RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Influence of on-site manure storage and land application strategy on the fate and transport of antimicrobials and antimicrobial resistance genes in the environment, **NPB #11-018**

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Industry Summary:

The objective of this study was to evaluate the role of manure storage on antimicrobial and antimicrobial resistance (AMR) genes in swine manure and to quantify the transport of these constituents in runoff after land application of swine manure. To meet these objectives, a series of field rainfall simulation experiments and laboratory storage experiments were performed. In the rainfall simulation experiments, swine manure different methods: broadcast, surface incorporation, and injection. We determined that antimicrobials and AMR genes were detected in fresh swine manure, and that the concentration of both antimicrobials and the relative abundance of AMR genes decreased after storage of manure under anaerobic conditions. This indicates that on-site storage or holding of swine manure prior to land application will reduce the amount of antimicrobials and AMR genes present in the manure. Although storage decreased the levels of all AMR genes, the AMR genes associated with chlortetracycline (*tetQ* and *tetX*) were found to decrease more substantially compared with tylosin AMR genes, *ermB* and *ermF*. Results from the field rainfall simulation experiments indicated that AMR genes could be vertically transported in soil with infiltrating rainfall. The manure application strategy and timing of rainfall were found to be significant for predicting antimicrobial concentrations in runoff, with higher concentrations observed in runoff from plots receiving broadcast and incorporated manure compared with injected manure. In addition, decreasing concentrations were observed with increased time since manure application. To our knowledge, this is the first study to systematically investigate how different manure land application strategies affect antimicrobial and AMR gene levels in agricultural runoff and provide evidence that both manure storage and land application strategy can influence observed concentrations.

Keywords

Antimicrobials, antimicrobial resistance, manure management, runoff, land application

Scientific Abstract

Land application of swine manure is important as it provides a source of nutrients and can increase soil productivity, improve water filtration and reduce the potential for soil erosion. Swine manure contains residues of antimicrobials and antimicrobial resistance (AMR) genes and the presence of these constituents has been documented in water bodies adjacent to animal production facilities. In this study, we investigated the role of antimicrobial administration, on-site manure storage, field application method (broadcast, incorporation, or

injection) and timing of rainfall on antimicrobial and AMR genes in manure, soil and runoff. Antimicrobial concentrations in runoff and manure were determined using liquid chromatography tandem mass spectroscopy and AMR genes were evaluated using qPCR. Results from laboratory storage experiments indicated that both AMR genes and antimicrobials in swine manure decreased over a 40-day period under anaerobic conditions. A half-life of approximately 10 days was determined for chlortetracycline and decreases in AMR genes *tetX, tetQ*, *ermB,* and *ermF* were also observed. Observed decreases in AMR genes were approximately 1 order of magnitude over the 40 day study period. Although sharp decreases in CTC and TYL concentrations were observed during manure storage, similar trends were not observed for AMR genes. Instead, the decreases in AMR genes were more moderate. There could be multiple explanations for the difference in the chemical and microbial profiles. In this study, antimicrobial metabolites were not analyzed. Because some metabolites may still have antimicrobial effects, the total selective pressure could remain high during the storage period. Results from the land application experiments indicate that antimicrobials and AMR genes can be transported in runoff after land application of swine manure. It was determined that application method and the timing of rainfall had a significant effect on the concentration of antimicrobials in runoff, but the effect of these variables on AMR gene transport was not as clear. AMR genes were determined to be transported vertically in the soil profile after three consecutive rainfall application experiments. In addition, mass loading rates for antimicrobials after land application were calculated. Mass loading rates for chlortetracycline ranged from 4.5 to 0.15 ug/m^2 for broadcast manure to 0.27 to 0.09 ug/m^2 for injected manure. Tylosin mass loading rates were highest for broadcast manure (280 to 56 ug/m^2) and lowest for injected manure plots (1.7 to 5.2 ug/m^2). Essentially no bacitracin was detected in runoff regardless of manure application strategy or rainfall timing. No bacitracin resistance genes were detected in manure.

