2020

Influence of Setback Distance on Antibiotics and Antibiotic Resistance Genes in Runoff and Soil Following the Land Application of Swine Manure Slurry

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Environmental Science & Technology

pubs.acs.org/est

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Influence of Setback Distance on Antibiotics and Antibiotic Resistance Genes in Runoff and Soil Following the Land Application of Swine Manure Slurry

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Cite This: *Environ. Sci. Technol.* 2020, 54, 4800–4809

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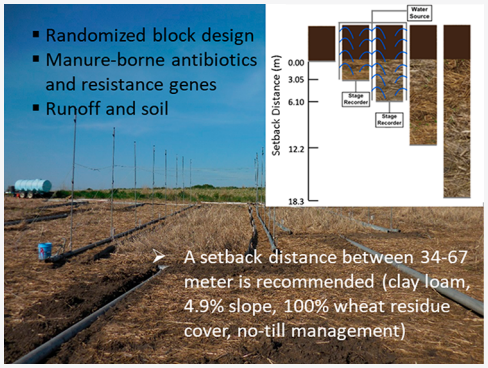
ABSTRACT: The environmental spread of antibiotics and antibiotic resistance genes (ARGs) from the land application of livestock wastes can be a potential public health threat. The objective of this study was to assess the effects of setback distance, which determines how close manure may be applied in relation to surface water, on the transport of antibiotics and ARGs in runoff and soil following land application of swine manure slurry. Rainfall simulation tests were conducted on field plots covered with wheat residues, each of which contained an upslope manure region where slurry was applied and an adjacent downslope setback region that did not receive slurry. Results show that all three antibiotics (chlortetracycline, lincomycin, and tiamulin) and seven out of the ten genes tested (*erm*(B), *erm*(C), *intI1*, *tet*(O), *tet*(Q), *tet*(X), and the 16S rRNA gene) decreased significantly in runoff with increased setback distance. Only bla_{TEM}, chlortetracycline, and tiamulin decreased significantly in surface soil with increased setback distance, while the other analytes did not exhibit statistically significant trends. By using linear regression models with field data, we estimate that a setback distance between 34−67 m may allow manure-borne antibiotics and ARGs in runoff to reach background levels under the experimental conditions tested.



INTRODUCTION

Antibiotic resistance in the environment poses a potential threat to public health.[1] One source of antibiotics and antibiotic resistance genes (ARGs) in the environment is manure from livestock receiving antibiotics for disease prevention and treatment[2] in concentrated animal feeding operations (CAFOs).[3] A range of unmetabolized antibiotics are released in manure together with antibiotic resistant bacteria (ARB) and ARGs.[4] When swine manure slurry is land applied as a soil amendment to supplement chemical fertilizers in soil nutrient programs, manure borne ARGs are introduced to the environment and can persist in the soil for up to several weeks.[6] With rainfall or irrigation, manure borne antibiotics and ARGs may contaminate surface water through runoff.[7]

Best management practices (BMPs) are important for minimizing the transport of manure-borne contaminants to surface water. Examples of BMPs are lagoon treatment, soil incorporation of manure during field application, vegetated filter strips, and eliminating high risk areas from manure application such as steeply sloping land and low lying land that tends to flood.[8,9] Another recommendation that is used to prevent water contamination is the use of setback distances. Setback distance refers to the minimum distance between an area where manure is land applied and a water source or residential/commercial area.[10] The Environmental Protection Agency (EPA) requires large CAFOs to implement a setback distance for manure application at least 100 ft (30.5 m) from surface waters and conduits to surface waters, or substitute with a 35 ft (10.7 m) vegetated buffer.[11] Several states currently regulate setback distances from manure application to landscape features such as wells, streams, ponds, and property lines. Missouri, for example, has a recommended setback distance of 300 ft (91.4 m) from wells, 100 ft (30.5 m) from streams, and 150 ft (45.7 m) from neighboring houses.[12] Iowa requires a 200 ft (61.0 m) setback distance from drinking water wells and an 800 ft (244 m) setback distance from high quality water sources for unincorporated manure application. The 200 and 800 ft setback distances can be decreased to 50 ft (15 m) with a buffer strip.[13] These regulations or recommendations on setback distances are often based on the standards on the concentration and mass of nutrients in precipitation-related discharges set forth by regulatory

Received: August 9, 2019
Revised: March 23, 2020
Accepted: March 24, 2020
Published: March 24, 2020

https://dx.doi.org/10.1021/acs.est.9b04834
Environ. Sci. Technol. 2020, 54, 4800–4809

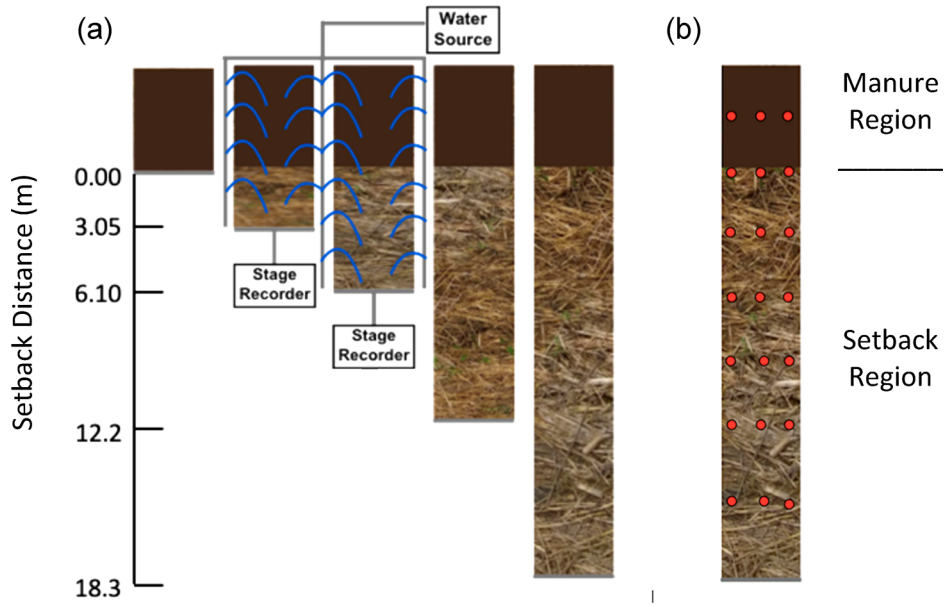


Figure 1. (a) Plots with various setback distances. For each setback distance, four replicate plots were randomly assigned. Two plots were run each week. Runoff samples were collected at the end of each plot when the surface runoff reached steady-state flow. (b) The locations of soil cores (red dots) collected in the manure region and at various setback distances in the setback region of the longest plot. Images are not drawn to scale.

agencies.[11] There are no such standards for antibiotics or ARGs.

The goal of this study is to determine the setback distance needed to minimize the transport of antibiotics and ARGs in agricultural runoff following the land application of swine manure slurry. Specifically, we (1) investigated the effectiveness of varying setback distances on the removal of antibiotics and ARGs in runoff and soil, (2) determined the effect of two back-to-back rainfall events on the concentration of antibiotics and ARGs in runoff, and (3) identified the vertical transport distance for antibiotics and ARGs in soil. Simulated rainfall tests were conducted on replicate plots in the field established using a randomized complete block design. Runoff and soil samples were collected from plots with varying setback distances. The results were analyzed using ANOVA with GLIMMIX to determine the effectiveness of setback distance on contaminant levels in runoff and soil. Outcomes of the study can be used in determining the setback distances needed to protect surface water quality from the antibiotics and ARGs in runoff from manure land application sites.

MATERIALS AND METHODS

Study Site. The study site was located at the University of Nebraska Rogers Memorial Farm, 18 km east of Lincoln, NE. The area chosen for this study had uniform crop residue and a slope of 4.9%. The area had previously been used to grow corn, sorghum, soybeans, and winter wheat. No manure had been applied to the study area since 1966. Winter wheat was harvested prior to the field tests, and glyphosate was applied for weed control. The wheat residue was not removed and gave the soil surface 100% coverage during the study period. The soil type was Aksarben silty clay loam (fine, smectite, mesic Typic Argiudoll), which is a benchmark soil for the corn belt.

Plot Setup. The field tests were conducted over a 10-week period in summer 2016. Twenty (20) plots were established in the study area using a randomized complete block design. Within each of the four blocks, five setback distances (0.0, 3.0, 6.1, 12.2, and 18.3 m) were randomly assigned to the plots. The longest plots that we could set up in the field measured 18.3 m in length. All plots were 3.7 m wide. At the top of each plot, a 3.7 m × 4.9 m area was designated as the manure region to receive swine manure slurry. Downslope from the manure region was the setback region, whose length was determined by the assigned setback distance (Figure 1a). At the end of each plot was a metal lip that could collect runoff and direct it through a flume, where flow could be measured by a stage recorder. During natural rainfall events, the plots were covered with plastic sheets.

