RESEARCH REPORT

PUBLIC HEALTH/WORKER SAFETY

Title: Influence of Setback Distance on Antibiotics and Antibiotic Resistance Genes in Soil and Runoff Following the Land Application of Swine Manure Slurry, **NPB # 16-072**

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Industry Summary

Setback distance is a commonly used measure to minimize the impacts manure land application on the quality of nearby surface water. The goal of this study was to determine the effectiveness of varying setback distances on reducing the concentrations of antibiotics and antibiotic resistance genes (ARGs) in runoff and soil following the land application of swine manure slurry. To achieve the goal, field tests and laboratory measurements were conducted.

Longer setback distances helped reduce the concentrations of antibiotics and ARGs in runoff. The three antibiotics tests were chlortetracycline, lincomycin, and tiamulin. The ARGs tested included those conferring resistance to tetracycline, macrolide, and penicillin. The concentrations of all three antibiotics in runoff decreased exponentially with increased setback distance, with tiamulin decreasing the fastest and lincomycin the slowest. The concentrations of seven genes tested also decreased exponentially with increased setback distance. Also, for most of the antibiotics and genes tested, their concentrations in the runoff from the second rainfall event were much lower than those in the runoff from the first rainfall event. Taken together, a setback distance of 50 m is deemed necessary to reduce the levels of ARGs in runoff to levels similar to those in runoff from control plots.

As expected, the manure application had significant impacts on the antibiotic and ARG concentrations in the soil within the manured region. For the soil within the setback region, setback distance affected antibiotics and ARGs differently. The concentrations of antibiotics in the surface soil within the setback region reduced significantly with increased setback distance. Because several ARGs were not detected in the surface soil within the setback region, no statistical conclusion was drawn for these ARGs. One gene that is often involved in horizontal gene transfer reduced significantly in surface soil with increased setback distances.

In addition to the individual ARGs tested, we also examined the resistome of the runoff and soil samples. Resistome refers to all the ARGs in a sample. Both the diversity and abundance of the resistome in runoff decreased significantly with increased setback distances. Manure application significantly enriched the resistome of the soil within the manured region. However, manure application appeared to have very limited impacts on the resistome of the soil within the setback region.

This project presents a comprehensive study about the effects of setback distances on the quality of runoff, in terms of antibiotics and ARGs, from soils receiving swine manure slurry. This study was set up to simulate a worst-case scenario where rainfalls occurred shortly after manure application. Information generated from this project can help regulators determine their recommendation about the setback distance needed to minimize the impacts of manure application on the quality of nearby surface water.

Keywords
Swine manure slurry, setback distance, runoff, soil, antibiotic, antibiotic resistance gene, resistome.

Scientific Abstract
Setback distance is a commonly used measure to minimize the impacts manure land application on the quality of nearby surface water. The objective of this project was to determine how varying setback distances affect the concentrations of antibiotics and ARGs in runoff and soil following the land application of swine slurry. To achieve this objective, a portable rainfall simulator was assembled over a series of plots with varying lengths. Manure was applied by broadcasting at the top of each plot (manured region) and the rainfall simulator was placed so that it covered the entire length of the plot (manured region and setback region). At the end of each plot, a trough collected runoff and directed it to a flume where flow was measured and runoff sampled. The rainfall simulation with sampling of runoff was repeated once the following day. Five days after the second rainfall simulation, soil cores were sampled at various setback distances. Each soil core was divided into three depths. The runoff and soil samples were analyzed for both antibiotics and ARGs.

Out of the ten genes tested, *erm*(B), *erm*(C), *tet*(O), *tet*(Q), *tet*(X), *intI1*, and the 16S rRNA gene showed statistically significant decreases in their concentrations in runoff with increased setback distance. The log concentrations of these seven genes in runoff decreased linearly with increased setback distances, with *tet*(O), *tet*(Q), *erm*(B), and *erm*(C) having slopes ranging from -0.0800 to -0.0838, the 16S rRNA gene and *intI1* having slopes ranging from -0.0638 and -0.0641, and *tet*(X) having a slope of -0.0715. For the three antibiotics tested, chlortetracycline, lincomycin, and tiamulin, their all showed statistically significant decreases in their concentration in runoff with increased setback distance. The log concentrations of these antibiotics in runoff decreased linearly with increased setback distances, with the slopes for chlortetracycline, lincomycin and tiamulin being -0.0639, -0.0505, and -0.0722. By using the linear equations for ARGs and antibiotics, we calculated the setback distance needed to reduce the ARG and antibiotic concentrations in runoff to values similar to those in runoff from control plots where no manure was land applied. That setback distance was 50 meter.

Among the genes tested, the 16S rRNA gene, *bla*$_{TEM}$, *intI1*, and *tet*(D) were the only genes that were consistently detected in the surface soil within the setback region. Among them, *bla*$_{TEM}$ was the only gene that decreased significantly with increased setback distance. The other six ARGs tested (*erm*(B), *erm*(c), *erm*(F), *tet*(O), *tet*(Q), and *tet*(X)) were only detected in surface soil with the short setback distances (e.g., less than 9 meters). Because their concentrations in surface soil at most of the setback distances tested were below the method detection limits, ANOVA tests could not determine the statistical significances of setback distance on their concentrations. Nonetheless, these six genes, plus *bla*$_{TEM}$, are believed to decrease significantly in surface soil with in the setback region with increased setback distance. Similarly, the concentrations of chlortetracycline, lincomycin, and tiamulin decreased significantly in the surface soil with in the setback region with increased setback distance.

As revealed from the high-throughput qPCR analyses, the number of ARGs in runoff dropped slightly from 140 to 111 as the setback distance increased from 0.0 to 18.3 meter. However, the sum of the relative abundance, a measure that sums up the relative abundance of all ARGs tested, dropped from 1.2 to 0.2 ARG copy per the 16S rRNA gene copy as the setback distance increased from 0.0 to 18.3 meter. The percentages of resistance genes against aminoglycoside and MSLB decreased in runoff samples as the setback distance increased. The resistome of the soil within the setback region was not substantially affected by the land application of manure in the manured region, in terms of the number of ARGs detected, the sum of the relative abundance, or the composition of the resistome.

Introduction

Pork production is a significant agricultural enterprise in the U.S., with the majority of producers located in the Midwest and North Carolina.[1] Swine manure provides a valuable source of nutrients including nitrogen and phosphorus, and has historically been used as a soil amendment for crop production. Rising fertilizer costs indicates that swine manure will continue to represent a valuable component of crop fertility programs.

There has been an increase in the numbers of livestock being raised in confinement housing over the past thirty years. Many of these systems are defined by regulatory standards as concentrated animal feeding operations (CAFOs) based upon a site capacity greater than 1,000 “animal units” (e.g., 2,500 swine).[2] The benefits of CAFOs to swine producers include economies of scale and enhanced production quality controls. Current swine industry practice is to house animals in confinement facilities with capture and storage of liquid or semi-liquid manure in pits or lagoons. At CAFOs, antibiotics and other pharmaceuticals are often used at sub therapeutic levels for growth promotion and prophylaxis and at therapeutic levels for disease treatment.[3,4] The antibiotics added in animal feed are often not completely absorbed in the animal gut, and antibiotic residues may be released with livestock wastes.[5] The antibiotic residues in the animal gut and in animal manure may result in the emergence of antibiotic resistance among commensal and pathogenic bacteria.

The benefits of land application of manure to agricultural fields is substantial as it provides a source of valuable nutrients and organic matter, resulting in increased soil productivity, improved water infiltration, and reduced soil erosion potential. However, the presence of antibiotic compounds and antibiotic resistant bacteria in manure introduces the potential for these

constituents to enter the environment when manure is land applied. Recent studies have attempted to relate the occurrence of antibiotic compounds and associated resistant bacteria to the distribution of livestock production in watersheds.[6] Residues of antibiotics and antibiotic resistance genes (ARGs), the genetic material that confers antibiotic resistance to bacteria, have been documented in water bodies adjacent to CAFO sites although the links between sources and occurrence have not yet been fully established.[7]

Currently several best management practices (BMPs) are used by farmers to minimize the spread of manure constituents from manured field into water bodies. Examples of these BMPs include lagoon treatment, incorporation of manure, detention ponds, parallel terraces, filter strips, and eliminating high risk areas from being used as farmland such as sloping land and low laying land that tends to flood.[8,9]

One BMP to restrict water contamination is the use of setback distances. The setback distance determines how close manure can be land applied to a water source or residential/commercial area.[10,11] Several states currently require or recommend setback distances from soil receiving manure to landscape features such as wells, streams, ponds, property lines etc. Missouri, for example, has a recommended setback distance of 300 ft from wells, 100 ft from streams, and 150 ft from neighboring houses.[12] Pennsylvania requires a manure-application setback distance of at least 100 feet (i.e., 30.5 meters) from surface waters.[13] These regulations or recommendations are often based on nutrient analysis. Some studies have investigated the transport of indicator bacteria (e.g., *E. coli*) in runoff.[14–17] However, it is unclear if the setback distance determined for nutrients would apply to other swine manure constituents, such as antibiotics and ARGs. Hence, there is a critical need to determine the proper setback distances to limit the transport of antibiotics and ARGs in agricultural runoff.

Objectives

The objective of this study was to determine how varying setback distances affect the concentrations of antibiotics and ARGs in runoff and soil following the land application of swine slurry.

Materials and Methods

Study Site Characteristics

The study was conducted at University of Nebraska Rogers Memorial Farm, 18 km east of Lincoln, NE. For the purposes of this experiment, the area chosen had uniform residue cover and a slope of 4.9%. It had previously been used for growing corn, sorghum, soybeans, and winter wheat. No manure had been applied to the study area since 1966. Winter wheat had been harvested in the summer of 2015 and glyphosate had been applied after harvest for weed control. The wheat residue was not removed and gave the soil surface 100% coverage. The soil type is Aksarben silty clay loam (fine, smectite, mesic Typic Argiudoll), which has a moderately low saturated hydraulic conductivity, 4.94-16.35 cm h^{-1}.[18]

Manure Collection and Characterization

The experiment was scheduled over a 10-week period in summer 2016. On the Monday of each week, fresh manure was collected from a commercial swine operation facility in southeast Nebraska. A sample of the manure was sent for chemical and physical analysis. The mean

measured values were 2.43 mg kg^{-1} for NO_3-N, 2.98 g kg^{-1} for NH_4-N, 5.52 g kg^{-1} for total Kjeldahl N, 2.54 g kg^{-1} for organic N, 2.89 g kg^{-1} for total phosphorus (TP), 4.4 mg kg^{-1} for boron, 2.22 g kg^{-1} for calcium, 1.95 g kg^{-1} for magnesium, 43.4 mg kg^{-1} for manganese, 2.64 g kg^{-1} for potassium, 0.92 g kg^{-1} for sodium, 131 mg kg^{-1} for zinc, 25.85 dS m^{-1} for EC, 7.81 for pH, and 5.35% for solids. The liquid slurry was surface broadcast at a rate of 3.90×10^4 kg ha^{-1}. The rate was based on annual nitrogen requirements for corn (151 kg N ha^{-1} $year^{-1}$ for an expected yield of 9.4 Mg ha^{-1}).[19]

Rainfall Simulation Field Test

The study site was divided into 20 plots and the experiment was set up as a randomized complete block design with a total of four blocks, each containing five setback distances: 0.0 m, 3.0 m, 6.1 m, 12.2 m, and 18.3 m. Within each block the distances were randomly assigned to the plots. Simulated rainfall came from a sprinkler system that uses 3 m sections of 10 cm diameter irrigation pipe on which 2 cm diameter risers were mounted. Sprinkler heads are located on the top of the risers. The rainfall simulation system was placed so that it covered the entire plot area (i.e., both the manured region and the setback region) in order to produce a uniform rainfall. The intensity of the rainfall was approximately 52 mm hr^{-1} and was recorded by rain gages placed along the plots. Irrigation water was extracted from an onsite irrigation well. The plots were 3.7 m wide and the length depended on the given setback distance. At the top of each plot, a 3.7 m by 4.9 m area received manure (i.e., manured region). The setback distance was measured from the bottom of the manured region (Figure 1A). At the end of each plot was a metal lip that collected runoff and lead it through a flume where flow was measured by a stage recorder. When the flow reached steady state, grab samples of runoff were collected in one 250-mL amber glass jar for antibiotics analysis and in two 1-L sterile plastic bottles for nutrient and microbial analyses. Samples were stored on ice and shipped back to the laboratory for further analysis.

