites were therefore combined for further analysis. Similarly, significant differences ($p < 0.01$) in concentrations were not identified between surface water sample locations and the data for these three sites were combined. Artificial subsurface drainage contained significantly more *ermB* copies 100 mL^{-1} ($p < 0.0001$) and *ermF* copies 100 mL^{-1} ($p < 0.01$) than surface water samples (Figs. 2 and 3). Mean concentrations above LOQs for both genes were one to two orders of magnitude greater in drainSage samples than surface water samples (Table 1).

ErmB was the most frequently detected gene in artificial subsurface drainage and surface water with detection in over 92% and 78% of

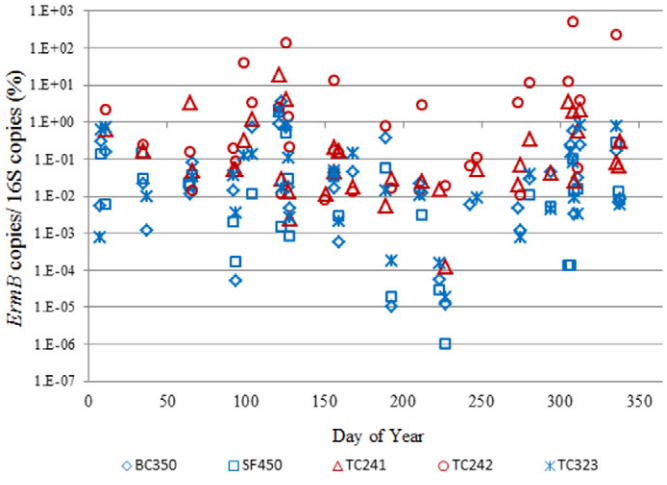

Fig. 2. Relative abundance of *ermB* (% of 16S rRNA abundance) in surface and subsurface drainage waters over time. Red symbols denote artificial drainage samples (TC241 and TC242), while blue symbols represent surface water samples (BC350, SF450, and TC323).

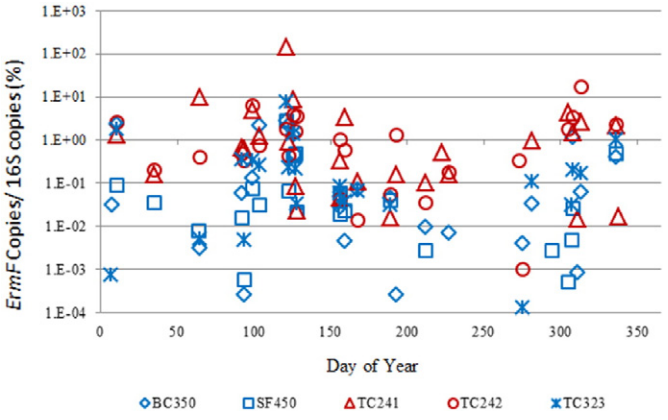

Fig. 3. Relative abundance of *ermF* (% of 16S rRNA abundance) in surface and subsurface drainage waters over time. Red symbols denote artificial drainage samples (TC241 and TC242), while blue symbols represent surface water samples (BC350, SF450, and TC323).

samples, respectively. Significant differences in drainage and surface water samples were identified based on temporal classifications. Surface water *ermB* drainage period concentrations were significantly greater ($p < 0.01$) than pre-fall manure application and frozen soil samples (Fig. 4a). Surface water *ermB* drainage concentrations were not significantly greater ($p > 0.01$) than post-fall manure concentrations. Additionally, when normalized to 16S rRNA, relative abundances of *ermB* were significantly greater ($p < 0.01$) in drainage period, post-manure and frozen soil surface water samples than pre-fall manure application samples (Fig. 5a). No significant temporal differences ($p > 0.01$) were identified in *ermB* concentrations in artificial drainage samples. However, *ermB* 16S rRNA ratios were significantly greater ($p < 0.01$) in post fall manure application artificial drainage samples compared to drainage period and pre fall manure samples (Fig. 5a).

ErmF was detected in over 70% of artificial subsurface drainage and 50% of surface water samples. Surface water *ermF* drainage period concentrations were significantly greater ($p < 0.01$) than pre fall manure application, post fall manure application and frozen soil samples. Similar results were obtained when normalized to 16S rRNA relative abundances, with drainage period samples containing significantly ($p < 0.01$) more *ermF* than any other temporal classification. Contrasting *ermB*, artificial drainage *ermF* concentrations were significantly greater ($p < 0.01$) during the drainage period when compared to frozen soil and pre fall manure periods (Fig. 4b). However, when normalized to 16S rRNA concentrations, only the relative abundances of *ermF* in artificial drainage were significantly greater than relative abundances identified during the pre-fall manure period (Fig. 5b).