Simulated rainfall was generated with a portable rainfall simulator sprinkler system[14] consisting of 3 m sections of 10 cm diameter irrigation pipes, on which 2 cm diameter risers were mounted. Sprinkler heads (Model 78C, Rain Jet Corporation) were located on the top of the risers. The rainfall simulation system was placed so that it covered the entire plot area (i.e., manure region plus setback region). Irrigation water was obtained from an onsite irrigation well. The intensity of the rainfall was approximately 52 mm hr^{-1}, and it was measured using rain gauges placed along the perimeters of the plots.

Manure Collection and Characterization. Manure slurry was collected from a commercial swine operation facility in southeast Nebraska each week. Manure slurry samples were sent to Wards Laboratory for characterization (Grand Island, NE). The mean values and standard deviations for NO_3^--N, NH_4^+-N, total Kjeldahl N, organic N, total phosphorus (TP), pH, and solids content of the slurry were 0.98 ± 0.64 mg kg^{-1}, 2.98 ± 0.33 g kg^{-1}, 5.52 ± 0.57 g kg^{-1}, 2.54 ± 0.67 g kg^{-1}, 6.95 ± 0.88 g kg^{-1}, 7.81 ± 0.19, and 5.35 ± 0.67%, respectively.

Rainfall Simulation Tests. Each week, two plots were used for rainfall simulation tests. In a typical week, rainfall simulations were conducted on the pair of plots on days 1 and 2 under existing antecedent soil moisture. On day 3, the manure slurry was collected and broadcast onto the manure region of the plots by hand at the rate of 3.90 × 10^4 kg liquid slurry ha^{-1}. The rate was based on an annual nitrogen requirements for corn of 151 kg N ha^{-1} $year^{-1}$ for an expected

https://dx.doi.org/10.1021/acs.est.9b04834
Environ. Sci. Technol. 2020, 54, 4800−4809

yield of 9.4 Mg ha^{-1}.[15] On days 4 and 5, rainfall simulation tests were conducted again on the plots.

During rainfall simulation tests when the runoff flow reached steady state based on hydrographs, 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.

For the plots with the longest setback distance, 5 days after the last rainfall simulation, three replicate soil cores were collected from the middle of the manure region as well as at 0.0, 3.0, 6.1, 9.1, 12.2, and 15.2 m setback distances in the setback region (Figure 1b). The soil cores were 30 cm long and were later divided into three layers: 0−10, 10−20, and 20−30 cm. Soil segments from the same depth of the triplicate cores were combined, transported on ice to the laboratory, and stored in freezers until further analysis. Plots with the longest setback distance (i.e., 18.3 m) were also used to generate controls for runoff (i.e., runoff from the plots prior to manure application) and soil (i.e., soil from the plots prior to manure application and any rainfall simulation).

ARG Analysis. Soil samples were thawed at 4 °C and homogenized by hand. Frozen runoff and manure slurry samples were thawed overnight at 4 °C. A 50 mL portion of runoff sample was filtered through sterile 0.22 μm filters, and 5 mL manure slurry sample was centrifuged. DNA was extracted from 0.25 g soil, filter paper, or centrifuge pellets using the Qiagen DNeasy PowerSoil Kit following the manufacturer protocol with the exception that two 40-s bead beating steps were used to lyse cells. Extracted DNA was purified with OneStep PCR Inhibitor Removal Kit (Zymo Research). Successful removal of PCR inhibitors was confirmed using end point PCR targeting the 16S rRNA gene.[16] Candidate ARGs detected positive in manure samples using end point PCR were selected for further qPCR analyses, which included bla_{TEM}, *erm*(B), *erm*(C), *erm*(F), *intI1*, *tet*(D), *tet*(O), *tet*(Q), and *tet*(X). Detailed qPCR procedure can be found in the Supporting Information file.

Antibiotic Analysis. On the basis of information given from the swine production facility, four antibiotics (chlortetracycline, lincomycin, penicillin G, and tiamulin), one antibiotic degradation product (penicillic acid), and one sweetener (neotame) were included in the quantification. The sweetener was fed to swine as a growth promoter. The procedure for antibiotic analysis using liquid chromatography tandem mass spectrometry was modified from an earlier study analyzing similar samples.[7] Detailed procedures are reported in the Supporting Information file. Method detection limits, determined by replicate extraction and analysis of low-level (0.005 μg/L) fortified water ranged from 0.0004 μg/L for penicillin G to 0.0240 μg/L for lincomycin, with recovery between 60 and 125%. Method detection limits in solid samples were determined from replicates in clean sand spiked at 1.0 ng/g and ranged between 0.4 and 2.0 ng/g with recoveries between 46 and 127%. A synthetic macrolide, oleandomycin, was added to all samples prior to extraction as a surrogate, and recovery averaged 70 ± 39% in all field samples. Quality controls analyzed at a frequency of not less than 1 in 20 (5%) of all field samples included laboratory duplicates, laboratory reagent (method) blanks, laboratory fortified blanks, and laboratory fortified matrix samples.

Statistical Analysis. Split plot in time ANOVA using GLIMMIX in SAS (Cary, NC) with setback distance as the whole plot factor and rainfall event as the "time" factor was used to determine the significance of setback distance on the log concentration of ARGs and antibiotics in runoff and soil. For runoff, treatment factors were setback distance and rainfall events after manure application (i.e., rainfall #1 and rainfall #2). For soil, treatment factors were setback distance and depth (i.e., 0−10 cm, 10−20 cm, 20−30 cm). For treatment factors identified by ANOVA as having significant impacts (i.e., $p < 0.05$), least significant difference (LSD) tests were further conducted. For ARGs/antibiotics significantly impacted by setback distances, multiple regression models were tested and linear regressions were chosen to estimate the relationship between log concentrations of ARGs/antibiotics in runoff and setback distances due to high R^2 values.

For statistical analyses that involve a large number of values below detection limits, which occurs mostly in soil samples, we used the following methods to treat these values. Only genes that had values higher than the detection limits in more than 50% of the samples were analyzed by ANOVA. Antibiotic concentrations below the detection limits were left blank in the input files for SAS analyses. Further, because no antibiotics were consistently detected in lower soil depths, only antibiotics in 0−10 cm soil depth were analyzed using ANOVA with setback distance as the only factor.

RESULTS

ARGs and Antibiotics in Manure. The 16S rRNA gene and nine other genes related to horizontal gene transfer, tetracycline resistance, macrolide resistance, and penicillin resistance were quantified for manure samples using qPCR (Table 1). Eight out of the ten genes were present in all ten manure samples. The *tet*(Q) gene was not detected in one manure sample, and *tet*(D) was detected in only one manure sample.

Table 1. Gene and Antibiotic Concentrations in Swine Manure Slurry Samples (Average ± Standard Error)

Analyte		Concentration	Number of Samples Detected Positive (n = 10)
Gene (copies/mL)	16S rRNA	$(6.9 \pm 1.1) \times 10^5$	10
	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)	1.6×10^2	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
Antibiotic (mg/kg ww)	Chlortetracycline	10.39 ± 1.45	10
	Lincomycin	0.23 ± 0.09	10
	Tiamulin	0.45 ± 0.08	10

Out of the six feed additives analyzed, penicillin G, penicillic acid, and neotame were not detected in any manure, runoff, or soil samples. In the manure slurry samples, the most abundant antibiotic was chlortetracycline at 10.39 ± 1.45 mg kg^{-1} ww. Lincomycin and tiamulin were also detected in all ten manure slurry samples, at levels about 2 orders of magnitude lower than chlortetracycline (Table 1).

https://dx.doi.org/10.1021/acs.est.9b04834
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ARGs in Runoff. The effects of setback distance and rainfall events on the gene concentrations in runoff are reported in Table 2. Out of the ten genes analyzed, the concentrations of the 16S rRNA gene, *erm*(B), *erm*(C), *intI1*, *tet*(O), *tet*(Q), and *tet*(X) in runoff were significantly impacted by setback distance ($p < 0.05$). The ARGs bla_{TEM}, *erm*(F), and *tet*(D) were not significantly impacted by setback distance ($p > 0.05$). For the seven genes that were significantly affected by setback distance, LSD tests showed that their concentrations in runoff decreased as setback distance increased (Table 2).

The least-squares means in runoff from rainfall #1, for the seven genes significantly affected by setback distance, were plotted against the setback distances in Figure 2a−c. The linear regression equations, along with the R^2 values, are reported in Table 3. On the basis of the values of the slopes, the seven genes appeared to be classified into three groups. The 16S rRNA gene and *intI1* both decreased with a slope of 0.064 (log copies per mL^{-1} per meter of setback distance). The gene *tet*(X) decreased with a slope of 0.072, while *erm*(B), *erm*(C), *tet*(O), and *tet*(Q) decreased most rapidly with a slope between 0.080 and 0.084. After normalizing to the 16S rRNA gene, the relative abundance of the remaining six genes are plotted in Figure S1.

Two genes, *erm*(C) and *tet*(O), exhibited a significant decrease in their concentrations in runoff during rainfall #2 versus runoff during rainfall #1 ($p < 0.05$, Table 2). The concentrations of these two genes in runoff generated from both rainfalls were plotted against setback distances in Figure S2a,b. At the $p < 0.10$ level, the concentrations of *erm*(B), *intI1*, and *tet*(Q) in runoff during rainfall #2 were also significantly lower than those in runoff during rainfall #1.