The experiment was conducted in the summer of 2016 and each week two plots were established and tested. In a typical week, rainfall simulation tests were conducted on the pair of plots on Monday and Tuesday to ensure homogenous antecedent soil moisture conditions. Runoff samples were collected from the two rainfall simulations as controls. On Wednesday, swine manure slurry was obtained from the production facility and then broadcast onto the top area of the two plots (i.e., the manured region). On Thursday one rainfall simulation took place on the plots and runoff was collected at the end of each plot (i.e., the end of the setback region). On Friday the same procedure as Thursday was repeated.

For the plots with the longest setback distance (i.e., 18.3 m), five days after the last rainfall simulation when the soil has dried (i.e., the Wednesday of the following week), soil cores were taken from the center of the manured region as well as at 0.0 m, 3.0 m, 6.1 m, 9.1 m, 12.2, and 15.2 m into the manured region (Figure 1B). For each distance, three replicate soil cores were taken as shown in Figure 1B. The soil cores were 30 cm deep and were divided into three layers: 0-10 cm, 10-20 cm, and 20-30 cm. Soil segments of the same depth from the replicate soil cores at each distance were combined, placed in re-sealable zipper storage bags, transported on ice back to the laboratory for further analysis. Soil cores were also collected from four control plots that received no manure.

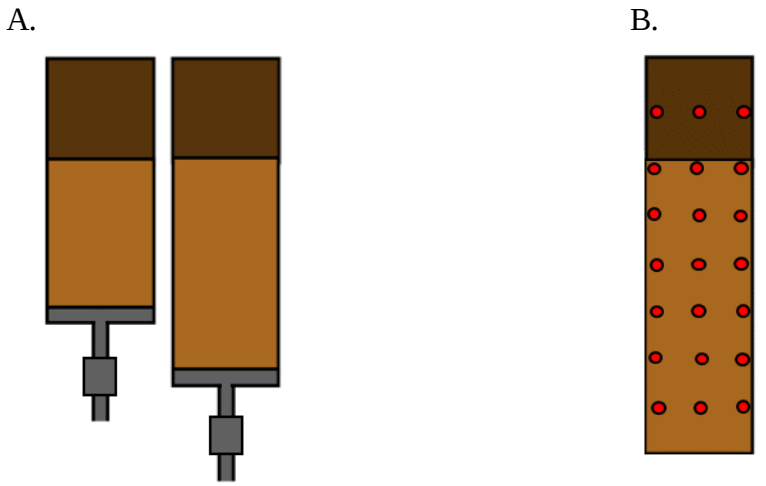


Figure 1**.** Illustrations of plots used in this experiment. (A) Two different setback distances where the dark brown color indicates the manured region where manure was broadcast, the light brown color indicates the setback region, and the gray areas indicate the metal lip and the runoff collection system. (B) Soil sampling locations where soil cores were collected from a total of seven distances, including one distance within the manured region and six setback distances within the setback region. At each distance, three replicate cores were collected.

ARG Analysis for Manure, Runoff, and Soil

Soil samples from each depth were thawed in 4°C and quickly homogenized by hand. Approximately 4 grams of soil were used to measure the soil moisture content gravimetrically, and 0.25 grams were used to extract DNA using the Qiagen DNeasy PowerSoil Kit. Frozen runoff samples were thawed overnight at 4°C and thoroughly mixed before a 50 ml of sample was filtered through an autoclaved filtration setup with sterile 0.22 µm filter paper. The filter papers were folded into rolls and then placed into Qiagen DNeasy PowerSoil kits for DNA extraction. Frozen manure slurry samples were also thawed overnight at 4°C, and 5 mL of well mixed manure was filtered in the same fashion as the runoff and filter papers were used for DNA extraction.

All three types of samples were extracted using the Qiagen DNeasy PowerSoil Kit following the manufacturer protocol with the exception that two 40-second bead beating steps were used in the lysis procedure. Successful extractions were confirmed using NanoDrop spectrometer and endpoint PCR targeting the 16S rRNA gene. Regular PCR was conducted on the DNA extracts from the manure samples to determine the ARGs to be included in qPCR analysis. ARGs frequently detected in manure samples and chosen for qPCR analysis were bla_{TEM}, *erm*(B), *erm*(C), *erm*(F), *intI1*, *tet*(D), *tet*(O), *tet*(Q), and t*et*(X). Synthesized DNA fragments (Integrated DNA Technologies) were used as qPCR standards. The qPCR reactions were performed on a Mastercycler ep realplex 2 thermocycler (Eppendorf, Hamburg, Germany) using KiCqStart® SYBR® Green qPCR ReadyMix™ and KiCqStart® Probe qPCR ReadyMix™ (Sigma-Aldrich, St. Louis, MO). Assay setup and cycling conditions were adopted from previously reported studies (Table 1) with some optimizations.[20–27]. Primer sequences and reaction conditions are listed in Table 1 and Table 2.

Table 1. Primers and probes used in qPCR assays

Target gene	Primer	Sequence (5'-3')	Target size (bp)	Annealing temp. (°C)	Reference
16s rRNA	BACT1369F	CGG TGA ATA CGT TCY CGG	142	56	[26]
	PROK1492R	GGW TAC CTT GTT ACG ACT T			
*bla*TEM	*bla*TEM-FW	CAC TAT TCT CAG AAT GAC TTG GT	85	60	[25]
	*bla*TEM-RV	TGC ATA ATT CTC TTA CTG TCA TG			
	Probe	CCA GTC ACA GAA AAG CAT CTT ACG G			
erm(B)	*erm*(B)-FW	GGT TGC TCT TGC ACA CTC AAG	191	65	[27]
	erm(B)-RV	CAG TTG ACG ATA TTC TCG ATT G			
erm(C)	*erm*(C)-FW	AAT CGT GGA ATA CGG GTT TGC	293	63	[27]
	erm(C)-RV	CGT CAA TTC CTG CAT GTT TTA AGG			
erm(F)	*erm*(F)-FW	TCT GGG AGG TTC CAT TGT CC	412	65	[27]
	erm(F)-RV	TTC AGG GAC AAC TTC CAG C			
*intI*1	qINT-3	TGC CGT GAT CGA AAT CCA GAT CCT	109	60	[24]
	qINT-4	TTT CTG GAA GGC GAG CAT CGT TTG			
tet(D)	*tet*(D)-FW	GAA TGC CTG CAC CTT TCT GAT G	346	62	[23]
	tet(D)-RV	GGC AAT AAA TCC GGC GAA AA			
tet(O)	*tet*(O)-FW	ACG GAR AGT TTA TTG TAT ACC	171	50.3	[20,22]
	tet(O)-RV	TGG CGT ATC TAT AAT GTT GAC			
tet(Q)	*tet*(Q)-FW	AGA ATC TGC TGT TTG CCA GTG	167	63	[22]
	tet(Q)-RV	CGG AGT GTC AAT GAT ATT GCA			
tet(X)	*tet*(X)-FW	AGC CTT ACC AAT GGG TGT AAA	278	60	[21]
	tet(X)-RV	TTC TTA CCT TGG ACA TCC CG			

* Primer sequence from Aminov et al., 2001 and annealing temperature from Pei et al., 2006

Table 2. Primers used in endpoint PCR assays (if different from qPCR primers)

Table 2. Primers used in endpoint PCR assays (if different from qPCR primers)					
Target gene	Primer	Sequence (5'-3')	Target size (bp)	Annealing temp. (°C)	Reference
16s rRNA	27F	AGA GTT TGA TCM TGG CTC AG	1,484	55	[28]
	1492R	GGW TAC CTT GTT ACG ACT T			
tet(D)	*tet*(D)-FW	AAA CCA TTA CGG CAT TCT GC	787	55	[29]
	tet(D)-RV	GAC CGG ATA CAC CAT CCA TC			
tet(O)	*tet*(O)-FW	AAC TTA GGC ATT CTG GCT CAC	515	55	[29]
	tet(O)-RV	TCC CAC TGT TCC ATA TCG TCA			

Antibiotic Analysis of Runoff and Soil

Based on information given from the swine production facility four antibiotics, one antibiotic degradation product, and one sweetener were included in the antibiotic analysis. The four

antibiotics were chlortetracycline (CTC), lincomycin (LCM), penicillin G, and tiamulin (TIA). The degradation product was penicillic acid and the sweetener was neotame. The sweetener, neotame, was included in the analysis since it was fed to the swine as a growth promoter. Compounds in all samples were quantified using methods previously developed by co-PI Dr. Dan Snow.[30,31] All samples were analyzed on a Quattro Micro triple quadrupole mass spectrophotometer coupled with a Waters 2695 high pressure liquid chromatograph (HPLC) and an autosampler. Electrospray ionization (ESI) in positive or negative ion mode was used for compound detection. The analytical method was optimized to detect multiple antibiotics in the samples.

Statistical Analysis

Split plot in time ANOVA using GLIMMIX in SAS (Cary, NC) was used to determine the significance of setback distance on the concentration of antibiotics and ARGs in runoff. Treatment factors were setback distance (i.e., 0.0 m, 3.0 m, 6.1 m, 12.2 m, and 18.3 m) and rainfall events (i.e., rainfall #1 and rainfall #2). For several ARGs, their concentrations in soils were frequently below the detection limits. Hence, only three genes could be analyzed by the two-way ANOVA using GLIMMIX: the 16S rRNA gene, bla_{TEM}, and *intI1*. Antibiotics in soil was analyzed by one-way ANOVA using GLIMMIX and the treatment factor was setback distance. No antibiotics were consistently detected in the lower soil depths. For treatment factors that had significant impacts (i.e., $p<0.05$), least significant difference (LSD) tests were further conducted.

High Throughput qPCR Analyses

For the high throughput qPCR (HT-qPCR) analyses, DNA was extracted from manure, soil and runoff samples using the DNeasy PowerLyzer PowerSoil DNA Isolation kit (Qiagen Inc, Germantown, MD) and purified using the ZYMO OneStep™ PCR Inhibitor Removal Kit (Irvine, CA). To meet the concentration requirement for high throughput qPCR analysis (i.e., >15ng/μL), multiple DNA extracts of the same sample were pooled together and concentrated using NaCl and ethanol precipitation followed by resuspension in the buffer from the DNA Isolation kit. Final DNA extracts were quantified using the Invitrogen Qubit Flourometer (Life Technology, Grand Island, NY).