Introduction

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

Over the past thirty years, increasing numbers of livestock produced for human consumption are raised in concentrated animal feeding operations (CAFOs). CAFOs are generally defined as facilities with more than 1000 animal 'units' (e.g. 2500 swine) confined on site. 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. Antimicrobials and other pharmaceuticals are often used at CAFOs at subtherapeutic levels for growth promotion and prophylaxis (Gaskins et al. 2002) and at therapeutic levels for disease treatment. The antimicrobials added in animal feed are often not completely absorbed in the animal gut, resulting in the potential for antimicrobial resistance among commensal bacteria and pathogenic bacteria (Salyers et al. 2004).

The benefits of land application of manure to agricultural fields is substantial as it provides a source of valuable fertilizer nutrients and organic matter, resulting in increased soil productivity, improved water infiltration, and reduced soil erosion potential. However, the presence of antimicrobial compounds and antimicrobial resistant (AMR) bacteria in treated manure introduces the potential for these constituents to enter the environment when manure is applied to soil. Recent studies reporting the occurrence of antimicrobial compounds and associated antimicrobial resistant (AMR) genes have attempted to relate these components to the distribution of livestock production in watersheds. Residues of antimicrobials and AMR genes, the genetic material that confers antimicrobial resistance to bacteria, has been documented in water bodies adjacent to CAFO sites though the links between sources and occurrence have not yet been established (Koike et al. 2007, Dolliver et al. 2008).

As with nutrients, trace contaminant movement in runoff and erosion can be quantified on small plots with simulated rainfall or larger field plots with runoff events resulting from natural rainfall. Because many factors may influence the fate and transport of antimicrobials and AMR bacteria from land-applied manure including site management (application method and timing), source management (applied versus soil nutrients), it is important to measure the effects of these variables and establish conditions under which transport occurs.

Significant gaps remain regarding our understanding of antimicrobial residues and AMR bacteria/genes in animal wastes related to antimicrobial administration in animal feed, as well as in our knowledge about the fate and transport of these compounds in the environment. The reported research will provide key information about the quantity of antimicrobial residues and AMR bacteria/gene in animal wastes as a function of antimicrobial dosages in animal feeds, and on the environmental fate of these pollutants, both during swine manure storage as well as following application to land. This information is critical in developing on-farm manure management practices and watershed management strategies that will reduce the potential for transport of these constituents to water after land application**.**

Objectives

Specific objectives of the research include:

1) Measure the presence of specific commonly-used antimicrobials and AMR bacteria/genes in swine wastes as a function of antimicrobial administration.

2) Quantify the fate and transport of these antimicrobials, antimicrobial resistance genes and antimicrobial resistant bacteria in soil and surface runoff after land applications of surface applied, incorporated and injected swine manure.

3) Evaluate the potential for transport of these antimicrobials and antimicrobial resistant bacteria at the watershed scale using current nutrient-based best management practices (BMPs), and identify practices to minimize transport of these constituents and their risk to the environment.

Materials and Methods

Manure Collection

Swine slurry was collected at the USDA Meat Animal Research Center in Clay Center, Nebraska. All animals were housed in mechanically-ventilated barns. Animals fed chlortetracycline (CTC) were feeder pigs. Manure from the animals was pushed through slots in the pen floor and was collected in channels under the pen. Typically, 2000 L of well water was discharged periodically (approx. 1 hr) through the trough to flush the manure slurry to a common lagoon system. The slurry collection point for this experiment was accessed through a cover just outside the building at the point where the two channels in the building jointed together. To ensure sufficient solids content of the slurry, the flush system was turned off over-night to allow solids to collect in the trough system. Slurry was collected the following morning using plastic buckets at the beginning of the first flush to ensure sufficient concentration of antibiotic in the slurry. These feeder pigs were fed a corn and soybean-based diet that included 66.2 mg active ingredient kg^{-1} ration.

The animals that were fed tylosin (TYL) were sows and gilts and were housed in the same type of building and manure handling system as the animals fed chlortetracycline. The same strategy was used to unsure sufficient concentration of antibiotic in the slurry by turning off the flush system to allow solids to build-up over-night. The collection point for this building was at a central collection point for several buildings. This point was located 4 m below the ground level and had three separate pipes discharging to this collection point. Each pipe was discharge from different swine housing complexes. To isolate and collect slurry from the facility with animals being fed tylosin, the flush systems to these other housing complexes were turned off and the collection well was allowed to drain. Temporary dams were put in place in front of the two inlet pipes coming from the non-target housing complexes and the effluent pipe leading to the common lagoon system. This allowed the water level to build-up in the collection well sufficient to allow an industrial submersible comminutor to pump to the surface for collection. Care was taken to collect slurry at the beginning of the flush cycle. These sows and gilts were fed a corn and soybean-based diet that included 75.0 mg active ingredient kg^{-1} ration.