Eight out of the ten genes analyzed were consistently detected in runoff from simulated rainfalls from control plots (Table S5). ANOVA tests showed that manure amendment had a significant effect ($p < 0.05$) on the ARG concentrations in runoff at the longest setback distance tested (18.3 m) for *erm*(B), *erm*(C), *erm*(F), *intI1*, *tet*(O), and *tet*(Q). For all six genes, the concentrations were higher in runoff from amended plots receiving manure. Because *erm*(C) and *tet*(O) in runoff from control plots were below detection limits, *p*-values were not established for them in ANOVA (Table S5). Nonetheless, they were significantly affected by manure amendment. No significant effect from manure amendment was observed on the 16S rRNA gene, bla_{TEM}, *tet*(D), or *tet*(X) ($p > 0.05$).

With the use of the equations in Table 3, a setback distance of 36−58 m is estimated to be necessary to lower the ARG levels in runoff down to background levels, which were defined as either ARG levels in runoff from control plots or qPCR detection limits. The longest setback distance tested in this study was 18.3 m. This distance, for the soil type and rainfall conditions presented here, effectively reduced the ARG concentrations by about 1.2−1.5 logs.

Antibiotics in Runoff. The effects of setback distance and rainfall events on the concentrations of antibiotics in runoff are reported in Table 2. The concentrations of all three antibiotics in runoff decreased significantly with increased setback distance ($p < 0.05$). The log transformed values of antibiotic concentrations in runoff are plotted against setback distance in Figure 2d. The relationship can be described using linear trendlines, and the linear equations are reported in Table 3. Tiamulin concentration in runoff decreased the most rapidly followed by chlortetracycline and then lincomycin. Using the approach similar to the one used to estimate the safe setback

Table 2. Means and *p*-Values for the Effects of Setback Distance and Rainfall Events on the Concentration of ARGs and Antibiotics in Runoff[f]

	16S rRNA	bla_{TEM}	*erm*(B)	*erm*(C)	*erm*(F)	*intI1*	*tet*(D)	*tet*(O)	*tet*(Q)	*tet*(X)	CTC[c]	LIN[d]	TIA[e]
	(copy/mL)	(copy/mL)	(copy/mL)	(copy/mL)	(copy/mL)	(copy/mL)	(copy/mL)	(copy/mL)	(copy/mL)	(copy/mL)	(μg/L)	(μg/L)	(μg/L)
Setback Distance (m)[a,b]													
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	25.0 a	9.94 a	0.950 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	8.14 b	4.09 a	0.235 b
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	6.22 bc	4.69 a	0.208 b
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	2.44 cd	0.52 b	0.034 c
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	1.30 d	0.36 b	0.010 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	8.01 a	2.30	0.195 a
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	3.45 b	1.82	0.063 b
***p*-values for:**													
distance	0.025	0.442	0.044	0.019	0.076	0.009	0.520	0.030	0.033	0.041	0.001	0.007	<.001
rainfall	0.265	0.901	0.088	<.001	0.146	0.083	0.153	<.001	0.081	0.244	0.002	0.642	0.005
distance × rainfall	0.725	0.571	0.752	0.781	0.812	0.795	0.601	0.692	0.649	0.640	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, 7.9×10^6 was calculated using the 16S rRNA gene concentrations of all runoff samples at distance 0.00 m from both rainfall events. [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]CTC, chlortetracycline. [d]LIN, lincomycin. [e]TIA, tiamulin. [f]Each treatment combination had four replicate plots.

https://dx.doi.org/10.1021/acs.est.9b04834
Environ. Sci. Technol. 2020, 54, 4800−4809

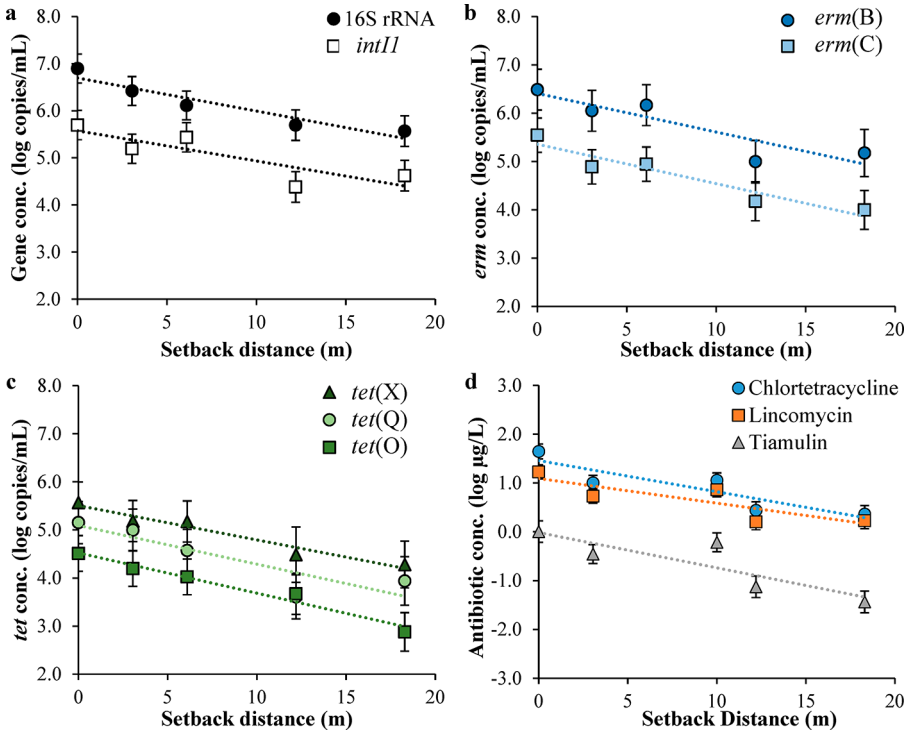

Figure 2. Means of log concentration of (a) the 16S rRNA gene and *intI1*, (b) erythromycin resistance methylase (*erm*) genes, (c) tetracycline resistance (*tet*) genes, and (d) antibiotics in runoff from manure-amended plots after the rainfall #1. The error bars represent the standard errors based on the ANOVA analysis with GLIMMIX. The trendlines are linear.

Table 3. Linear Regression Equations and R^2 for the Log Concentrations of ARGs and Antibiotics in Runoff[a] As a Function of Setback Distances

Analytes		Linear Eq[b] (ax + b)	R^2	Setback Distance Requirement[c] (m)
Gene (log copies/mL)	16S rRNA	−0.064x + 6.737	0.913	43
	erm(B)	−0.080x + 6.408	0.810	57
	erm(C)	−0.082x + 5.356	0.909	39
	intI1	−0.064x + 5.574	0.729	58
	tet(O)	−0.084x + 4.522	0.970	40
	tet(Q)	−0.081x + 5.092	0.785	39
	tet(X)	−0.071x + 5.506	0.953	36
Antibiotic (log μg/L)	Chlortetracycline	−0.066x + 1.426	0.862	56
	Lincomycin	−0.054x + 1.079	0.824	67
	Tiamulin	−0.079x − 0.016	0.905	34

[a]On the basis of weighted averages from rainfall #1. Only compounds with a significant reduction due to length are shown here. [b]x is setback distance in meters. [c]Setback distance needed to lower the analyte concentrations in runoff to the background levels, which were defined by the analyte concentrations in runoff from the control plots or by the detection limit. The distance requirement was calculated using the linear equations in the table.

distance for ARGs, it was found that a setback distance of 34−67 m would be necessary to lower the antibiotic concentrations in runoff to background levels (Table 3).

The concentrations of two antibiotics, chlortetracycline and tiamulin, in runoff during rainfall #2 were significantly lower than those in runoff during rainfall #1 ($p < 0.05$, Table 2, Figure S2c,d). The concentration of lincomycin in runoff did not significantly differ between the two rainfall events ($p = 0.642$). ANOVA tests showed that manure amendment had significant effects on the concentrations of all three antibiotics in runoff (Table S5).

ARGs in Soil. The effects of setback distance and soil depth on gene concentrations in soil are presented in Table 4. Among the ten genes tested, four genes were consistently detected above their detection limits in soil samples at most of the setback distances tested, the 16S rRNA gene, bla_{TEM}, *intI1*, and *tet*(D). Among them, bla_{TEM} was significantly affected by the setback distance ($p < 0.05$) and its concentration in soil dropped significantly between setback distances of 0.0 and 6.1 m (Table 4). The concentrations of bla_{TEM} in 0−10 cm soil were plotted against setback distances in Figure 3a.

The concentrations of all four genes consistently detected in soil were all significantly affected by soil depth (Table 4). With the exception of bla_{TEM}, the other three genes had higher concentrations in 0−10 cm soil than in 20−30 cm soil.