Log-linear correlation relationships between daily flowrates (daily average on the day of sampling) and resistance gene concentrations varied greatly among sampling locations and resistance genes (Table 2). In the smaller artificial drainage network (TC242) flowrates strongly correlated with concentrations for both genes, while R values from the larger artificial drainage network (TC241) were the smallest of any sampling location. The strongest correlations were identified at TC323, which contained the smallest range of average daily flowrates for the stream sampling locations.

4. Discussion

Mean concentrations for *ermB* and *ermF* in the main artificial subsurface drainage outlets were two to three orders of magnitude greater than concentrations in subsurface drainage from manure-treated plots reported by Garder et al. (2014) and Luby et al. (2016). The higher resistance gene concentrations in the major artificial subsurface drainage outlets compared to plot scale drainage concentrations may be attributed to the more frequent manure applications from swine confinements utilizing antibiotics, in the drainage area. Studies by Luby et al. (2016) and Garder et al. (2014) utilized plots under two-year corn soybean crop rotations, with nitrogen application in the form of swine manure only prior to the corn growing season. Previous studies have identified log scale reductions of ARGs in manure-treated agricultural soils within a year of application (Garder et al., 2014; Fahrenfeld et al. 2014; Marti et al., 2014; Luby et al., 2016). However, 53% of the combined subwatersheds analyzed in this study planted corn following corn at least once within the four-year study period. Additionally, 16% of the combined subwatersheds were maintained in corn during the entire length of the study. For the state of Iowa corn-soybean rotations occupy 47% of the agricultural land, soybeans rotated with two or more years of corn occupy an additional 25%, and continuous corn is grown on 3% of agricultural land (Tomer et al., 2017). Increasing the frequency of corn planting and manure application in a field may diminish the soil's natural ability to attenuate resistance gene additions to soil from swine manure. Furthermore, surface intakes installed to prevent ponding in cropped potholes within the watershed directly route surface water into drainage networks (Tomer et al., 2010). Such hydrologic routing impedes any natural filtering performed by the soil before reaching the drainage line, therefore permitting the direct transfer of pollutant reservoirs to surface waters.

Trends in *ermB* and *ermF* concentrations in artificial drainage and surface waters are likely influenced by manure application timing and seasonal drainage patterns. The strong correlations observed between average daily surface water flowrates and *erm* gene concentrations at the majority of sampling locations suggest surface water resistance gene concentrations are highly dependent on subsurface flow additions. Green et al. (2006a, 2006b) estimated that 71% of total discharge from the watershed from 1996 to 2004 was from tile drainage. Although tile drainage contributes the bulk of discharge in the watershed, the majority of the drainage occurs during spring through the middle of summer. Ninety-two percent and 71% of the total artificial drainage occurred between April 15th and July 15th during the study period at TC241 and TC242, respectively.

Significantly greater concentrations ($p < 0.01$) of *ermB* and *ermF* in surface water during periods of high drainage in the watershed were

Table 1
Detection frequency and concentrations of *erm* genes in tile drainage and surface water.

			ermB		*ermF*	
Location[a]	Drainage area (ha)	n[b]	% > LOQ[c]	Mean (>LOQ) ± SD[d] (gene copy 100 mL^{-1})	% > LOQ	Mean (>LOQ) ± SD (gene copy 100 mL^{-1})
BC350	18,130	43	88.3	$2.17 \times 10^4 \pm 5.60 \times 10^4$	58.1	$5.70 \times 10^4 \pm 1.70 \times 10^4$
SF450	5820	42	78.5	$1.06 \times 10^4 \pm 2.00 \times 10^4$	52.3	$3.64 \times 10^4 \pm 5.86 \times 10^4$
TC323	18,380	38	88.3	$1.75 \times 10^4 \pm 3.35 \times 10^4$	50.5	$5.86 \times 10^4 \pm 8.51 \times 10^4$
TC241	1043	40	92.5	$1.89 \times 10^5 \pm 6.85 \times 10^5$	70.0	$5.39 \times 10^5 \pm 1.51 \times 10^6$
TC242	150	35	97.2	$1.30 \times 10^6 \pm 4.91 \times 10^6$	72.2	$3.04 \times 10^5 \pm 6.55 \times 10^5$

[a] Samples from TC241 and TC242 were collected directly from main artificial drainage outlets and BC350, SF450 and TC323 samples were collected directly from each stream.
[b] Number of samples from each sampling location.
[c] Percent of samples above limits of quantification.
[d] Standard deviation.