Purified DNA samples were shipped to Michigan State University's Research Technology Support Facility Genomic Core for HT-qPCR processing. The HT-qPCR reactions were performed using the Wafergen SmartChip Real-time PCR system (Fremont, CA) to determine the diversity and abundance of ARGs. The HT qPCR array consisted of 144 primer sets, including 143 primer sets that target major classes of ARGs and mobile genetic elements (MGEs), and one targeting the 16S rRNA gene. Technical triplicates were tested as a method of quality control. A threshold cycle (C_T) of 28 was used as the detection limit for HT-qPCR results. The relative abundance of ARGs with respect to the 16S rRNA gene was calculated using the following equations:

$$\Delta C_T = C_{T,\,ARG} - C_{T,\,16S} \quad (1)$$

$$RA_{ARG} = 2^{-\Delta CT} \quad (2)$$

$$SRA = \Sigma\, RA_{ARG,i} \quad (3)$$

where $C_{T,\,ARG}$ and $C_{T,\,16S}$ are the threshold cycles for an ARG and the 16S rRNA gene, respectively; RA_{ARG}, is the relative abundance of an ARG; SRA is the sum of relative abundance of all ARGs in a sample. Most of the calculations were conducted using Microsoft Excel 2016. Heatmap graphs were produced using RStudio with the ggplot2 package.

Results

ARGs in Swine Manure Slurry

A total of nine genes related to horizontal gene transfer, tetracycline resistance, macrolide resistance, and penicillin resistance were analyzed with qPCR. The 16S rRNA gene was used to estimate the size of the microbial community. Eight out of the ten genes tested were present in all ten manure samples. ARG *tet*(Q) was detected in all but one manure sample and *tet*(D) was detected in only one manure sample. Gene copy numbers for each of the ten swine manure slurry samples used in the experiment are reported in Table 3. Gravimetric analysis showed that the average solid contents in the swine manure slurry was 5.35%.

Table 3. Gene concentrations in swine manure slurry (average ± standard error).

Gene	Gene Concentration (copies/mL slurry)	Number of Samples[a]
bla_{TEM}	$(8.1 \pm 4.4) \times 10^3$	10
erm(B)	$(8.6 \pm 2.1) \times 10^4$	10
erm(C)	$(8.9 \pm 3.3) \times 10^4$	10
erm(F)	$(9.5 \pm 2.0) \times 10^4$	10
intI1	$(3.3 \pm 1.0) \times 10^4$	10
tet(D)	$(1.6 \pm 0) \times 10^2$	1
tet(O)	$(9.6 \pm 2.3) \times 10^2$	10
tet(Q)	$(3.3 \pm 0.8) \times 10^3$	9
tet(X)	$(7.2 \pm 3.3) \times 10^4$	10
16s rRNA	$(6.9 \pm 1.1) \times 10^5$	10

[a] Number of samples with values higher than detection limits.

Antibiotics in Swine Manure Slurry

Based on the information obtained from the facility operator, chlortetracycline, lincomycin, tiamulin, neotame, penicillin G, and penicillic acid were quantified in the manure slurry samples. Chlortetracycline, lincomycin, and tiamulin were detected in the swine manure slurry (Table 4). Chlortetracycline was the most abundant antibiotic, followed by tiamulin and lincomycin. Neotame, penicillin G, and penicillic acid were not detected.

Table 4. Antibiotic concentrations in swine manure slurry (average ± standard error).

Compound	Concentration (mg/L slurry)	Number of Samples[a]
Chlortetracycline	11.0 ± 1.5	10
Lincomycin	0.23 ± 0.09	10
Tiamulin	0.48 ± 0.08	10

[a] Number of samples with values higher than detection limits.

ARGs in Runoff

The statistical analysis results are reported in Table 5 on the effects of two treatment factors, setback distance and rainfall event, on the ARG levels in runoff. Out of the ten genes tested, *erm*(B), *erm*(C), *tet*(O), *tet*(Q), *tet*(X), *intI1*, and the 16S rRNA gene showed a statistically significant decrease in their concentrations in runoff with increasing setback distance. The three other genes, bla_{TEM}, *erm*(F), and *tet*(D), did not significantly decrease in runoff with increasing setback distance. At the $p<0.05$ level, two ARGs, *erm*(C) and *tet*(O), showed a significant decrease between rainfall #1 and #2. At the $p<0.10$ level, three other genes, *erm*(B), *intI1*, and *tet*(Q), also showed a significant decrease between rainfall #1 and #2.

In addition to the plots that received swine manure slurry, we also conducted rainfall simulation tests on control plots that received no swine manure slurry. For *erm*(B), *erm*(F), *intI1* and *tet*(Q), their concentrations in the runoff from manured plots were significantly higher than those from the control plots (Table 6). Besides, because the concentrations in runoff from the control plots were below detection limit, the ANOVA tests did not return *p* values for *erm*(C) and *tet*(O), indicating that the manure application significantly increased the gene concentrations in runoff. According to the ANOVA analyses, the concentrations of four genes (i.e., 16S rRNA, bla_{TEM}, *tet*(D), and *tet*(X)) in runoff were not significantly affected by the application of manure.

Table 5. Split plot in time ANOVA on the effects of setback distance and rainfall events on the concentration of genes in runoff.

	16S rRNA	bla_{TEM}	*erm*(B)	*erm*(C)	*erm*(F)	*intI1*	*tet*(D)	*tet*(O)	*tet*(Q)	*tet*(X)
	(copy/mL)	(copy/mL)	(copy/mL)	(copy/mL)	(copy/mL)	(copy/mL)	(copy/mL)	(copy/mL)	(copy/mL)	(copy/mL)
Setback distance (m)										
0.0	7.9×10^6 a	1.2×10^4	1.8×10^6 a	1.6×10^5 a	8.0×10^5	4.6×10^5 a	1.5×10^2	1.7×10^4 a	1.5×10^5 a	8.0×10^5 a
3.0	2.6×10^6 ab	7.0×10^3	6.0×10^5 ab	4.3×10^4 ab	3.4×10^5	1.4×10^5 a	9.2×10^1	7.8×10^3 ab	5.2×10^4 a	2.7×10^5 ab
6.1	1.3×10^6 bc	4.2×10^3	3.6×10^5 abc	2.6×10^4 abc	1.8×10^5	1.4×10^5 a	4.2×10^1	2.6×10^3 abc	1.4×10^4 ab	1.1×10^5 abc
12.2	5.0×10^5 bc	2.7×10^3	4.5×10^4 bc	5.9×10^3 bc	2.6×10^4	1.7×10^4 b	4.1×10^1	1.4×10^3 bc	3.9×10^3 b	5.4×10^4 bc
18.3	3.7×10^5 c	3.0×10^3	2.2×10^4 c	4.9×10^3 c	8.5×10^4	2.1×10^4 b	5.1×10^1	3.6×10^2 c	2.9×10^3 b	2.0×10^4 c
Rainfall event										
1	1.7×10^6	4.5×10^3	4.0×10^5	5.1×10^4 a	2.7×10^5	1.2×10^5	4.9×10^1	7.2×10^3 a	2.9×10^4	8.7×10^4
2	1.1×10^6	4.2×10^3	1.1×10^5	9.4×10^3 b	9.7×10^4	5.5×10^4	8.5×10^1	1.1×10^3 b	9.4×10^3	1.7×10^5
p-values for:										
Distance	0.025	0.442	0.044	0.019	0.076	0.009	0.520	0.030	0.033	0.041
Rainfall	0.265	0.901	0.088	<.001	0.146	0.083	0.153	<.001	0.081	0.244
Rainfall × Distance	0.725	0.571	0.752	0.781	0.812	0.795	0.601	0.692	0.649	0.640

[a] Values reported under "Setback distance" and "Rainfall event" are treatment averages, which were calculated based on the data for one particular treatment level. For example, 7.9×10^6 was calculate using the 16S rRNA gene concentration of all runoff samples at distance 0.00 m from both rainfall events.

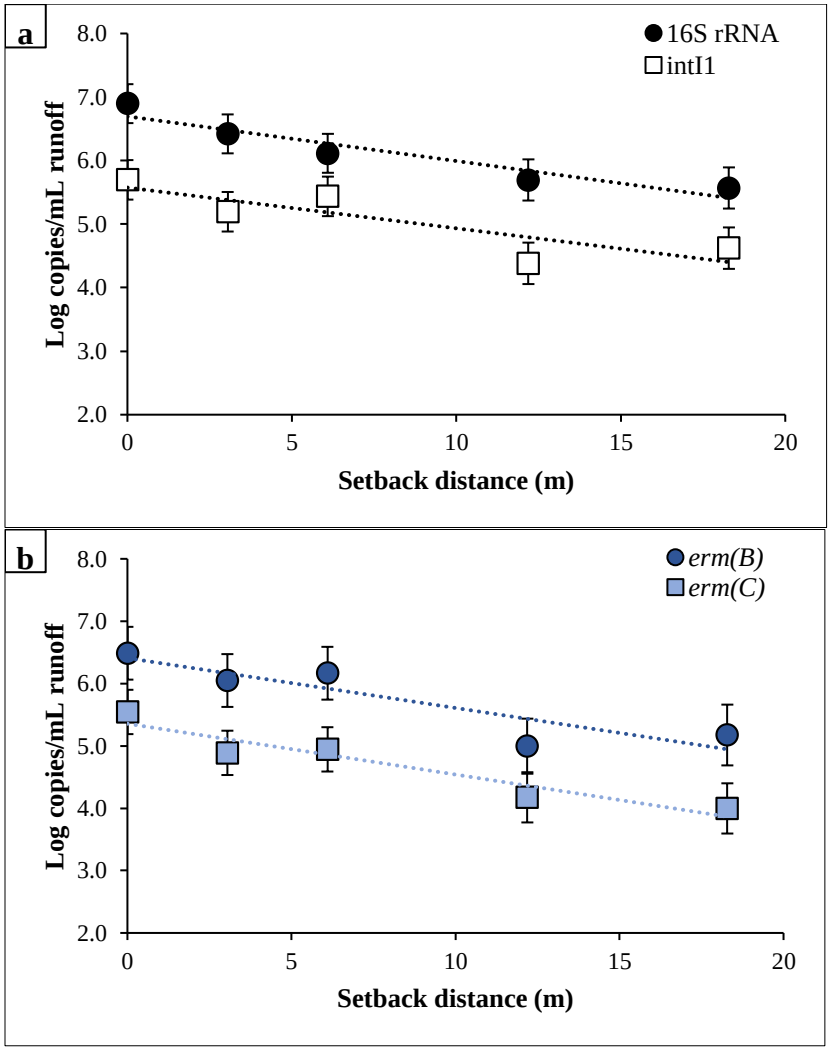
[b] In LSD tests, a value followed by a single letter (e.g., a) is significantly different at the 0.05 level from a value followed by a different single letter (e.g., b). A value followed by a letter combination (e.g., ab) are not significantly different at the 0.05 level from another value followed by a single letter (e.g., a).

Table 6 Impact of manure amendment on ARG concentration in runoff at the 18.3 m setback distance.

	16S rRNA (copy/mL)	*bla*$_{TEM}$ (copy/mL)	*erm*(B) (copy/mL)	*erm*(C) (copy/mL)	*erm*(F) (copy/mL)	*intI1* (copy/mL)	*tet*(D) (copy/mL)	*tet*(O) (copy/mL)	*tet*(Q) (copy/mL)	*tet*(X) (copy/mL)
Manure amendment[a]										
Amended plots	4.0×10^5	2.8×10^3	7.6×10^4 a	3.7×10^3	5.2×10^4 a	2.8×10^4 a	2.0×10^1	4.5×10^2	3.5×10^3 a	3.9×10^4
Control plots	9.5×10^3	4.4×10^2	6.9×10^1 b	BDL[b]	4.0×10^2 b	7.4×10^1 b	3.0×10^1	BDL	9.6×10^1 b	1.3×10^3
p-values:	0.113	0.172	0.004	N/A[c]	0.029	0.004	0.526	N/A	0.009	0.561

[a]Values are from rainfall #1 and #2.
[b]BDL, below detection limit, indicates that the gene was not detected in the runoff samples.
[c]N/A, not applicable, indicates that there was too little information to calculate a *p*-value.