ANOVA tests showed that manure amendment had significant effects on *intI1* ($p = 0.003$) and no significant effect on the concentrations of the 16S rRNA gene, bla_{TEM}, and *tet*(D) in soil (Table S6). Because the other six genes were not detected in the soil from the control plots, *p*-values were not established for them in ANOVA. Nonetheless, they were significantly affected by manure amendment.

https://dx.doi.org/10.1021/acs.est.9b04834
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Table 4. Means and *p*-Values for the Effects of Setback Distance and Soil Depth on the Concentration of Antibiotic Resistance Genes and Antibiotics in Soil

	16S rRNA	bla_{TEM}	*erm*(B)	*erm*(C)	*erm*(F)	*intI1*	*tet*(D)	*tet*(O)	*tet*(Q)	*tet*(X)	CTC[e]	LIN[f]	TIA[g]
	(copy/g dw)	(copy/g dw)	(copy/g dw)	(copy/g dw)	(copy/g dw)	(copy/g dw)	(copy/g dw)	(copy/g dw)	(copy/g dw)	(copy/g dw)	(ng/g dw)	(ng/g dw)	(ng/g dw)
Setback Distance (m)[a,b]													
0.0	2.4×10^7	3.9×10^4 a	3.3×10^4	BDL[c]	1.7×10^4	7.1×10^4	9.8×10^3	BDL	2.9×10^4	BDL	9.49 a	BDL	0.114 a
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	3.21 b	BDL	0.023 b
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	1.72 c	BDL	0.016 b
9.1	1.0×10^7	1.3×10^4 b	BDL	BDL	BDL	1.2×10^4	1.8×10^4	BDL	BDL	BDL	1.03 d	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	1.09 d	BDL	0.009 b
15.2	1.6×10^7	1.6×10^4 b	BDL	BDL	BDL	2.3×10^4	BDL	BDL	BDL	BDL	0.716 e	BDL	0.023 b
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	N/A	N/A	N/A
10–20	1.5×10^7 a	2.5×10^4 a	BDL	BDL	BDL	2.7×10^4 b	BDL	BDL	BDL	7.8×10^4	N/A	N/A	N/A
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	N/A	N/A	N/A
***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	<0.001	N/A	0.014
depth	0.021	0.050	N/A	N/A	N/A	<0.001	0.030	N/A	N/A	N/A	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	N/A	N/A	N/A

[a]Values reported under "Setback Distance" and "Depth" are treatment averages, which were calculated based on the data for one particular treatment level. For example, 2.4×10^7 was calculated using the 16S rRNA gene concentrations of all replicate soil samples at distance 0.0 m 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 ANOVA. [e]CTC, chlortetracycline. [f]LIN, lincomycin. [g]TIA, tiamulin.

https://dx.doi.org/10.1021/acs.est.9b04834
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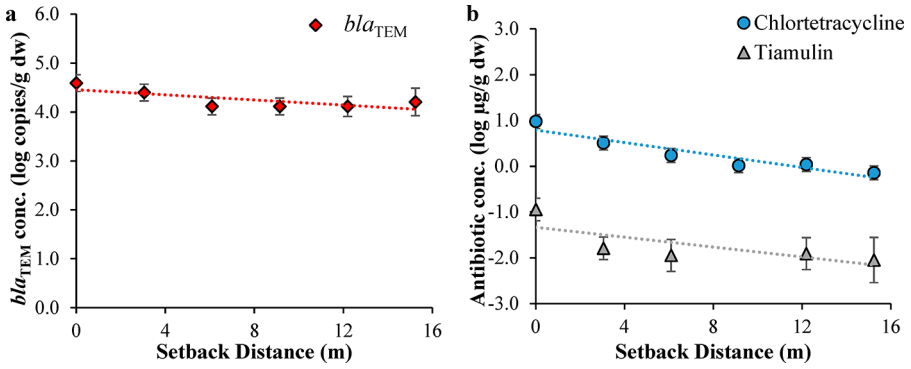

Figure 3. Means of the log concentration of (a) bla_{TEM} and (b) antibiotics in 10−20 cm soil in the setback region. The error bars represent the standard errors based on the ANOVA analysis using GLIMMIX. The trendlines are linear after the data has been log transformed.

Antibiotics in Soil. 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 ($p < 0.05$) (Table 4, Figure 3b). Lincomycin was detected in the surface soil of the manure region but not in the surface soil of the setback region.

ANOVA tests showed that manure amendment had significant effects on the concentration of chlortetracycline in soil ($p = 0.017$, Table S6). Since lincomycin and tiamulin were not detected in the soil from the control plots, p-values were not established for them in ANOVA. Nonetheless, they were significantly affected by manure amendment.

DISCUSSION

ARGs in Runoff. Information on the transport of ARGs in runoff as a function of setback distance is very limited in the literature. However, studies that investigated the transport of bacteria in runoff can provide useful references for this study. In one study, *E. coli* cells in runoff, which were originally sprayed on bare soil, decreased by about 0.062 log CFU per mL per meter of increased setback distance on plots with either 2 or 6% slope.[17] The use of free bacteria could potentially overestimate the transport distance for bacteria, which often attach to larger manure particles, in runoff.[18,19] In another study, *E. coli*, PRD1 bacteriophage, and *Cryptosporidium parvum*, which were originally inoculated in bovine fecal pats prior to land application, decreased in runoff by 0.058 to 0.208 log per mL per meter of increased setback distance with a minimum slope of 18%.[20] The rates of ARG reduction in runoff reported in this study (Table 3) were within the range reported in these other studies.

Several mechanisms could account for the reduction of ARG concentrations in runoff with increased setback distance. The most important mechanism is dilution. As manure particles, which up to 50% of manure-borne bacteria are attached to,[21−23] were flushed away from the manure region, additional water was added to the runoff from rainfall over the setback region, diluting ARG concentrations in runoff. Given the experimental conditions used in this study (i.e., the size of the manure region and the setback region), it was estimated that dilution alone could reduce ARG concentration in runoff by 0.036 logs per mL per meter of increased setback distance.

Another potentially important mechanism is sedimentation. According to a batch study using clay loam soil, approximately 35% of bacteria tended to attach to particles >2 μm in diameter, particularly those with a dimension of 16−30 μm.[18] Manure particles larger than 40 μm can settle within the first five meters of a setback distance.[24] Microbes that are not attached to larger manure particles are either attached to smaller particles,[18] which tend to settle slower, or are free as single cells.[22] Consequently, swine manure slurry containing smaller particles may lead to higher bacterial concentrations in runoff than would solid manure, such as cattle manure.[25,26]

Filtration by the crop residues may also contribute to the reduction of ARGs in runoff. Vegetative filter strips (VFSs) can significantly reduce the concentration of fecal coliforms in runoff. One study found that a 30-m VFS reduced bacterial concentration in runoff by 67−84%,[27] while another study reported that a 6.1-m VFS could remove fecal coliforms by 100%.[28] Similar to a VFS, crop residues in the field can reduce the runoff velocity and increase sedimentation and filtration.[29] Hence, it is plausible to expect the crop residues, which completely covered the test plots in this study, would contribute to the reduction of ARGs in runoff.

We believe that reporting the absolute abundance of ARGs is more appropriate for this study than relative abundance normalized to the 16S rRNA gene. As shown in Table 3, the slopes of the ARGs are slightly lower (i.e., more negative) than the 16S rRNA. Had we normalized these genes to the 16S rRNA gene, the relative abundance would decrease at higher slopes (i.e., less negative) than the absolute abundance along setback distance. This would underestimate the effects of setback distance on controlling ARG levels in runoff. Also, in this work, we did not specifically separate genes in intracellular DNA from those in extracellular DNA, because our previous study shows that on average ARGs in extracellular DNA accounted for less than 0.5% of the total ARGs in swine treatment lagoons, an environment similar to the pits where we collected swine manure slurry for this study.[30]

ARGs in Soil. After manure application and simulated rainfall, the concentration of ARGs in soil generally decreased with increasing soil depth. Most of the genes were not detected beneath the top 10 cm soil layer or past the first 6 m of setback distance (Table 4). The exception were the genes that were consistently detected in original soil (i.e., the 16S rRNA gene, bla_{TEM}, *intI1*, and *tet*(D)). These results are consistent with another study where no increase in ARG concentrations was observed at 5−20 cm soil depth following repeated rainfall events on soil receiving dairy manure through surface application.[6]

Variability of Genes. Out of the genes that decreased significantly in runoff with increasing setback distance, the 16S

https://dx.doi.org/10.1021/acs.est.9b04834
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rRNA gene and *intI1* had the least steep slopes (Table 3). This was likely due to the fact that these two genes were naturally occurring in soil (Table S6). Hence, in addition to the soil in the manure region (i.e., manure-amended soil), the soil in the setback region also contributed these genes to runoff.[31]

Setback distance had no significant impacts on the concentrations of bla_{TEM} ($p = 0.442$, Table 2) or *tet*(D) ($p = 0.520$) in runoff. The gene bla_{TEM} was the only gene that decreased significantly in soil as a function of setback distance, primarily due to the drop in the first 6.1 m within the setback region (Table 4). The results from runoff and soil together suggest that some of the bacterial host(s) of the bla_{TEM} gene quickly settled to soil while other host(s) of the gene largely remained in runoff. The gene *tet*(D) was the only gene that was not consistently detected in swine manure slurry (Table 1). In the meantime, the average concentration of *tet*(D) in the original soil was 1.6×10^4 copies per g soil dry weight (dw). Hence, the manure likely was not the main source of this ARG in the runoff.