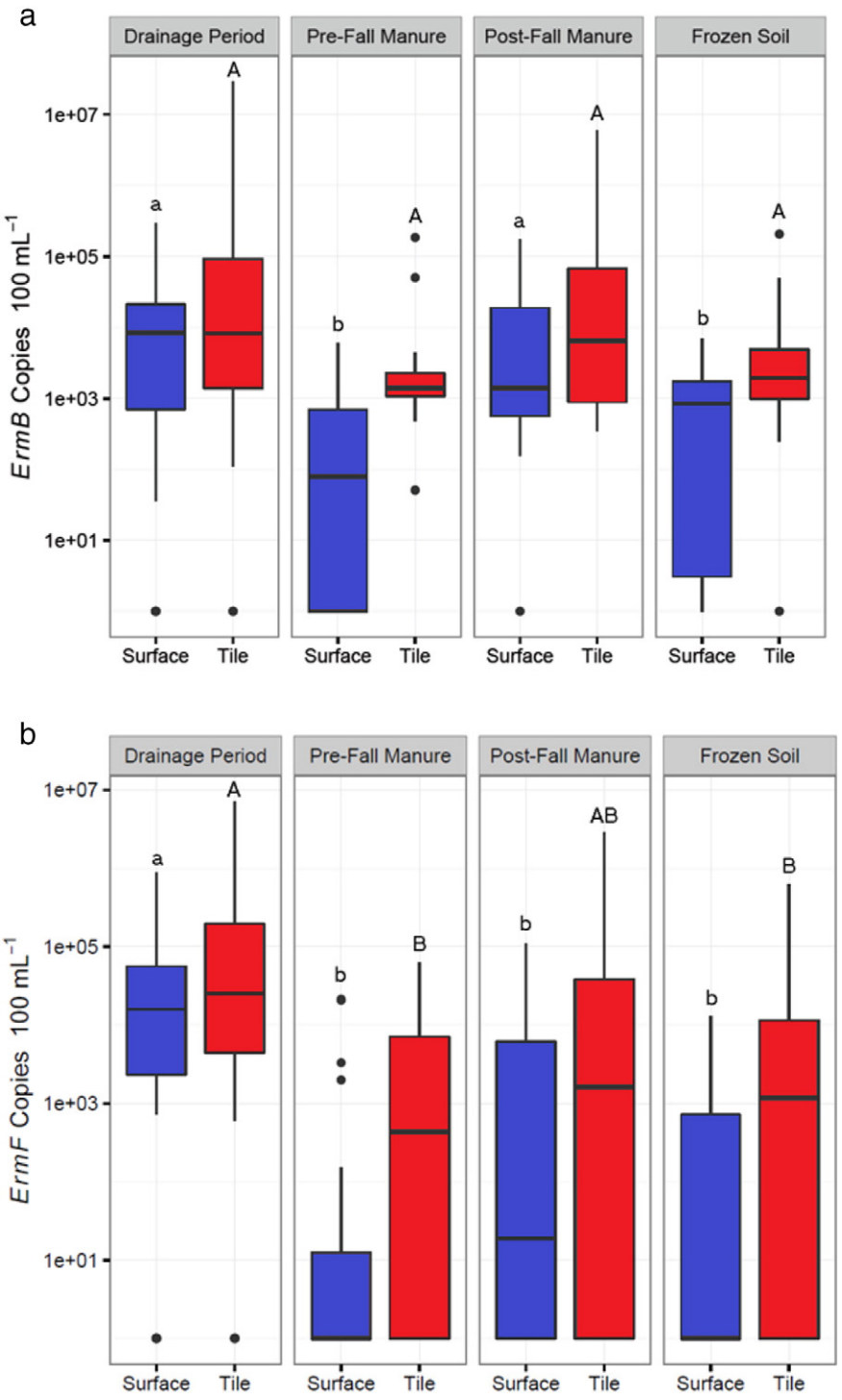

Fig. 4. a: Concentrations of *ermB* (copies 100 mL^{-1}) in surface water and artificial drainage water by season. Samples with *ermB* below LOQs are represented as a value of 1. Significantly different surface water gene concentrations ($p < 0.01$) are denoted by different lowercase letters. Significantly different tile water gene concentrations ($p < 0.01$) are denoted by different uppercase letters. The center bar in the colored box represents the median and the top of the colored box represents 75th percentile, while the bottom of the box represents the 25th percentile. Whiskers represent minimum and maximum values and dots represent outliers, which are either three time the interquartile range or more above the 75th percentile or three times the interquartile rate or more below the 25th percentile. b: Concentrations of *ermF* (copies 100 mL^{-1}) in surface water and artificial drainage water by season. Samples with *ermF* below LOQs are represented as a value of 1. Significantly different surface water gene concentrations ($p < 0.01$) are denoted by different lowercase letters. Significantly different tile water gene concentrations ($p < 0.01$) are denoted by different uppercase letters.

likely influenced by artificial drainage contributions. Similar rises in surface water *Escherichia coli* concentrations during periods of high artificial drainage contributions within the South Fork Iowa River Watershed were identified by Tomer et al. (2008). However, concentrations of *E. coli* in surface water were consistently higher compared to artificial subsurface drainage outlets indicating overland flow as the major pathway of *E. coli* to surface waters. Additionally, significantly greater relative abundances of *ermB*-16S rRNA were identified in post-fall manure drainage samples ($p < 0.01$), while Tomer et al. (2008) showed no significant increases in *E. coli* concentrations in drainage following typical manure application timing, further suggesting different environmental sources were responsible for transporting *E. coli* and ARGs to surface water.