For the seven genes that exhibited significant decreases in their concentrations in runoff from manured plots, their concentrations as a function of the setback distance are visualized in Figure 2. When plotted on a semi-log scale (i.e., Log copies per mL runoff), the decreases followed straight lines. The profiles were simulated using linear trend lines and the equations are reported in Table 7. The *tet*(O), *tet*(Q), *erm*(B), and *erm*(C) genes decreased at similar rates, with slopes ranging from 0.0800 to 0.0838. The 16S rRNA gene and *intI1* decreased at similar rates, with slopes ranging from 0.0638 to 0.0641. The *tet*(X) gene decreased with setback distance at the rate of 0.0715.



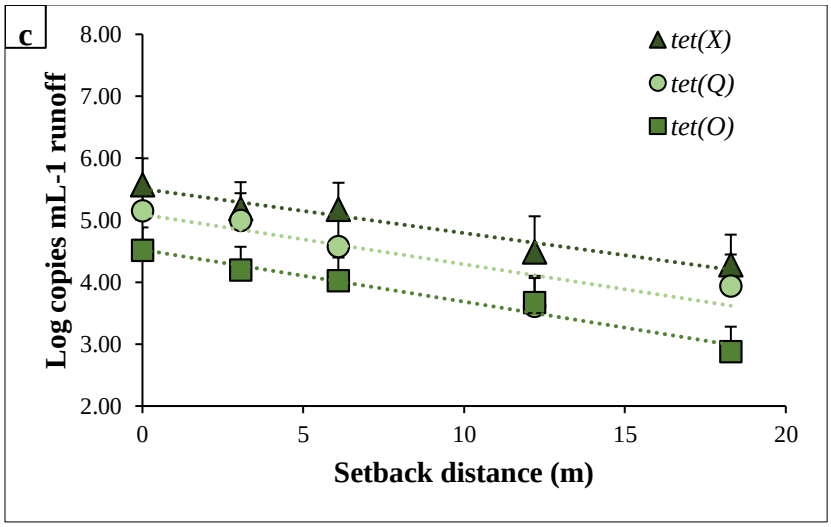


Figure 2**.** Weighted average concentration of (A) the 16S rRNA gene and *intI1*, (B) erythromycin resistance methylase (*erm*) genes, and (C) tetracycline resistance *(tet*) genes in runoff from manure-amended plots after the first rainfall. The error bars represent the standard errors based on the GLIMMIX analysis of replicates and distance.

Table 7. Linear equations and R^2 for the genes in runoff.[a]

Gene	$kx^b + m$ (log copies/mL)	R^2
erm(B)	-0.0800x + 6.408	0.810
erm(C)	-0.0816x + 5.356	0.909
intI1	-0.0641x + 5.574	0.729
tet(O)	-0.0838x + 4.522	0.970
tet(Q)	-0.0805x + 5.092	0.785
tet(X)	-0.0715x + 5.506	0.953
16S rRNA	-0.0638x + 6.737	0.904

[a] Based on weighted averages from rainfall #1. Only genes with a significant reduction due to setback distance are shown here.
[b] x is setback distance in meter.

Antibiotics in Runoff

The same three antibiotics detected in swine manure slurry were also detected in the runoff samples from the manured plot during both rainfall events. Split plot in time ANOVA with GLIMMIX showed that the reduction due to setback distance had significant impacts on the concentrations of all three antibiotics in runoff (Table 8). The concentrations of chlortetracycline and tiamulin were significantly higher in the runoff during the rainfall #1 than those in the runoff during rainfall #2. The concentrations of lincomycin in runoff were similar between the two rainfall events.

For all three antibiotics, their concentrations in the runoff from manured plots were significantly higher than those from the control plots (Table 8). For chlortetracycline and tiamulin, because

they were not detected in the runoff from the control plots, ANOVA test could not return *p* values for the analyses.

Table 8. Split plot in time ANOVA on the effects of setback distance and rainfall events on the concentration of antibiotics in runoff.

	Chlortetracycline (µg/L)	Lincomycin (µg/L)	Tiamulin (µg/L)
Setback distance (m)			
0.0	25.0 a	9.94 a	0.950 a
3.0	8.14 b	4.09 a	0.235 b
6.1	6.22 bc	4.69 a	0.208 b
12.2	2.44 cd	0.522 b	0.034 c
18.3	1.30 d	0.359 b	0.010 c
Rainfall event			
1	8.01 a	2.30	0.195 a
2	3.45 b	1.82	0.063 b
p-values for:			
Distance	0.001	0.007	<.001
Rainfall	0.002	0.642	0.005
Distance × Rainfall	0.496	0.655	0.456

[a] Values reported under "Setback distance" and "Rainfall event" are treatment averages, which were calculated based on the data for one particular treatment level. For example, 25.0 was calculate using the chlortetracycline concentration of all runoff samples at distance 0.00 m from both rainfall events.
[b]In LSD tests, a value followed by a single letter (e.g., a) is significantly different at the 0.05 level from a value followed by a different single letter (e.g., b). A value followed by a letter combination (e.g., ab) are not significantly different at the 0.05 level from another value followed by a single letter (e.g., a).

Table 9. Impact of manure amendment on antibiotic concentrations in runoff from both rainfalls.

	Chlortetracycline (µg/L)	Lincomycin (µg/L)	Tiamulin (µg/L)
Manure Amendment			
Amended Plots	1.48	1.11 a	0.015
Control Plots	ND[a]	0.003 b	ND
p-values:	N/A[b]	<0.001	N/A

[a]ND, not detected.
[b]N/A, not applicable, not sufficient values detected to run ANOVA.

The concentrations of antibiotics in runoff from Rainfall #1 were plotted against setback distances in Figure 3. The concentrations of all three antibiotics followed an exponential decrease, shown as straight lines on the semi-log graph. The linear equations and their R^2 values are reported in Table 10. Tiamulin and lincomycin decreased at similar rates, with slopes being

0.0961 and 0.1034, respectively. Chlortetracycline had the lowest decrease rate, with a slope of 0.0655. No antibiotics were detected in the runoff from the control plots.

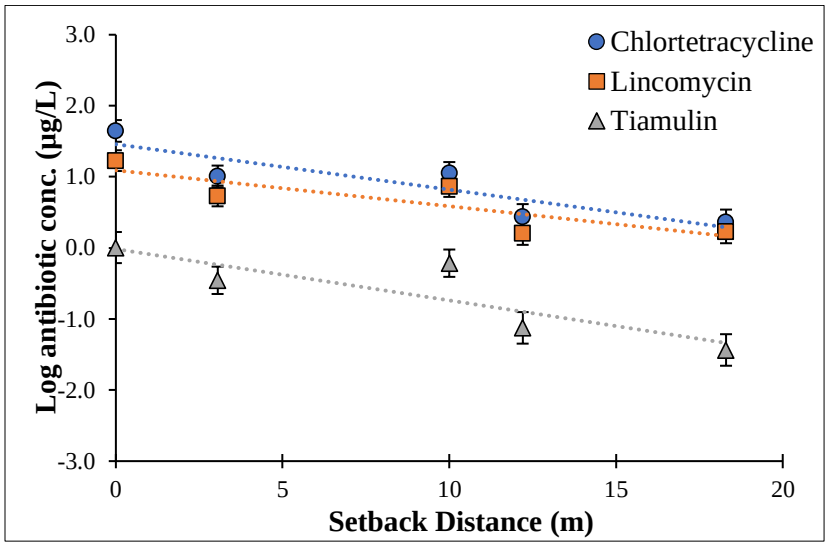


Figure 3. Weighted average concentration of chlortetracycline, lincomycin, and tiamulin in runoff after rainfall one. The error bars represent standard errors based on ANOVA of replicates and distance using GLIMMIX.

Table 10. Linear equations and R^2 for the GLIMMIX analysis on antibiotics in runoff.[a]

Compound	kx + m (log µg/L)[b]	R^2
Chlortetracycline	-0.0639x + 1.459	0.799
Lincomycin	-0.0505x + 1.092	0.718
Tiamulin	-0.0722x - 0.016	0.744

[a] Based on weighted averages from rainfall one.
[b] x is setback distance in meter.

ARGs in Soil

The effects of setback distance and soil depth on gene concentrations in soil are presented in Table 11. Among the ten genes tested, six genes were consistently detected below their detection limits at all depths and all distances. Most of the detected values were for soil samples from top layers at locations close to the manured region. For these six genes, ANOVA did not return *p*-values for the two treatment factors. The remaining four genes, *bla*$_{TEM}$, *tet*(D), *intI1*, and the 16S rRNA gene, were consistently detected at most setback distances. The gene *bla*$_{TEM}$ was significantly affected by the setback distance ($p < 0.05$) and the major drop occurred at around a setback distance of 3.0 m. The concentrations of *bla*$_{TEM}$ in top soil were plotted against setback distances in Figure 4. The impacts of setback distance on the other three genes were not significant at the $p < 0.05$ level.

The concentrations of all four genes in soil were significantly affected by soil depth. With the exception of *bla*$_{TEM}$, the other three genes exhibited a clear trend with higher concentrations in top soil and lower concentrations in bottom soil (Table 11)..

Table 11. ANOVA on the effects of setback distance and depth on the concentration of genes in soil within the setback region.

	16S rRNA (copy/g dw)	*bla*$_{TEM}$ (copy/g dw)	*erm*(B) (copy/g dw)	*erm*(C) (copy/g dw)	*erm*(F) (copy/g dw)	*intI1* (copy/g dw)	*tet*(D) (copy/g dw)	*tet*(O) (copy/g dw)	*tet*(Q) (copy/g dw)	*tet*(X) (copy/g dw)
Setback Distance (m)[a,b]										
0.0	2.4×10^7	3.9×10^4 a	3.3×10^4	BDL	1.7×10^4	7.1×10^4	9.8×10^3	BDL	2.9×10^4	BDL
3.0	1.8×10^7	2.5×10^4 ab	1.2×10^4	BDL	8.7×10^3	2.5×10^4	1.0×10^4	BDL	BDL	BDL
6.1	4.4×10^6	1.3×10^4 b	1.4×10^4	BDL	BDL	4.3×10^4	1.4×10^4	BDL	BDL	BDL
9.1	1.0×10^7	1.3×10^4 b	BDL[c]	BDL	BDL	1.2×10^4	1.8×10^4	BDL	BDL	BDL
12.2	7.2×10^6	1.3×10^4 b	BDL	BDL	BDL	3.4×10^4	1.8×10^4	BDL	BDL	BDL
15.2	1.6×10^7	1.6×10^4 b	BDL	BDL	BDL	2.3×10^4	BDL	BDL	BDL	BDL
Depth (cm)										
0-10	1.8×10^7 a	1.3×10^4 b	1.8×10^4	BDL	BDL	9.1×10^4 a	2.5×10^4 a	BDL	BDL	BDL
10-20	1.5×10^7 a	2.5×10^4 a	BDL	BDL	BDL	2.7×10^4 b	BDL	BDL	BDL	BDL
20-30	5.5×10^6 b	1.8×10^4 ab	BDL	BDL	BDL	1.1×10^4 c	9.3×10^3 b	BDL	BDL	BDL
p - values for:										
Distance	0.205	0.017	N/A[d]	N/A	N/A	0.070	0.757	N/A	N/A	N/A
Depth	0.021	0.050	N/A	N/A	N/A	< 0.001	0.030	N/A	N/A	N/A
Distance × Depth	0.410	0.093	N/A	N/A	N/A	0.221	0.452	N/A	N/A	N/A

[a]Values reported under "Setback" and "Depth" are treatment averages, which were calculated based on the data for one particular treatment level. For example, 1.7×10^7 was calculate using the 16S rRNA gene concentration of all replicate soil samples underneath the manured region for all three depths.