Antibiotics in Manure. Chlortetracycline, lincomycin, and tiamulin were detected in manure slurry, while penicillin was not detected. This was not surprising since β-lactam antibiotics, including penicillin, have been found to have short half-lives in manure (5 days)[32] during manure storage. Tetracyclines have half-lives that range between 82 and 150 days.[33,34] A study examining tiamulin degradation during manure storage found no degradation of tiamulin over the 180-day experiment and suggested that the use of this antibiotic should be avoided due to its environmental persistence.[5] Swine treatment lagoons sampled in Iowa and Ohio exhibited similar trends to what was found in this study. Tetracyclines and macrolides were detected in the 10−500 $\mu g\ L^{-1}$ range, while penicillin was detected below or close to the detection limit.[35]

Antibiotics in Runoff and Soil. Chlortetracycline, lincomycin, and tiamulin had distinctive physiochemical properties. Tiamulin has very low solubility in water[36] and will therefore likely have a high adsorption coefficient. Chlortetracycline has a higher adsorption coefficient than lincomycin (500−1800 L kg^{-1} vs 20−200 L kg^{-1}).[37,38] Difference in physiochemical properties can explain the different behaviors of the antibiotics in runoff and soil.

The setback distances required for antibiotics in runoff to drop to the background levels are longer than those required for ARGs (Table 3), because mechanisms like sedimentation and filtration, which apply to both free and attached microbes, are less relevant to the soluble fraction of the antibiotics. Dilution still plays an important role: dilution itself can cause a reduction in concentration at 0.036 log per mL per meter of setback distance. Adsorption also played a potentially important role for the transport of the soluble portion of antibiotics in runoff. The adsorption coefficient of lincomycin was smaller than the other two antibiotics; consequently, lincomycin had a lower reduction rate in runoff with setback distance (Table 3). However, it is recognized that the estimated setback distances needed to achieve background levels are considerably beyond the experimental setback distances in the experiment and may not be estimated very precisely.

Parallel to this study that focused on ARGs and antibiotics, a companion study was conducted using the same field setup and was focused on the effects of setback distance on nutrients (i.e., dissolved phosphorus, ammonia, total nitrogen, etc.).[15] Compared to the slopes for antibiotics in Table 3, those for the nutrients in runoff had a wider range of reduction rates with setback distance (i.e., 0.001 to 0.086 logs per meter of increased setback distance). Compared to antibiotics, nutrients generally had lower reduction rates with setback distance, likely because nutrients occurred more extensively in the field than antibiotics (i.e., contributing from both manure and setback regions).

The hydrophobicity of chlortetracycline and tiamulin leads to the occurrence of these compounds in the surface soil of the setback region (Figure 3b), which presumably resulted from the transport of manure particulates to which these antibiotics were adsorbed. The low sorption coefficient explains why lincomycin was not detected in surface soil (Table 4). The half-lives for chlortetracycline, lincomycin, and tiamulin in soil are 24,[39] 18,[40] and 16 days,[41] respectively. Given the time frame of our field testing, degradation would not significantly affect the antibiotic concentrations in soil in this study.

Soil texture can affect the vertical transport of antibiotics following manure application. In this study, where the soil in the field was silty clay loam, no antibiotics were detected below a 10 cm depth at any setback distance following manure application. Another study also reported no or low levels of tetracycline and oxytetracycline in loamy soil deeper than 5 cm following irrigation on soil amended with cattle manure.[42] In contrast, chlortetracycline can occur in relatively high concentrations in sandy soil (91.6% sand) as deep as 30 cm following liquid swine manure application.[43]

The setback distances determined in this study was based on our experimental conditions: cropland management (no-til), location (southeast Nebraska), slope (4.9%), soil type (silty clay loam), crop residue coverage (100%), rainfall (52 mm hr^{-1}, 1 and 2 days after manure application), etc. To make more general recommendations on setback distances, additional field tests are needed on plots with different soil types, slopes, cropping, and management conditions. Finally, we would like to acknowledge that the setback distances reported in Table 3 were obtained from extrapolation of linear regression models, a potential limitation of the study.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.9b04834.

Antibiotic analytical methods; PCR and qPCR information, antibiotic and ARG concentrations in two rainfall events; antibiotic and ARG concentrations between control and treatment plots (PDF)

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https://dx.doi.org/10.1021/acs.est.9b04834
Environ. Sci. Technol. 2020, 54, 4800−4809

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The study was funded by the National Pork Board (#16-072) and National Science Foundation (CBET-1351676). M.C.H. and N.A.M. were partially supported by the Robert B. Daugherty Water for Food Global Institute at the University of Nebraska and USDA (#2017-68003-26497), respectively.

■ REFERENCES

(1) Pruden, A.; Alcalde, R. E.; Alvarez, P. J. J.; Ashbolt, N.; Bischel, H.; Capiro, N. L.; Crossette, E.; Frigon, D.; Grimes, K.; Haas, C. N.; Ikuma, K.; Kappell, A.; LaPara, T.; Kimbell, L.; Li, M. Y.; Li, X.; McNamara, P.; Seo, Y.; Sobsey, M. D.; Sozzi, E.; Navab-Daneshmand, T.; Raskin, L.; Riquelme, M. V.; Vikesland, P.; Wigginton, K.; Zhou, Z. An environmental science and engineering framework for combating antimicrobial resistance. *Environ. Eng. Sci.* **2018**, *35*, 1005−1011.
(2) Berendonk, T. U.; Manaia, C. M.; Merlin, C.; Fatta-Kassinos, D.; Cytryn, E.; Walsh, F.; Burgmann, H.; Sorum, H.; Norstrom, M.; Pons, M. N.; Kreuzinger, N.; Huovinen, P.; Stefani, S.; Schwartz, T.; Kisand, V.; Baquero, F.; Martinez, J. L. Tackling antibiotic resistance: the environmental framework. *Nat. Rev. Microbiol.* **2015**, *13*, 310.
(3) EPA. Regulatory Definitions of Large CAFOs, Medium CAFO, and Small CAFOs.
(4) Cheng, W.; Chen, H.; Su, C.; Yan, S. Abundance and persistence of antibiotic resistance genes in livestock farms: a comprehensive investigation in eastern China. *Environ. Int.* **2013**, *61*, 1−7.
(5) Schlüsener, M. P.; Von Arb, M. A.; Bester, K. Elimination of macrolides, tiamulin, and salinomycin during manure storage. *Arch. Environ. Contam. Toxicol.* **2006**, *51*, 21−28.
(6) Fahrenfeld, N.; Knowlton, K.; Krometis, L. A.; Hession, W. C.; Xia, K.; Lipscomb, E.; Libuit, K.; Green, B. L.; Pruden, A. Effect of manure application on abundance of antibiotic resistance genes and their attenuation rates in soil: field-scale mass balance approach. *Environ. Sci. Technol.* **2014**, *48*, 2643−2650.
(7) Joy, S. R.; Bartelt-Hunt, S. L.; Snow, D. D.; Gilley, J. E.; Woodbury, B. L.; Parker, D. B.; Marx, D. B.; Li, X. Fate and transport of antimicrobials and antimicrobial resistance genes in soil and runoff following land application of swine manure slurry. *Environ. Sci. Technol.* **2013**, *47*, 12081−12088.
(8) Kaini, P.; Artita, K.; Nicklow, J. W. Optimizing structural best management practices using SWAT and genetic algorithm to improve water quality goals. *Water Resour. Manag.* **2012**, *26*, 1827−1845.
(9) Oun, A.; Kumar, A.; Harrigan, T.; Angelakis, A.; Xagoraraki, I. Effects of biosolids and manure application on microbial water quality in rural areas in the US. *Water* **2014**, *6*, 3701−3723.
(10) EPA. *Managing Manure Nutrients at Concentrated Animal Feeding Operations.* (2004).
(11) U.S Environmental Protection Agency. NPDES permit writer's manual for concentrated animal feeding operations. In Water. (**2012**).
(12) Cromley, S.; Lory, J. Setback Distances for Land Application of Manure. Available at: https://extension2.missouri.edu/g9219.
(13) Iowa Department of Natural Resources. *Separation Distances for Land Application of Manure from Open Feedlots & Confinement Feeding Operations, including SAFOs.* (**2008**).
(14) Schulz, E. F.; Yevjevich, V. M. Experimental investigation of small watershed floods. *Complet. Rep. Ser. (Colorado State Univ. Nat. Resour. Center)*; no. 18 (**1970**).
(15) Gilley, J. E.; Bartelt-Hunt, S. L.; Eskridge, K. M.; Li, X.; Schmidt, A. M.; Snow, D. D. Setback distance requirements for removal of swine slurry constituents in runoff. *Trans. ASABE* **2017**, *60*, 1885−1894.
(16) Suzuki, M. T.; Giovannoni, S. J. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl. Environ. Microbiol.* **1996**, *62*, 625−630.
(17) Abu-Ashour, J.; Lee, H. Transport of bacteria on sloping soil surfaces by runoff. *Environ. Toxicol.* **2000**, *15*, 149−153.
(18) Oliver, D. M.; Clegg, C. D.; Heathwaite, A. L.; Haygarth, P. M. Preferential attachment of Escherichia coli to different particle size fractions of an agricultural grassland soil. *Water, Air, Soil Pollut.* **2007**, *185*, 369−375.
(19) Muirhead, R. W.; Collins, R. P.; Bremer, P. J. Interaction of Escherichia coli and soil particles in runoff. *Appl. Environ. Microbiol.* **2006**, *72*, 3406−3411.
(20) Ferguson, C. M.; Davies, C. M.; Kaucner, C.; Krogh, M.; Rodehutskors, J.; Deere, D. A.; Ashbolt, N. J. Field scale quantification of microbial transport from bovine faeces under simulated rainfall events. *J. Water Health* **2007**, *5*, 83−95.
(21) Soupir, M. L.; Mostaghimi, S.; Dillaha, T. Attachment of Escherichia coli and Enterococci to particles in runoff. *J. Environ. Qual.* **2010**, *39*, 1019−1027.
(22) Muirhead, R. W.; Collins, R. P.; Bremer, P. J. Erosion and subsequent transport state of Escherichia coli from cowpats. *Appl. Environ. Microbiol.* **2005**, *71*, 2875−2879.
(23) Guber, A. K.; Pachepsky, Y. A.; Shelton, D. R.; Yu, O. Effect of bovine manure on fecal coliform attachment to soil and soil particles of different sizes. *Appl. Environ. Microbiol.* **2007**, *73*, 3363−3370.
(24) Gharabaghi, B.; Rudra, R. P.; Goel, P. K. Effectiveness of vegetative filter strips in removal of sediments from overland flow. *Water Qual. Res. J. Can.* **2006**, *41*, 275−282.
(25) Thurston-Enriquez, J. A.; Gilley, J. E.; Eghball, B. Microbial quality of runoff following land application of cattle manure and swine slurry. *J. Water Health* **2005**, *3*, 157−171.
(26) Blaustein, R. A.; Pachepsky, Y. A.; Hill, R. L.; Shelton, D. R. Solid manure as a source of fecal indicator microorganisms: release under simulated rainfall. *Environ. Sci. Technol.* **2015**, *49*, 7860−7869.
(27) Fajardo, J. J.; Bauder, J. W.; Cash, S. D. Managing nitrate and bacteria in runoff from livestock confinement areas with vegetative filter strips. *J. Soil Water Conserv.* **2001**, *56*, 185−191.
(28) Lim, T. T.; Edwards, D. R.; Workman, S. R.; Larson, B. T.; Dunn, L. Vegetated filter strip removal of cattle manure constituents in runoff. *Trans. ASAE* **1998**, *41*, 1375.
(29) Steiner, J. L. Crop residue effects on water conservation. *Manag. Agric. residues 76*, (**1994**).
(30) Zhang, Y.; Snow, D. D.; Parker, D.; Zhou, Z.; Li, X. Intracellular and extracellular antimicrobial resistance genes in the sludge of livestock waste management structures. *Environ. Sci. Technol.* **2013**, *47*, 10206−10213.
(31) Tyrrel, S. F.; Quinton, J. N. Overland flow transport of pathogens from agricultural land receiving faecal wastes. *J. Appl. Microbiol.* **2003**, *94*, 87−93.
(32) Boxall, A. B. A., Fogg, L. A.; Blackwell, P. A.; Kay, P.; Pemberton, E. J.; Croxford, A. Veterinary medicines in the environment. in *Reviews of environmental contamination and toxicology* 1−91 (Springer, 2004).