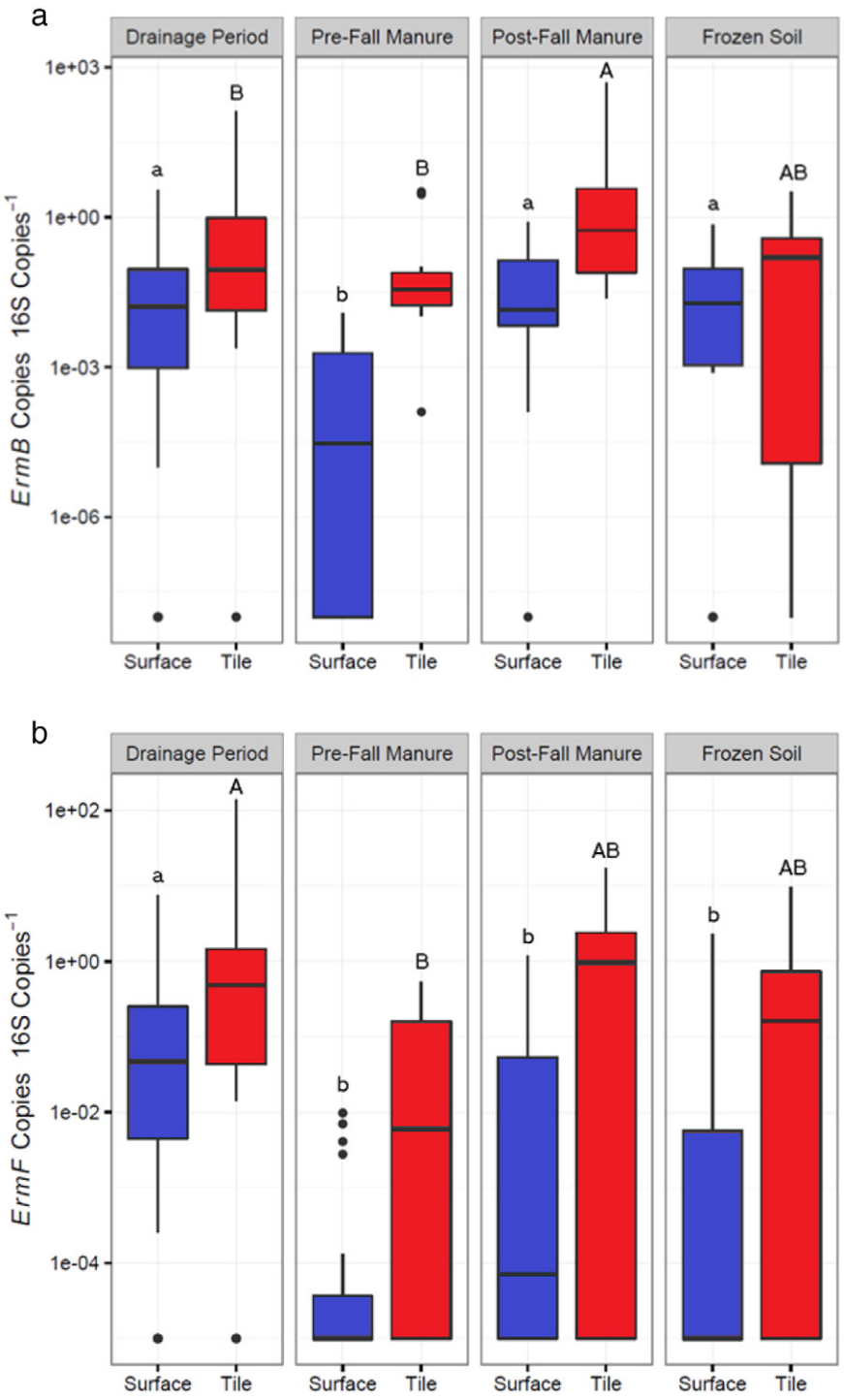

Fig. 5. a: Surface water and artificial drainage *ermB*-16S rRNA relative abundance percentages by season. Samples with *ermB* below LOQs are represented as a value of 1×10^{-8}. Significantly different surface water gene concentrations ($p < 0.01$) are denoted by different lowercase letters. Significantly different tile water gene concentrations ($p < 0.01$) are denoted by different uppercase letters. b: Surface water and artificial drainage *ermF*-16S rRNA relative abundance percentages by season. Samples with *ermF* below LOQs are represented as a value of 1×10^{-5}. Significantly different surface water gene concentrations ($p < 0.01$) are denoted by different lowercase letters. Significantly different tile water gene concentrations ($p < 0.01$) are denoted by different uppercase letters.

Differences identified between fecal indicator bacteria and ARG transport through the watershed are indicative of ARGs residing in a lasting soil environmental reservoir. The significantly higher concentrations of *erm* genes in main subsurface artificial drainage compared to plot scale concentration identified by Luby et al. (2016) and Garder et al. (2014) suggest either a higher frequency of ARG inputs into the environment from differing swine antibiotic regimens or the transfer of ARGs from fecal sources to naturally occurring environmental bacteria. Other potential ARG sources within the watershed may include human sources from leaky septic tanks or bovine fecal material deposited directly in surface waters. Three wastewater treatment plants and two sanitary sewer overflows are located within the watershed. Furthermore, numerous studies have identified populations of ARGs in environments free of anthropogenic influences (Bhullar et al., 2012; Brown and Balkwill, 2009; Miteva et al., 2004), indicating naturally occurring background levels of antibiotic resistance across environmental

Table 2