[b]Values followed by a letter combination sharing one or more letters are not statistically different at the $p < 0.05$ level based on LSD tests.

'[c]BDL, below detection limit, indicates that there were too few values above detection limit to estimate an average.

[d]N/A, not applicable, indicates that there were too few values to run successful ANOVA.

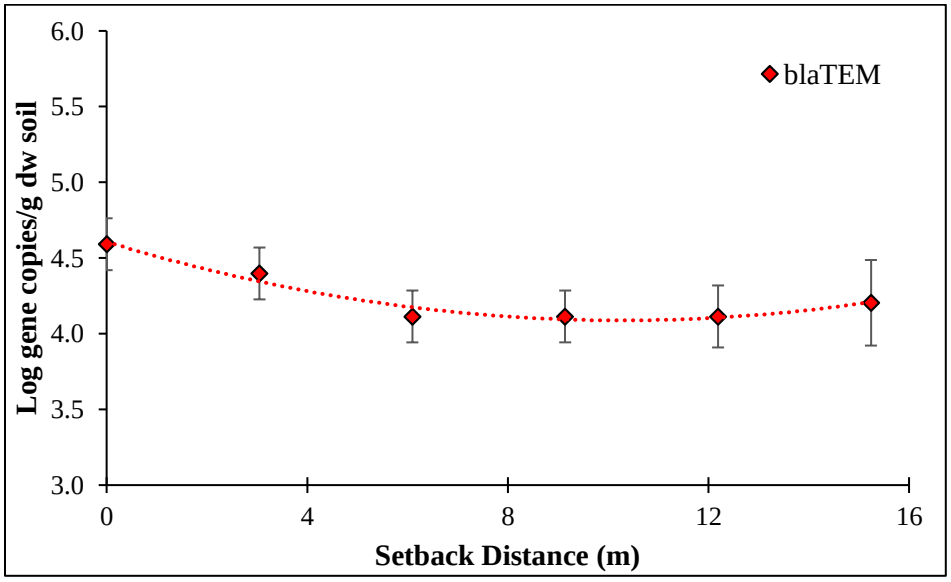


Figure 4. Weighted average concentration of *bla*$_{TEM}$ in soil at 0-10 cm depth. The error bars indicate standard error based on the GLIMMIX analysis of replicates and distance.

Manure application had significant impacts on the levels of ARGs in the plots (Table 12). Four genes (i.e., 16S rRNA Gene, *bla*$_{TEM}$, *intI1*, and *tet*(D)) were detected positive in soil samples in both the control and the amended plots. Among them, *intI1* levels in soil were significantly affected manure application. The six remaining genes had few values above the detection limits. As a result, ANOVA tests could not return treatment average or *p* values. These six genes and *intI1* in soil were caused by manure amendment.

Table 12. Impact of manure amendment on gene concentrations in soil within the manured region.

	16S rRNA (copy/g dw)	*bla*$_{TEM}$ (copy/g dw)	*erm*(B) (copy/g dw)	*erm*(C) (copy/g dw)	*erm*(F) (copy/g dw)	*intI1* (copy/g dw)	*tet*(D) (copy/g dw)	*tet*(O) (copy/g dw)	*tet*(Q) (copy/g dw)	*tet*(X) (copy/g dw)
Manure Amendment										
Amended Plots	5.8×10^7	2.4×10^4	1.6×10^6	1.3×10^6	7.4×10^5	2.9×10^6	1.8×10^4	3.5×10^4	7.7×10^5	1.2×10^6
Control Plots	1.1×10^8	2.0×10^4	BDL[a]	BDL	BDL	1.5×10^4	1.6×10^4	BDL	BDL	BDL
p-values:	0.443	0.558	N/A[b]	N/A	N/A	0.003	0.466	N/A	N/A	N/A

[a]BDL, below detection limit, indicates that there were too few values above detection limit.

[b]N/A, not applicable. There were not sufficient number of values above detection limit to run ANOVA.

Antibiotics in Soil

Chlortetracycline was the most abundant antibiotic in soil. It was detected, albeit at values just above the detection limit, at all depths of soils prior to manure application. Neither lincomycin nor tiamulin was present above their detection limits in any depths of the soils prior to manure application. The other feed additives neotame, penicillin G, and penicillic acid were not detected at any distance or depth.

Because no antibiotics were detected in soil past a depth of 10 cm, setback distance was the only treatment factor subject to ANOVA analysis. Chlortetracycline and tiamulin were detected in the surface soil at almost all setback distances and decreased significantly with increased setback distance (Table 13). The concentrations of these two antibiotics in surface soil are plotted against setback distances in Figure 5. Lincomycin was not detected in the surface soil at any setback distance.

Table 13. ANOVA results for the effects of setback distance on the concentration of antibiotics in top soil within the setback region.

	Chlortetracycline	**Lincomycin**	**Tiamulin**
	(ng/g dw)	(ng/g dw)	(ng/g dw)
Setback (m)			
0.0	9.49 a	ND[a]	0.114 a
3.0	3.21 b	ND	0.023 b
6.1	1.72 c	ND	0.016 b
9.1	1.03 d	ND	ND
12.2	1.09 d	ND	0.009 b
15.2	0.72 e	ND	0.023 b
p - values:	< 0.001	N/A[b]	0.014

[a]ND, not detected.

[b]N/A, not applicable, indicates that there were too few values to run successful ANOVA.

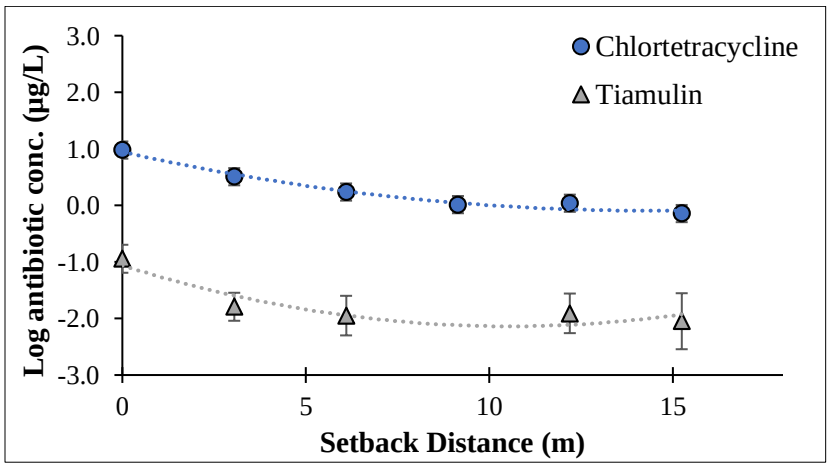


Figure 5. Weighted average concentration of chlortetracycline and tiamulin in soil at 0-10 cm depth. The error bars indicate standard error based on the GLIMMIX analysis of replicates and distance.

The effects of manure application on antibiotic levels in soil within the manured region were evident (Table *14*). ANOVA analyses showed that the impacts of manure amendment was significant on the concentration of chlortetracycline in soil in the manured region. For lincomycin and tiamulin, because they were not detected in the soil of the control plots, ANOVA did not return treatment averages or *p* values for these two antibiotics.

Table 14. Impacts of manure amendment on antibiotic concentrations in soil within the manured region.

	Chlortetracycline	Lincomycin	Tiamulin
	(ng/g dw)	(ng/g dw)	(ng/g dw)
Manure Amendment			
Amended Plots	51.2	0.75	6.35
Control Plots	0.62	ND[a]	ND
p-values:	0.017	N/A[b]	N/A

[a]ND, not detected.
[b]N/A, not applicable, indicates that there were too few values to run successful ANOVA.

High Throughput qPCR Results
A total of 134 ARGs were detected among all the samples (

Table *15*). On average, 95 ARGs were detected in manure samples. Thirty-one (31) ARGs were detected in the runoff samples from plots prior to manure application, while an average of 126 ARGs were detected in runoff samples from the plots after manure application. Thirteen (13) ARGs were detected in soil samples before manure application, compared to 111 ARGs detected in soil receiving manure (i.e., the manured region, Figure 1). For soil samples downslope from the manured regions (i.e., the setback region, Figure 1), 44 ARGs occurred at 0-m distance, and an average of 10 ARGs were detected across the setback distances from 3-m to 15.2-m.

Table 15. The number of ARGs detected in runoff and soil samples before and after manure application.

	Runoff Samples	Soil Samples
Control plots receiving no manure		
	31	13
Amended plots receiving manure		
Manured Region	-	111
Setback Region		
0 m	130	44
3 m	131	8
6.1 m	131	10
9.1 m	-	12
12.2 m	123	9
15.2 m	-	10
18.3 m	111	-

For each ARGs detected positive on the high-throughput qPCR platform, its relative abundance was calculated using Equation (2). The sum of the relative abundance for all ARGs was calculated, according to Equation (3), for rainfall sample, and plotted against setback distances in Figure 6. Among the runoff samples from amended plots, a decreasing trend in the sum of the relative abundance of all ARGs is observed. Runoff samples were only collected from the control plots with the longest setback distance tested (i.e., 18.3 m). The sum of the relative abundance of all ARGs in the runoff sample from the control plots was lower than the counterpart from the amended plots.

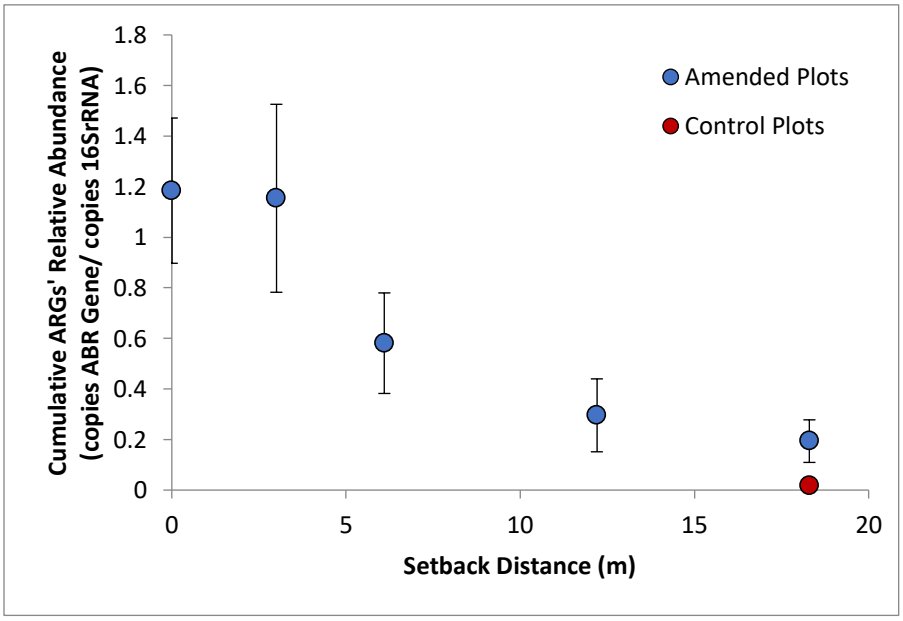


Figure 6. The sums of the relative abundance of all ARGs in runoff samples as a function of setback distances.