(33) Stone, J. J.; Clay, S. A.; Zhu, Z. W.; Wong, K. L.; Porath, L. R.; Spellman, G. M. Effect of antimicrobial compounds tylosin and chlortetracycline during batch anaerobic swine manure digestion. *Water Res.* **2009**, *43*, 4740−4750.
(34) Winckler, C.; Grafe, A. Use of veterinary drugs in intensive animal production. *J. Soils Sediments* **2001**, *1*, 66.
(35) 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. Antimicrobial residues in animal waste and water resources proximal to large-scale swine and poultry feeding operations. *Sci. Total Environ.* **2002**, *299*, 89−95.
(36) Compound Summary for CID 656958. PubChem Available at: https://pubchem.ncbi.nlm.nih.gov/compound/Tiamulin. (Accessed: 11th April **2018**).
(37) Teixidó, M.; Granados, M.; Prat, M. D.; Beltrán, J. L. Sorption of tetracyclines onto natural soils: data analysis and prediction. *Environ. Sci. Pollut. Res.* **2012**, *19*, 3087−3095.
(38) Wang, C.; Teppen, B. J.; Boyd, S. A.; Li, H. Sorption of lincomycin at low concentrations from water by soils. *Soil Sci. Soc. Am. J.* **2012**, *76*, 1222−1228.
(39) Carlson, J. C.; Mabury, S. A. Dissipation kinetics and mobility of chlortetracycline, tylosin, and monensin in an agricultural soil in Northumberland County, Ontario, Canada. *Environ. Toxicol. Chem.* **2006**, *25*, 1−10.
(40) Kuchta, S. L.; Cessna, A. J.; Elliott, J. A.; Peru, K. M.; Headley, J. V. Transport of lincomycin to surface and ground water from manure-amended cropland. *J. Environ. Qual.* **2009**, *38*, 1719−1727.
(41) Schlüsener, M. P.; Bester, K. Persistence of antibiotics such as macrolides, tiamulin and salinomycin in soil. *Environ. Pollut.* **2006**, *143*, 565−571.
(42) Aga, D. S.; O'Connor, S.; Ensley, S.; Payero, J. O.; Snow, D.; Tarkalson, D. Determination of the persistence of tetracycline antibiotics and their degradates in manure-amended soil using enzyme-linked immunosorbent assay and liquid chromatography-mass spectrometry. *J. Agric. Food Chem.* **2005**, *53*, 7165−7171.
(43) Hamscher, G.; Sczesny, S.; Höper, H.; Nau, H. Determination of persistent tetracycline residues in soil fertilized with liquid manure by high-performance liquid chromatography with electrospray ionization tandem mass spectrometry. *Anal. Chem.* **2002**, *74*, 1509−1518.

https://dx.doi.org/10.1021/acs.est.9b04834
Environ. Sci. Technol. 2020, 54, 4800−4809

Supplementary Information

Influence of Setback Distance on Antibiotics and Antibiotic Resistance Genes in Runoff and Soil Following the Land Application of Swine Manure Slurry

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MATERIALS AND METHODS

ARG Analysis. Synthesized gBlocks gene fragments (Integrated DNA Technologies) were used as qPCR standards. The qPCR reactions were performed on an Eppendorf Mastercycler ep realplex 2 thermocycler (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 (Tables S1 and S2). Linear ranges and reaction efficiencies are reported in Table S3. Samples were considered below detection limit (BDL), if the results from at least two of the four replicate plots were below the detection limit and the results from the remaining plots were close to the detection limit.

Antibiotic Analyses. Swine slurry and soil were both processed as solid samples during antibiotic extraction. Prior to extraction, swine slurry was mixed with 0.5 g EDTA and clean sand in a 1:25 ratio by weight. Homogenized soil (10 g) and swine slurry/sand samples (5.2 g) were spiked with 100 ng surrogate oleandomycin and mixed with an aqueous buffer (14 mL of 100 mM ammonium citrate plus 4.0 g/L ammonia acetate adjusted with ammonium hydroxide to pH 6) along with 6 mL of acetonitrile. The mixtures were thoroughly shaken on a Burrell wrist-action shaker for 30 min and centrifuged for 10 min. The solids were extracted a second time using 4 mL aqueous buffer and and 16 mL acetonitrile. The supernatants from the two extraction steps were combined and then concentrated on a Labconco RapidVap N_2 sample concentrator (Labconco Corporation, Kansas City, MO) at 30°C until the volume was reduced by approximately half. Purified reagent water was then added to bring the final volume to 100 mL prior to solid phase extraction.

Water samples were measured into 100 mL aliquots, spiked with oleandomycin surrogate and vacuum filtered through pre-combusted 0.5 µm Gellman A/E binderless glass fiber filters in tandem with 200 mg Oasis HLB (Waters Corporation, Milford, MA) solid phase extraction (SPE) cartridges preconditioned with 5 mL acetonitrile followed by 5 mL high purity (ASTM Type I) reagent water. Aqueous soil and slurry extracts were extracted using the same cartridges. The SPE cartridges were eluted into borosilicate test tubes using 6 mL mixture of 1% 100 mM ammonium acetate (pH=4.0) plus 99% acetonitrile. The eluent was evaporated to dryness and concentrated extracts were reconstituted with 200 µL of mobile phase containing 100 ng doxycycline, penicillin V, and roxithromycin as internal standards. The 200 µL eluent samples were combined with 250 µL of mobile phase and then analyzed on an Agilent 1100 high pressure liquid chromatograph (HPLC) coupled with an Agilent 6410 triple quadrupole mass spectrophotometer (Agilent Technologies, Palo Alto, CA) using positive electrospray ionization.

