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

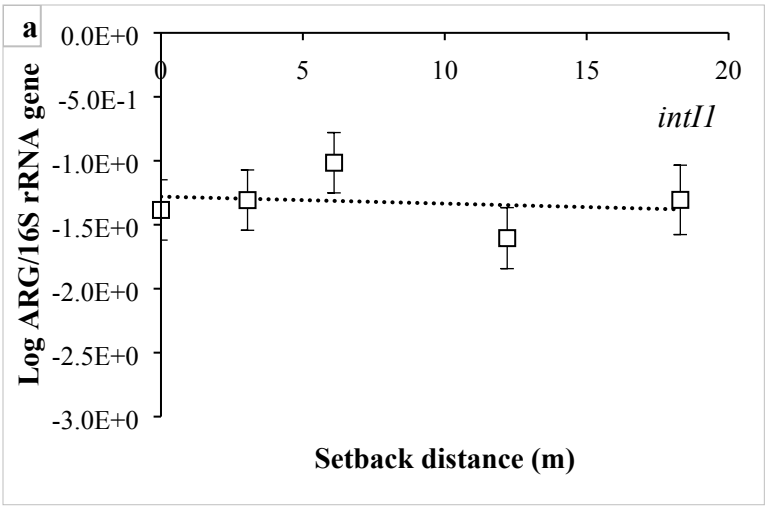
[d]LIN; lincomycin

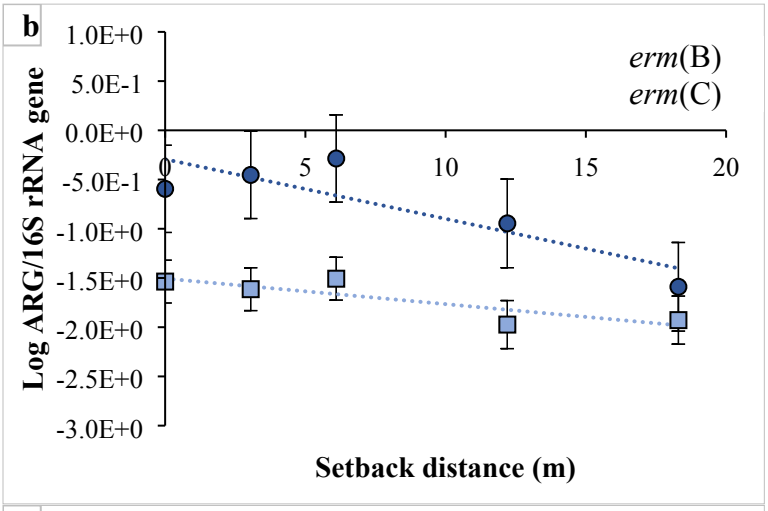
[e]TIA; tiamulin

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





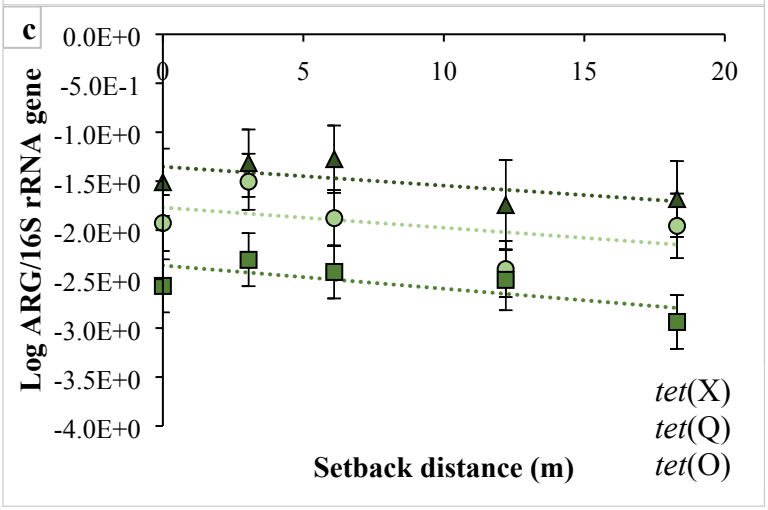


Figure S1. Means of log concentration of the relative abundance of (a) *intI1*, (b) erythromycin resistance methylase (*erm*) genes, and (c) tetracycline resistance (*tet*) genes in runoff from manure-amended plots after the rainfall #1. The error bars represent the standard errors based on the ANOVA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