Average daily flow rate ranges and resistance gene concentration log-linear regression for surface water and tile drainage sampling locations.

Gene	Site	n[a]	R	*p*	Flow range ($m^3 s^{-1}$)
ermB	BC350	38	0.79	2.59×10^{-5}	1.551–5.52
	SF450	33	0.87	4.42×10^{-7}	7.08–26.1
	TC323	32	0.98	2.20×10^{-16}	0.297–2.20
	TC241	32	0.51	0.1519	0.479–5.28
	TC242	35	0.96	8.05×10^{-15}	2.83×10^{-3}–3.40×10^{-2}
ermF	BC350	25	0.93	1.250×10^{-8}	1.705–5.23
	SF450	22	0.73	$1.171 \times 10–^{2}$	10.19–26.1
	TC323	23	0.96	1.499×10^{-10}	0.445–2.46
	TC241	22	0.58	0.1327	0.479–2.59
	TC242	26	0.95	1.150×10^{-10}	2.83×10^{-3}–3.40×10^{-2}

[a] Number of samples used in linear regressions, samples with resistance gene concentrations below limits of quantification were not included.

landscapes. Pei et al. (2006) identified significantly greater concentrations of ARGs in river sediments impacted by human and agricultural inputs when compared to pristine water sediments, however, the authors were unable to determine the relative impacts of the different sources. West et al. (2011) identified the transfer of plasmid derived *erm* and tetracycline resistance genes from environmental microorganisms to model microorganisms, but little is known regarding ARG dissemination throughout environmental microbiomes. Huerta et al. (2013), working in Spanish watersheds, reported correlations between macrolide antibiotic concentrations, *ermB*, and two bacteria phyla, but were unable to confirm the causal mechanism responsible for the correlations. Additionally, only tracking resistance genes do not provide information on the location and activity of the genes. To better characterize the impact of introducing ARGs into the soil environment additional studies are needed to link their presence with host identification and transfer mechanisms.

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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.2017.08.116.

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