Separation was performed on a 250 mm × 2.1 mm ID, 5 µm particle size HyPURITY™ C18 column (ThermoFisher, St. Louis, MO) at a temperature of 50°C and a gradient flow rate of 0.20 mL/min. Mobile phase solvents were: A) 1 mM ammonium citrate (pH=4) in 97% methanol / 3% water, and B) 1 mM ammonium citrate (pH=4) in water. Gradient details were: initial conditions at 0% A for 1.0 min, linear gradient to reach 75% A at 4 min and 100% A at 12 min, and 100% A until 22 min. The column was flushed with 2% formic acid in methanol for 3 min and then back to initial conditions (0% A) for 7 min. Total run time is 32 minutes.
Multi-reaction monitoring, using a pseudo-molecular ion [M+H]+ selected as the parent ion for fragmentation and corresponding fragment ion(s), were used for identification and quantitation. Ionization and collision energies are optimized based on procedures described by the instrument manufacturer. Desolvation gas was nitrogen (N_2) at 12 L/min, sheathe gas temperature was 350°C, nebulizer held at 40 psi, capillary voltage was 4 kV and cell accelerator voltage at 7 kV. Fragmentor and collision energies used for each standard and analyte are given in Table S4.

Table S1. Primers and probes used in qPCR assays

Target gene	Primer	Sequence (5'-3')	Target size (bp)	Annealing temperature (°C)	Reference
16s rRNA	BACT1369F	CGG TGA ATA CGT TCY CGG	142	56	[1]
	PROK1492R	GGW TAC CTT GTT ACG ACT T			
bla$_{TEM}$	*bla*$_{TEM}$-FW	CAC TAT TCT CAG AAT GAC TTG GT			
	bla$_{TEM}$-RV	TGC ATA ATT CTC TTA CTG TCA TG	85	60	[2]
	Probe	CCA GTC ACA GAA AAG CAT CTT ACG G			
erm(B)	*erm*(B)-FW	GGT TGC TCT TGC ACA CTC AAG	191	65	[3]
	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	[3]
	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	[3]
	erm(F)-RV	TTC AGG GAC AAC TTC CAG C			
*intI*1	qINT-3	TGC CGT GAT CGA AAT CCA GAT CCT	109	60	[4]
	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	[5]
	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	[6, 7]*
	tet(O)-RV	TGG CGT ATC TAT AAT GTT GAC			
tet(Q)	*tet*(Q)-FW	AGA ATC TGC TGT TTG CCA GTG	167	63	[6]
	tet(Q)-RV	CGG AGT GTC AAT GAT ATT GCA			
tet(X)	*tet*(X)-FW	AGC CTT ACC AAT GGG TGT AAA	278	60	[8]
	tet(X)-RV	TTC TTA CCT TGG ACA TCC CG			

*Primer sequence from Aminov et al. 2009 and annealing temperature from Pei et al. 2006.

Table S2. Primers used in endpoint PCR assays (if different from qPCR primers)

Target gene	Primer	Sequence (5'-3')	Target size (bp)	Annealing temperature (°C)	Reference
16S rRNA gene	27F	AGA GTT TGA TCM TGG CTC AG	1,484	55	[9]
	1492R	GGW TAC CTT GTT ACG ACT T			
tet(D)	*tet*(D)-FW	AAA CCA TTA CGG CAT TCT GC	787	55	[10]
	tet(D)-RV	GAC CGG ATA CAC CAT CCA TC			
tet(O)	*tet*(O)-FW	AAC TTA GGC ATT CTG GCT CAC	515	55	[10]
	tet(O)-RV	TCC CAC TGT TCC ATA TCG TCA			

Table S3. qPCR assay reaction conditions, linear ranges, and efficiencies

Target gene	Linear range (gene copies/μL)	R^2	Efficiency
16s rRNA	10^2-10^8	≥ 0.998	88%-94%
bla_{TEM}	10^1-10^8	≥ 0.990	82%-90%
erm(B)	10^1-10^8	≥ 0.995	85%-95%
erm(C)	10^2-10^8	≥ 0.999	86%-91%
erm(F)	10^1-10^8	≥ 0.993	84%-103%
*intI*1	10^1-10^8	≥ 0.995	84%-92%
tet(D)	10^1-10^8	≥ 0.998	80%-84%
tet(O)	10^1-10^8	≥ 0.994	97%-105%
tet(Q)	10^1-10^8	≥ 0.997	88%-101%
tet(X)	10^2-10^8	≥ 0.997	78%-88%

89 Table S4. Multiple reaction monitoring (MRM) transitions used and source conditions for
90 analytes, internal standards (*) and surrogate (**) compounds.
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Compound	Parent Ion (m/z)	Product Ion (m/z)	Fragmentor Voltage (V)	Collision Energy (eV)	Retention time (min) (
Chlortetracycline	479	462	110	16	12.92
Doxycycline*	445	428	120	15	13.02
Lincomycin	407	126	90	30	12.04
Neotame	379	172	150	20	14.63
Oleandomycin**	688.85	158.2	130	25	13.07
Penicillin G	335	160	70	5	13.21
Penicillin V*	351	160	70	5	13.58
Penillic acid	335	176	70	10	13.18
Roxithromycin*	837.5	158	170	35	14.48
Tiamulin	494.7	191.9	70	15	13.68

Table S5. Impact of manure application on the ARG (copy/mL) and antibiotic (μg/L) concentrations in runoff from plots with the 18.3 m setback distance.

	16S rRNA	***bla*$_{TEM}$**	***erm*(B)**	***erm*(C)**	***erm*(F)**	***intI1***	***tet*(D)**	***tet*(O)**	***tet*(Q)**	***tet*(X)**	**CTC[c]**	**LIN[d]**	**TIA[e]**
Manure Application													
Amended plots (with manure)	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}	1.48	1.11 a	0.015
Control plots (without manure)	9.5×10^{3}	4.4×10^{2}	6.9×10^{1} b	BDL[a]	4.0×10^{2} b	7.4×10^{1} b	3.0×10^{1}	BDL	9.6×10^{1} b	1.3×10^{3}	BDL	0.01 b	BDL
p-values:	0.113	0.172	0.004	N/A[b]	0.029	0.004	0.526	N/A	0.009	0.561	N/A	< 0.001	N/A

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

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

[c]CTC; chlortetracycline

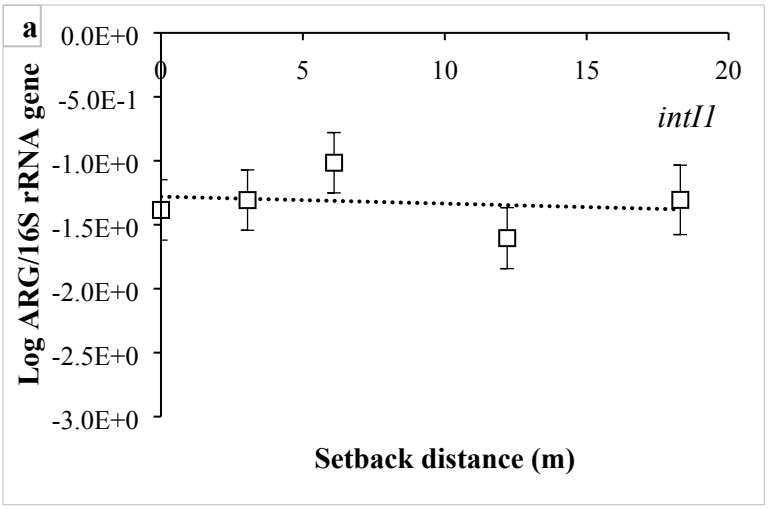
[d]LIN; lincomycin

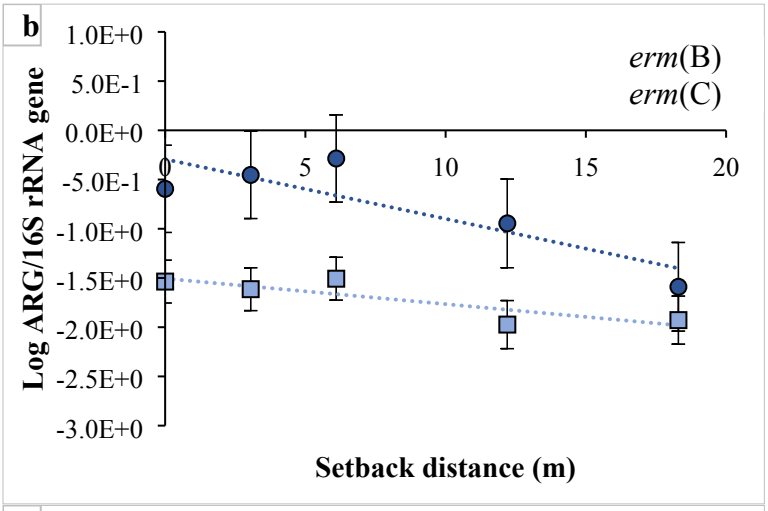
[e]TIA; tiamulin

Table S6. Impact of manure application on the ARG (copy/g dw) and antibiotic (ng/g dw) concentrations in soil.