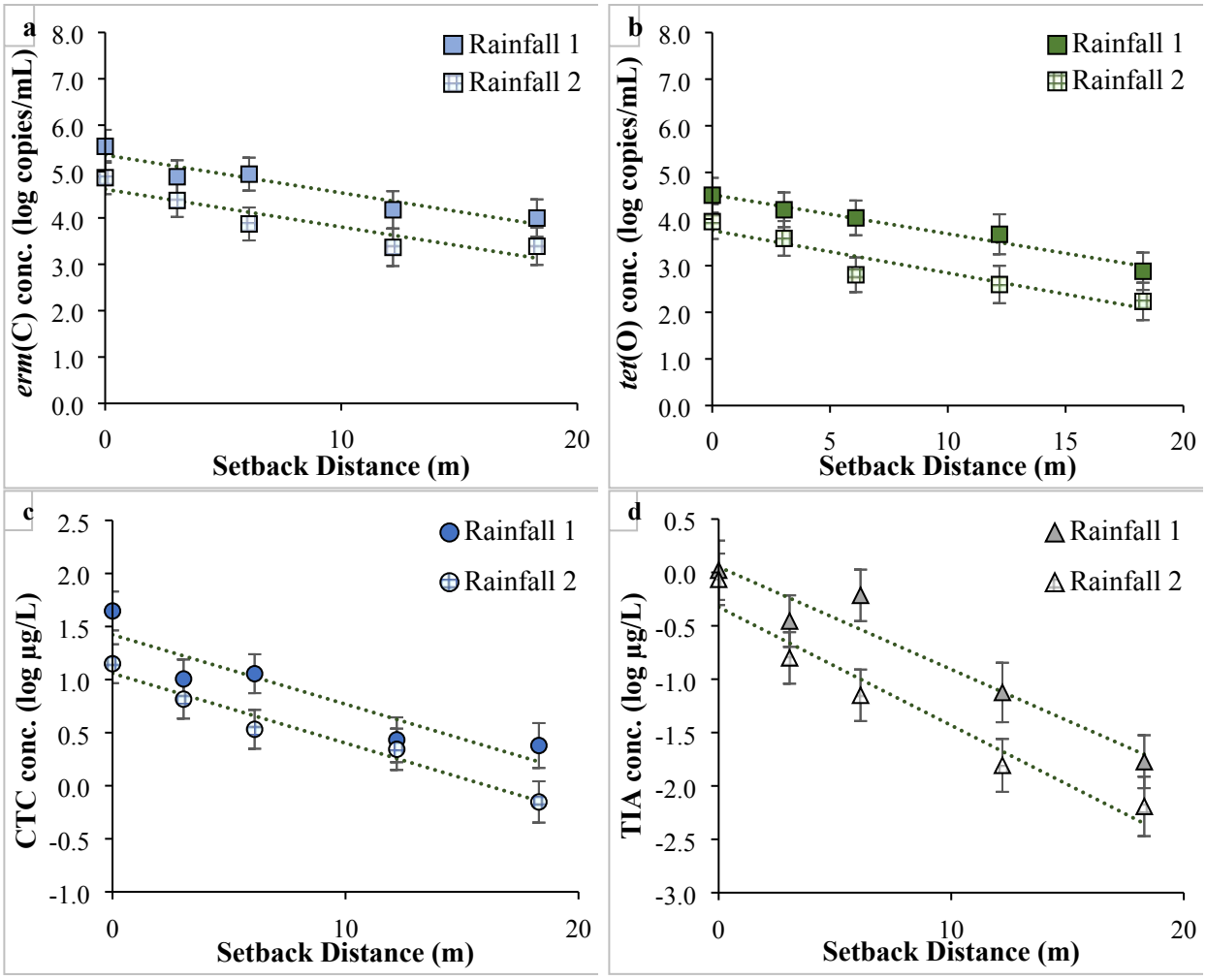


Figure S2. Weighted average concentration of (a) *erm*(C), (b) *tet*(O), (c) chlortetracycline (CTC), and (d) tiamulin (TIA) in runoff during rainfall #1 and rainfall #2. The error bars represent the standard errors based on the ANOVA analysis of replicates and distance using GLIMMIX.

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