In addition to the sum of the relative abundance of all ARGs, we also looked into the makeup of the resistome in the runoff samples (Figure 7). The percentages of resistance genes against aminoglycoside and MSLB decreased in runoff samples as the setback distance increased (Figure 7, A-E). On the contrary, the percentage of ARGs belonging to MDR increased as the setback distance increased (Figure 7, A-E). For the resistome in runoff from the control plots, the two major classes of ARGs were MGE and ARGs belonging to MDR.

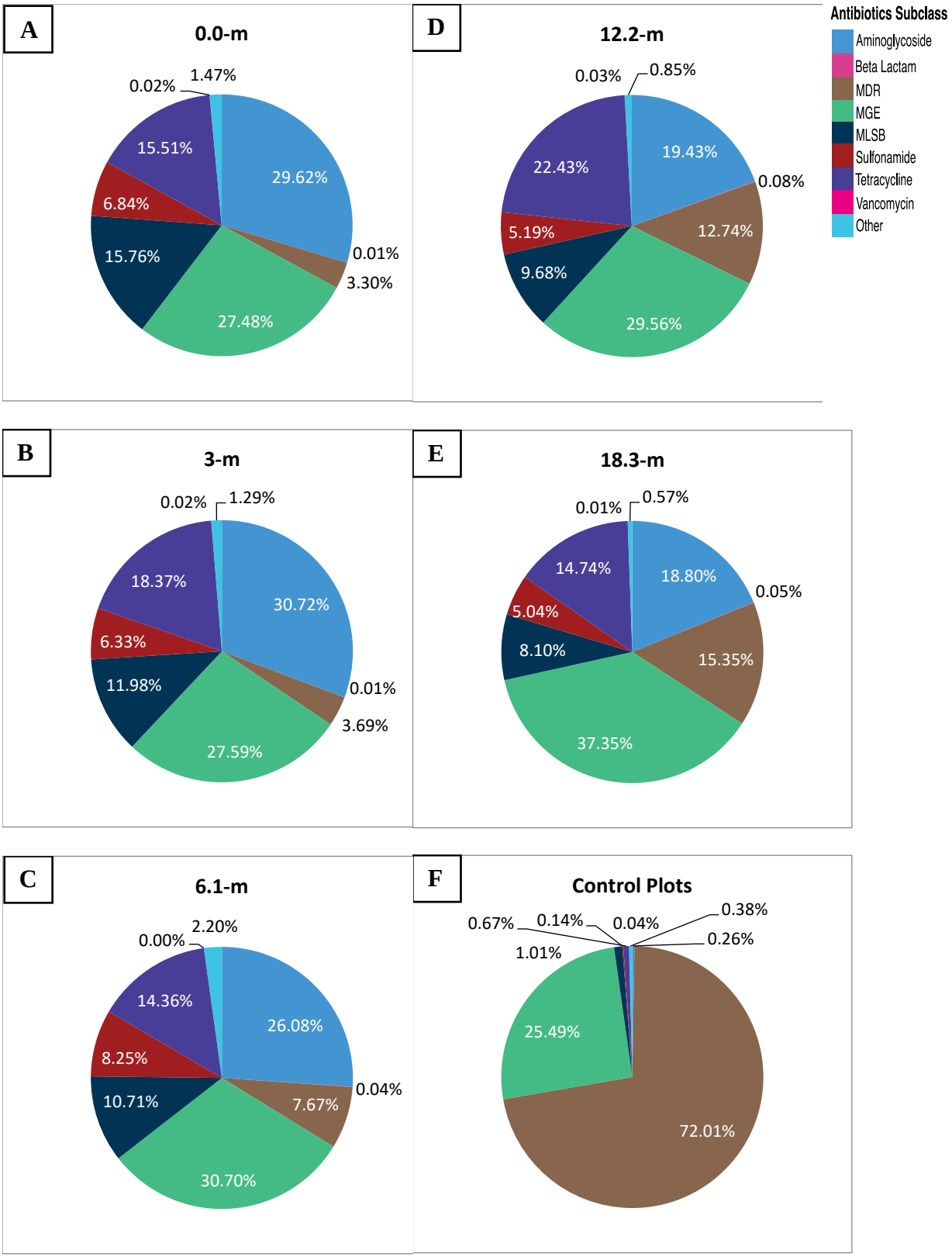


Figure 7. The makeup of the sum of the relative abundance of all ARGs in the runoff samples from various setback distances: (A) 0.0 m, (B) 3.0 m, (C) 6.1 m, (D) 12.2 m, and (E) 18.3 m. (F)

shows the makeup of the sum of the relative abundance in the runoff samples collected at the setback distance of 18.3 from the control plots. MDR, multidrug resistance; MLSB, Macrolide-Lincosamide-Streptogramin B; and MGE, mobile genetic elements.

The sum of the relative abundance for all ARGs was calculated for soil samples, and plotted against setback distances in Figure *8*. Among the soil samples in the setback region, a flat trend in the sum of the relative abundance of all ARGs is observed and maintained at a relatively low level. For the manured region, the addition of manure significantly increased the sum of the relative abundance of all ARGs.

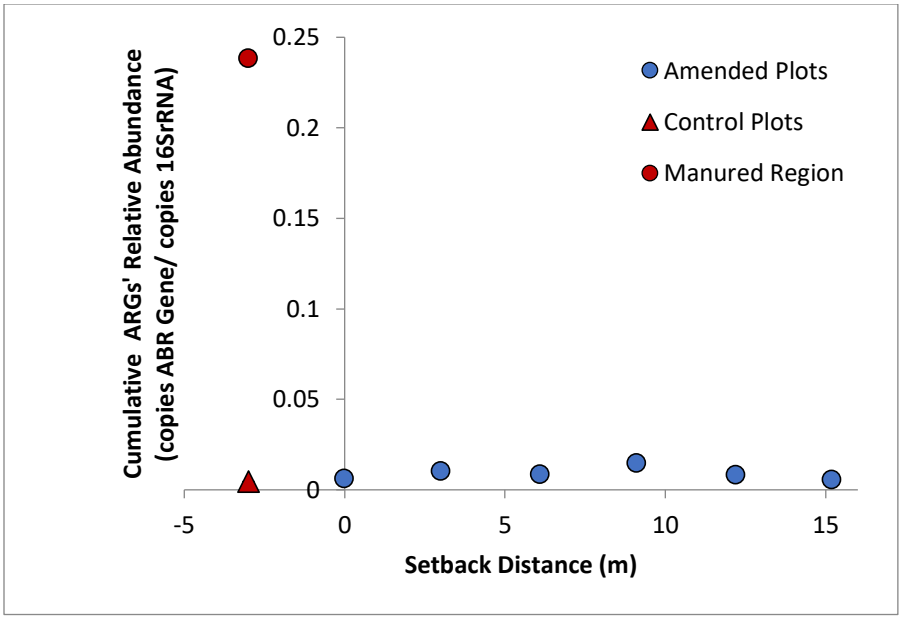


Figure 8. The sums of the relative abundance of all ARGs in soil samples as a function of setback distances. The SRA of ARGs in the manured region is also reported in this figure. Standard deviations were calculated based on duplicate samples and three technical replicates on the HT-qPCR platform. Error bars based on the standard deviations are too small to be visible in the figure.

The impact of manure application on the makeup of the resistome of the manured region is shown in Figure *9*. The resistome composition of soil in the manured region is very different before and after manure application. Before manure application, ARGs belonging to MDR were the predominant ARGs in soil. After manure application, ARGs belonging to MDR only consists of 10.42% of the resistome. Meanwhile, the percentages of tetracycline, aminoglycoside, sulfonamide, and MLSB increased substantially.

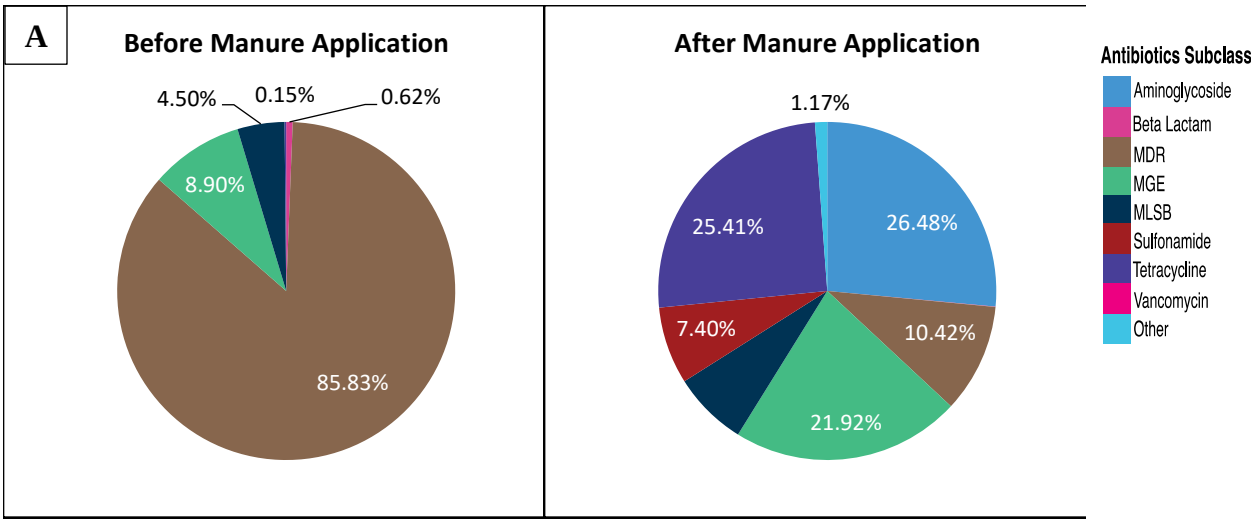


Figure 9. The composition of the resistome in soil before and after manure application. MDR, multidrug resistance; MLSB, Macrolide-Lincosamide-Streptogramin B; and MGE, mobile genetic elements.

Only the soil at the shortest setback distance appeared to be affected by the manure with respect to resistome composition (Figure *10*). The impact quickly diminished in soil at the subsequent setback distances. For example, resistance genes corresponding to aminoglycoside and tetracycline only presented in substantial percentages in the soil at the 0.0 m setback distance (i.e., soil immediately down slope from the manured region). No such resistance genes were present in substantial percentages at subsequent setback distances. For the soil samples at the setback distance 3.0 m and beyond, the main classes of resistance genes detected belonged to MDR, MGE and MLSB.