	16S rRNA	***bla*$_{TEM}$**	***erm*(B)**	***erm*(C)**	***erm*(F)**	***intI1***	***tet*(D)**	***tet*(O)**	***tet*(Q)**	***tet*(X)**	**CTC[c]**	**LIN[d]**	**TIA[e]**
Manure Application													
Amended plot (with manure)	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}	51.2	0.75	6.35
Control plot (without manure)	1.1×10^{8}	2.0×10^{4}	BDL[a]	BDL	BDL	1.5×10^{4}	1.6×10^{4}	BDL	BDL	BDL	0.62	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	0.017	N/A	N/A

[a]BDL, below detection limit, indicates that there were too few values above detection limit to estimate an average.
[b]N/A, not applicable, indicates that there were too few values for ANOVA to return a *p*-value.
[c]CTC; chlortetracycline
[d]LIN; lincomycin
[e]TIA; tiamulin





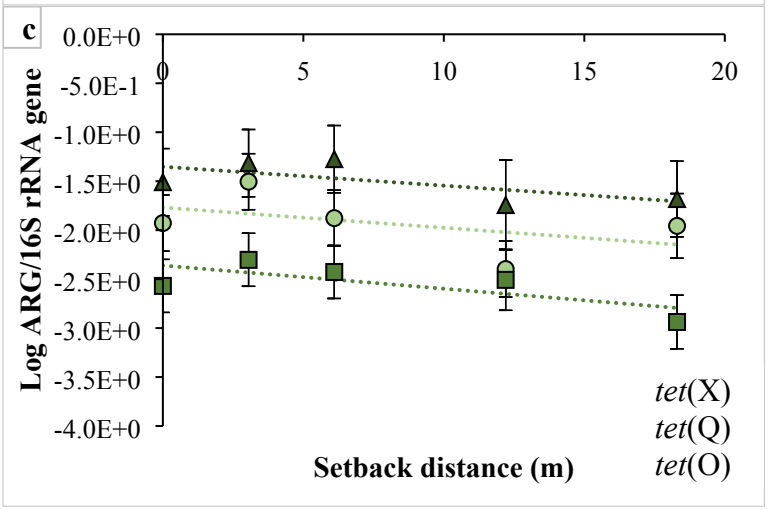


Figure S1. Means of log concentration of the relative abundance of (a) *intI1*, (b) erythromycin resistance methylase (*erm*) genes, and (c) tetracycline resistance (*tet*) genes in runoff from manure-amended plots after the rainfall #1. The error bars represent the standard errors based on the ANOVA analysis with GLIMMIX. The trendlines are linear.

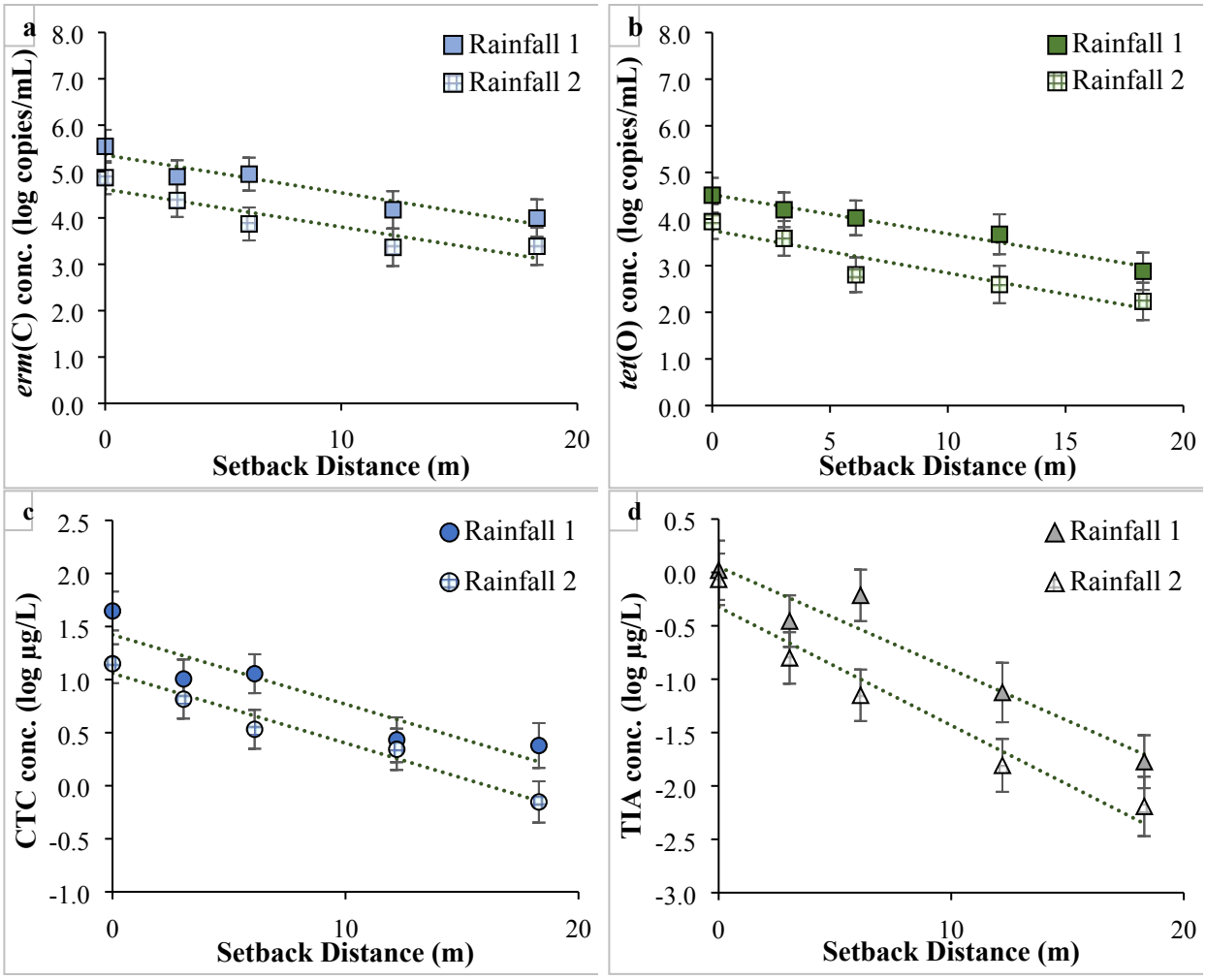


Figure S2. Weighted average concentration of (a) *erm*(C), (b) *tet*(O), (c) chlortetracycline (CTC), and (d) tiamulin (TIA) in runoff during rainfall #1 and rainfall #2. The error bars represent the standard errors based on the ANOVA analysis of replicates and distance using GLIMMIX.

References

1. Suzuki, M. T.; Taylor, L. T.; DeLong, E. F.,Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5 '-nuclease assays. *Appl. Environ. Microbiol.* **2000,** *66*, (11), 4605-4614.
2. Lachmayr, K. L.; Kerkhof, L. J.; DiRienzo, A. G.; Cavanaugh, C. M.; Ford, T. E.,Quantifying Nonspecific TEM beta-Lactamase (bla(TEM)) Genes in a Wastewater Stream. *Appl. Environ. Microbiol.* **2009,** *75*, (1), 203-211.
3. Koike, S.; Aminov, R. I.; Yannarell, A. C.; Gans, H. D.; Krapac, I. G.; Chee-Sanford, J. C.; Mackie, R. I.,Molecular ecology of Macrolide-Lincosamide-Streptogramin B methylases in waste lagoons and subsurface waters associated with swine production. *Microb. Ecol.* **2010,** *59*, (3), 487-498.
4. Rosewarne, C. P.; Pettigrove, V.; Stokes, H. W.; Parsons, Y. M.,Class 1 integrons in benthic bacterial communities: abundance, association with Tn402-like transposition modules and evidence for coselection with heavy-metal resistance. *FEMS Microbiol Ecol* **2010,** *72*, (1), 35-46.
5. Fan, W.; Hamilton, T.; Webster-Sesay, S.; Nikolich, M. P.; Lindler, L. E.,Multiplex real-time SYBR Green IPCR assay for detection of tetracycline efflux genes of Gram-negative bacteria. *Mol. Cell. Probes* **2007,** *21*, (4), 245-256.
6. Aminov, R. I.; Garrigues-Jeanjean, N.; Mackie, R. I.,Molecular ecology of tetracycline resistance: Development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. *Appl. Environ. Microbiol.* **2001,** *67*, (1), 22-32.
7. Pei, R. T.; Kim, S. C.; Carlson, K. H.; Pruden, A.,Effect of River Landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res.* **2006,** *40*, (12), 2427-2435.
8. Ghosh, S.; Ramsden, S. J.; LaPara, T. M.,The role of anaerobic digestion in controlling the release of tetracycline resistance genes and class 1 integrons from municipal wastewater treatment plants. *Appl. Microbiol. Biotechnol.* **2009,** *84*, (4), 791-796.
9. Suzuki, M. T.; Giovannoni, S. J.,Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl. Environ. Microbiol.* **1996,** *62*, (2), 625-630.
10. Ng, L. K.; Martin, I.; Alfa, M.; Mulvey, M.,Multiplex PCR for the detection of tetracycline resistant genes. *Mol. Cell. Probes* **2001,** *15*, (4), 209-215.