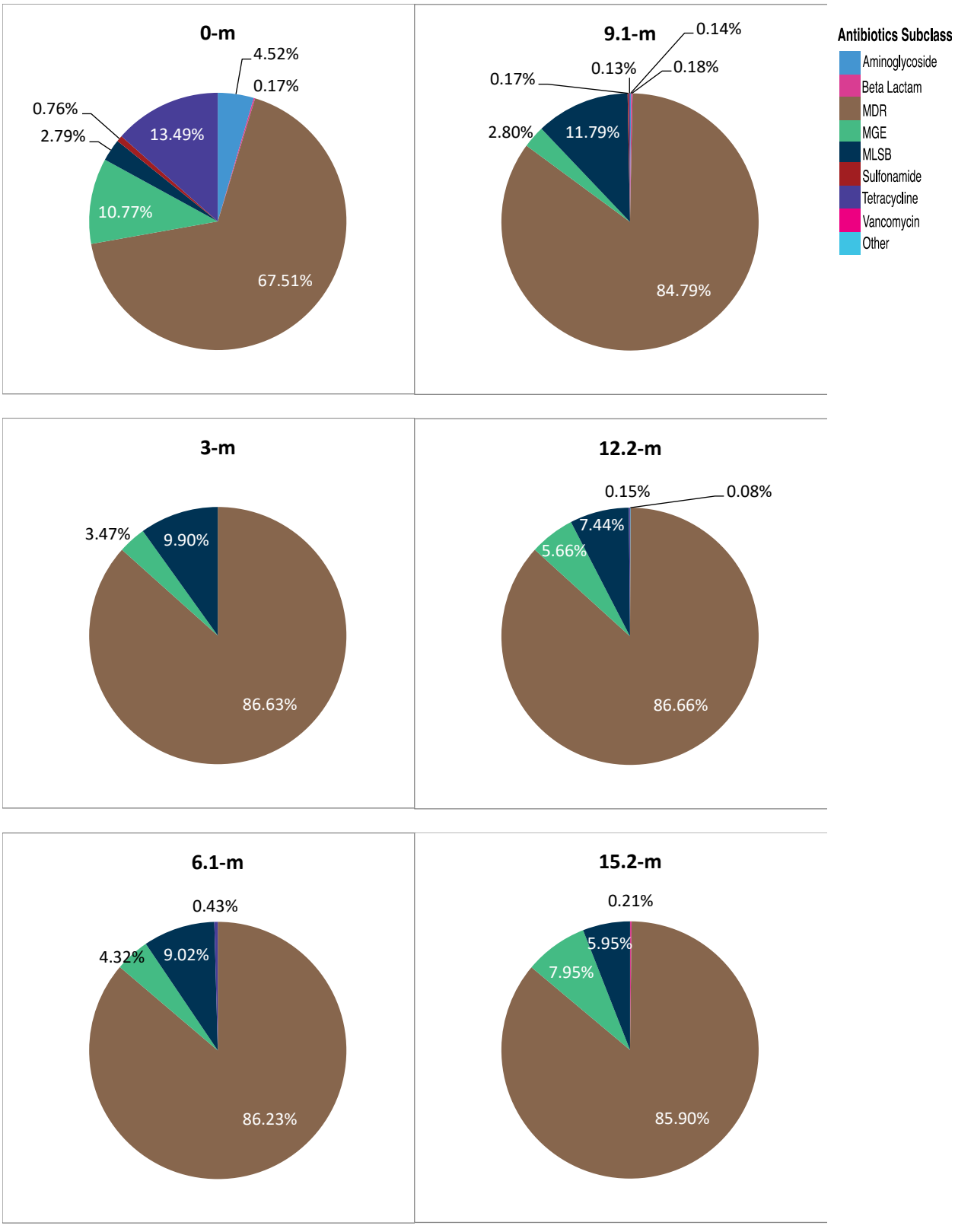

0-m
4.52%
0.17%
0.76%
2.79%
13.49%
10.77%
67.51%
9.1-m
0.14%
0.13%
0.18%
0.17%
2.80%
11.79%
84.79%
Antibiotics Subclass
Aminoglycoside
Beta Lactam
MDR
MGE
MLSB
Sulfonamide
Tetracycline
Vancomycin
Other
3-m
3.47%
9.90%
86.63%
12.2-m
0.15%
0.08%
7.44%
5.66%
86.66%
6.1-m
0.43%
4.32%
9.02%
86.23%
15.2-m
0.21%
5.95%
7.95%
85.90%

Figure 10. The composition of the resistome in soil across the setback distances of 0m, 3m, 6.1m, 9.1m, 12.2m and 15.2m. MDR, multidrug resistance; MLSB, Macrolide-Lincosamide-Streptogramin B; and MGE, mobile genetic elements.

Heatmap is another way to visualize the resistome composition, at the individual gene level, of different samples. The three heatmaps shown below provided a comprehensive picture on the resistome of three sample types: manure (Figure *11*), runoff (Figure 12), and soil (Figure 13). The x-axis of the heatmaps shows the samples, while the y-axis shows individual ARGs grouped by resistance gene families. The color of each cell corresponds to the relative abundance calculated using Equation 2.

The resistome composition of the swine manure was relatively stable over the course of the field test. During the 10-week field test, fresh manure slurry was collected weekly from the swine facility. We sent six of the ten manure samples for the high-throughput qPCR analyses. Results show that the resistome composition was relatively stable among these manure samples.

Manure land application significantly altered the resistome composition in runoff. In Figure 12, the first column was the resistome composition of runoff from a control plot without manure application. The rest of the columns show the resistome composition of runoff from manured plots with various setback distances. The runoff from manured plots contained many ARGs that were absent in the runoff from control plots. Among the runoff from manured plots, longer setback distances resulted in runoff containing ARGs with lower relative abundance, as reflected in the gradual change of colors.

Manure application significantly altered the resistome composition in the soil within the manured region, however it had limited impacts on the soil within the setback region. In Figure 13, the first two columns show the soil resistome of the manured region before and after manure application. The sharp contrast shows that manure application significantly increased the variety and relative abundance of ARGs in soil. The other columns show the resistome of soil in setback region at various distances. Other than the soil at the shortest setback distance (i.e., 0.0 m), which exhibited some increase in the variety and abundance of ARGs, the soils at other setback distances were not affected by manure application in the manured region.

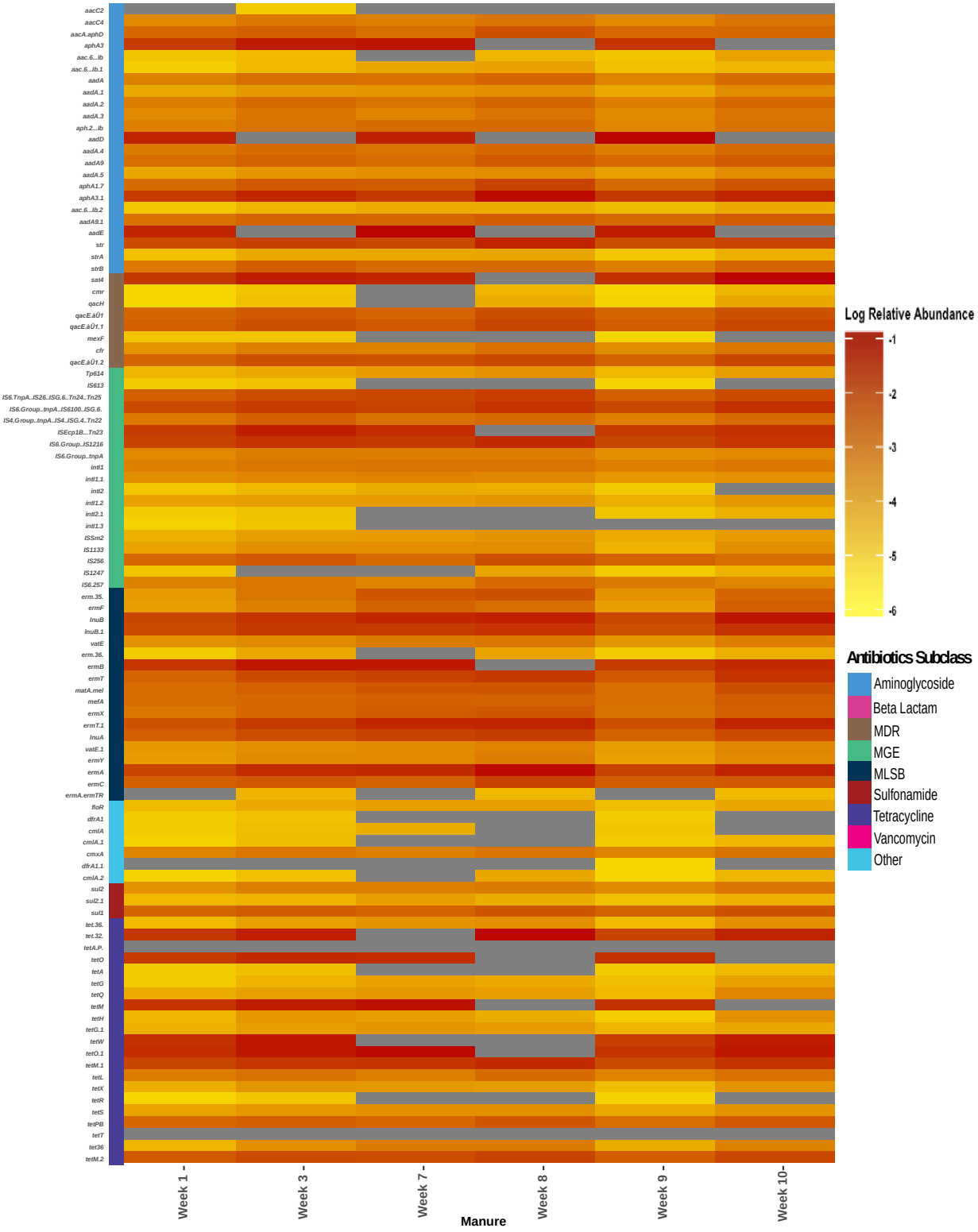


Figure 11. A heatmap showing the relative abundance of individual genes in manure samples collected from multiple weeks. MDR, multidrug resistance; MLSB, Macrolide-Lincosamide-Streptogramin B; and MGE, mobile genetic elements.

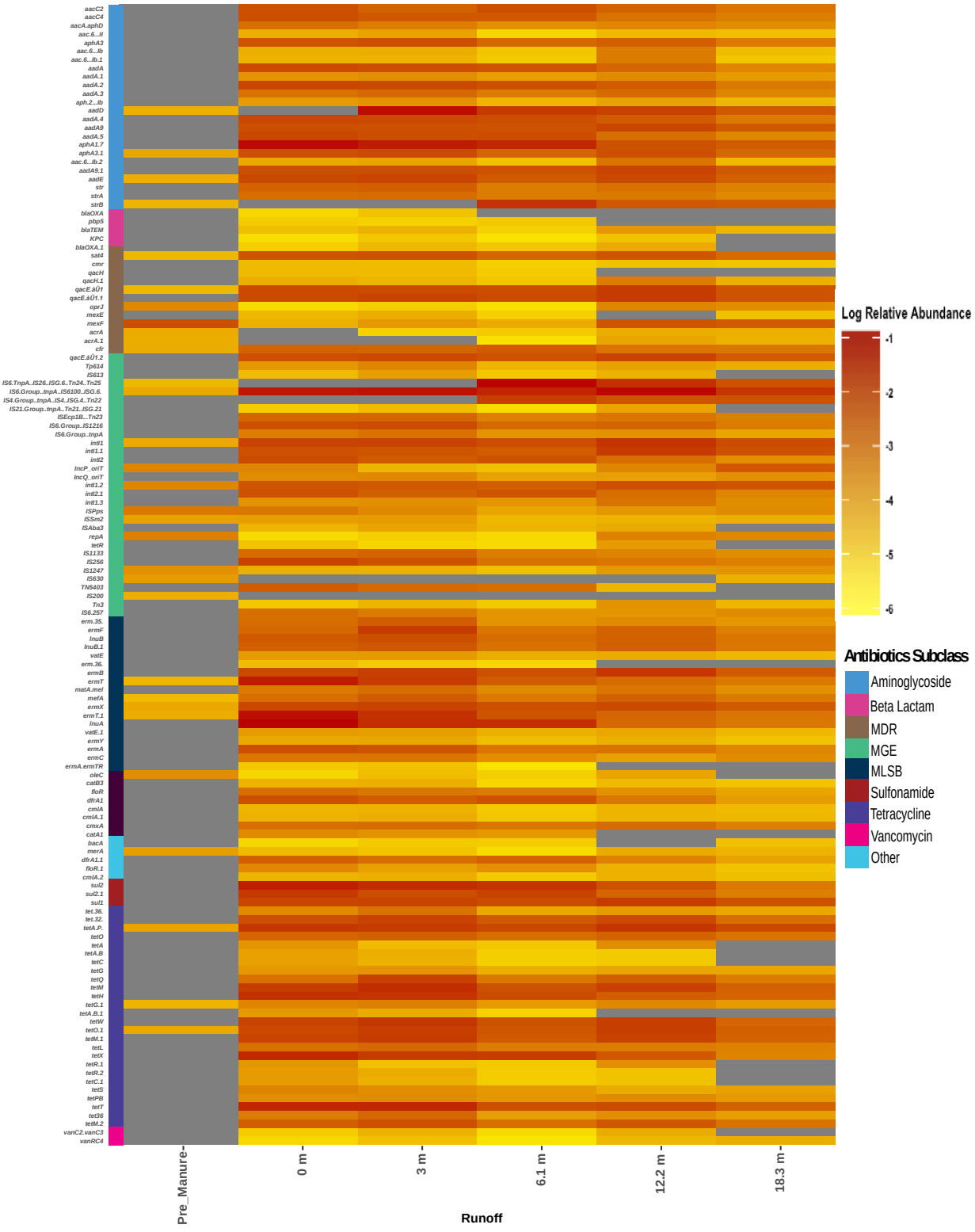


Figure 12. A heatmap showing the relative abundance of individual genes in runoff samples collected from different setback distances. MDR, multidrug resistance; MLSB, Macrolide-Lincosamide-Streptogramin B; and MGE, mobile genetic elements.

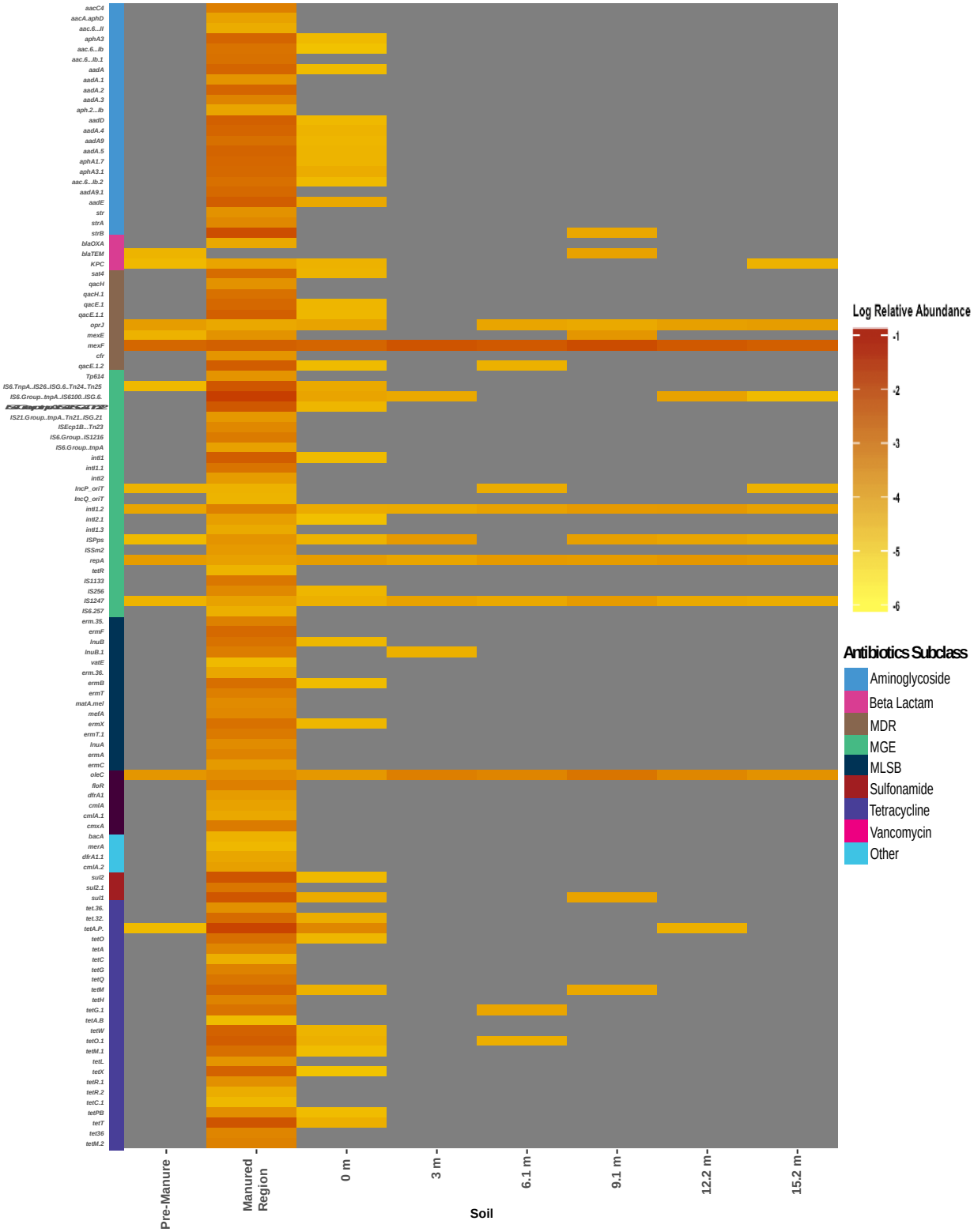


Figure 13. A heatmap showing the relative abundance of individual genes in soil samples collected from different setback distances. The relative abundance of each gene is color coded. The gray color means no detection of the ARGs in the soil at certain setback distances. MDR,

multidrug resistance; MLSB, Macrolide-Lincosamide-Streptogramin B; and MGE, mobile genetic elements.

Discussion

ARGs

There are several possible mechanisms that contribute to the reduction of the ARG concentrations in runoff with increased setback distance. The log percentage reduction, due to dilution from water falling on the setback region, would be 0.0355 log per meter of increased setback distance. All seven genes whose concentrations in runoff were significantly affected by setback distances had slopes steeper than 0.0355 (Table 7). This suggest mechanisms other than dilution also played significant roles in reducing gene concentrations in runoff as setback distance increased. One of such mechanisms can be infiltration, which was verified in a separate study.

Out of the seven genes that decreased significantly with increased setback distance, the 16S rRNA gene and *intI1* had the least steep reduction with setback distances, having slopes at 0.0638 and 0.0641 log per meter, respectively (Table 7). This could be because the two genes occurred naturally in soil (Table 12). Hence, in addition to the manured region, the setback region also contributed these two genes to the runoff. In contract, the other five genes (i.e., *erm*(B), *erm*(C), *tet*(O), *tet*(Q), and *tet*(X)) did not occur naturally in soil (Table 12). In other words, manure-borne bacteria applied in the manured region were the only source of these ARGs in runoff. As a result, their reduction along the setback distance was more pronounced (Table 7).

A companion study with the same runoff samples investigated the concentrations of manure-borne nutrients in runoff with respect to setback distance.[19] The study showed that a setback distance of 12.2 m could effectively reduce the nutrient concentrations in runoff to values similar to the background. Nutrients analyzed included dissolved phosphorous, ammonia, total nitrogen, etc. In this study, it is estimated that a setback distance of 50 meters would bring the level of ARGs in runoff down to the background level (i.e., runoff from control plots receiving no manure). This was estimated using the equations listed in Table 7 and solving for the distance that correspond to ARG levels in runoff from control plots. Compared to the antibiotics and ARGs in this study, nutrients reached background levels at a much shorter setback distance. This is likely because nutrients were naturally abundant in soil, resulting in high levels of nutrients in the runoff from control plots.

Several studies have examined the impacts of manure application, setback distance, and vegetated filter strips (VFS) on the concentrations of *E. coli* in both runoff and soil.[14,17,32,33] Abu-Ashour et al found that on bare soil plots, 2% and 6% slope respectively, *E. coli* was found up to a distance of 20 m in both soil and runoff for the plot with 2% slope and 30 m in soil and 35 m in runoff for the plot with 6%.[14] However, in that study *E. coli* cells were sprayed on the soil and might not have the same type of attachment or infiltration as do microbes in manure. Thus, they might have different release rates from the soil matrix.

Because the HT-qPCR assay included more than 130 ARGs, it can provide a reasonably comprehensive picture of the resistome of the samples, revealing valuable information on the impacts of manure application on the resistomes of soil and in runoff water. Compared to the

resistome of the soil in the setback region, the resistome of runoff was more significantly impacted by manure application, in terms of both the number of ARGs detected (

Table *15*) and the relative abundance of the ARGs (Figure 6 vs. Figure 8).

For the resistome in runoff, increasing setback distance did not significantly reduce the number of ARGs detected (

Table *15*). It is reasonable to expect that most of the bacteria carrying ARGs would flow with runoff within the range tested (i.e., 0-18.3 m setback distance). Some ARG-carrying bacteria were held back by surface soil or plant residues, resulting in the disappearance of some ARGs in the runoff collected at the longest setback distance (i.e., 18.3 m). In contrast to the relatively small reduction in ARG numbers, the sum of the relative abundance of all ARGs detected was more significantly affected by increasing setback distance (Figure 6): the value at 18.3 m is only about one sixth of the value at 0.0 m. It is noted that at even the longest setback distance tested, the sum of the relative abundance for runoff from the manured plots was still higher than that for runoff from the control plots, suggesting that a longer setback distance would be needed to diminish the impacts of manure application on the microbial quality of runoff water.

For the resistome in soil, the impacts of manure application were largely limited to the manured region. The setback region, except for the area very close to the manured region, was essentially unaffected by manure application (

Table *15* and Figure 8). In this study, soil samples were collected five days after the second rainfall simulation. Hence, the results suggest that even though some ARG-carrying bacteria in runoff may be trapped in soil within the setback region, they were not substantial enough to alter the resistome of the soil days after the rainfall event.

Antibiotics

The antibiotics in the runoff decreased at approximately the same rates as the genes. A prior study evaluating antimicrobial transport from swine slurry amended plots with simulated rainfall found concentrations of 0.07-2.7 μg L^{-1} lincomycin in the runoff.[34] These values are similar to the ones from this study, which ranged from 0.36 to 9.9 μg L^{-1}. The concentration of tiamulin detected in the manure, 8.48 ± 1.42 mg kg^{-1} dw, was higher than that of lincomycin, 4.26 ± 1.64 mg kg^{-1} dw. In the soil samples, lincomycin was only present in the top soil on the broadcast area while runoff contained a higher concentration of lincomycin than tiamulin. Based on the data it seems that lincomycin has a higher solubility than tiamulin.

A previous study tested the impacts of rainfall events on the concentration of chlortetracycline and tylosin in runoff. It was found that between event intervals of 24 h the concentration of tylosin did decrease significantly while the concentration of chlortetracycline did not.[31] The low antibiotic concentrations at deeper depths are consistent with other studies. One study found that oxytetracycline, with a k_d of 680-1030 L kg^{-1}, did not occur at depths below 5 cm.[30] Reported values of k_d for chlortetracycline is 500-1800 L kg^{-1} for a silty clay loam,[36] which is similar to the soil texture in this study.[37] The half-life for chlortetracycline on soil was reported as 24-25 days.[38,39] The half-life of tiamulin in soil is reported as 16 days.[35]

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