The animals that were fed bacitracin were replacement gilts. These animals were housed in the same type of building as the other two: however, the manure handling system was a pull-plug system. The manure was allowed to collect in pits under the slotted pen and was drained once a week by pulling a plug and allowing it to drain. After draining, the plug was replaced and well water was allowed to refill the pit to approximately a 0.5 m depth. Slurry was collected from this facility by removing a grate and simply dipping a plastic bucket in and collecting the slurry. These replacement gilts were fed a corn and soybean-based diet that included 39.7 mg active ingredient kg^{-1} ration.

Each week, swine slurries were collected and transported to the land application site in 20-L plastic buckets. A subsample of the swine slurry was analyzed for solids and nutrient analyses at Ward Laboratories (Kearney, NE, USA) and for antimicrobial and antimicrobial resistance genes at the University of Nebraska-Lincoln.

Field rainfall simulation experiments

Thirty-six plots were established across the slope using a randomized block design (Figure 1). Each of the experimental treatments which included tillage (broadcast, incorporated, or injected) and antibiotic (bacitracin, chlortetracycline, or tylosin) were replicated three times. Rainfall simulation tests were performed over a 5-week period.

Small plots (0.75 m x 2.0 m) were constructed using 20 cm-wide sheet metal frames driven approximately 10 cm into the soil. Swine slurry was applied to meet the 1-yr N requirement for corn (151 kg N ha^{-1} yr^{-1} for an expected yield of 9.4 Mg ha^{-1}). When calculating manure application rates, it was assumed that the first year N availability from swine slurry was 70% of the total amount of nitrogen measured in the slurry.

Swine slurry was weighed at the field site, then land-applied. Because the study plots had been cropped in no-till corn and remained undisturbed following harvest the previous fall, there was considerable crop residue on the ground surface.

Rainfall simulation procedures adopted by the National Phosphorus Research Project were employed in this study (Sharpley and Kleinman 2003). A portable rainfall simulator based on the design by Humphry et al. (2002) was used to apply rainfall to the paired plots. The distance between paired plots was approximately 5 m to accommodate the tandem disk used for tillage. The simulator was used to apply rainfall for 30-min at an intensity of 70 mm hr^{-1}. Two additional rainfall simulation tests were conducted for the same duration and intensity at approximately 24-hr intervals.

Plot borders channeled runoff into a sheet metal lip that emptied into a collection trough located across the bottom of each plot. The trough diverted runoff into plastic buckets. A sump pump was then used to transfer runoff into larger plastic storage containers. The storage containers were weighed at the completion of each run to determine total runoff mass. Accumulated runoff was agitated immediately before sample collection. The runoff samples were collected within a few minutes following completion of the rainfall simulation tests and stored on ice prior to transport to the University of Nebraska-Lincoln.

Field Soil Samples

Undisturbed soil samples were collected from the surface of each check plot prior to manure application and from the surface of the incorporated and broadcast plots after manure application. Soil samples were also collected from the check plots, incorporated manure and broadcast manure plots after completion of the rainfall simulation experiments. Soil samples were stored on ice prior to transport to the University of Nebraska-Lincoln.

Laboratory Manure Storage Experiments

Manure was collected from the MARC facility from separate locations were animals were administered chlortetracycline, bacitracin and tylosin. As a smaller amount of manure was required for these laboratory experiments, manure samples were collected directly from fresh manure on the facility floor and the flushing

procedure described above to collect swine manure slurry was not used. Three manure samples were collected, one from each antibiotic source. Sacrificial reactors consisting of 100 mL glass amber wide mouth jars were used and manure and water were added in a 2:1 (w/w) ratio for a total mass of 75 g (Masse et al. 2000). Reactors were sparged with nitrogen for approximately 5 minutes and incubated at 37°C for up to 40 days. Reactors were sacrificed at various times in duplicate. Samples were frozen at -20°C until analysis. Samples were then analyzed for antibiotics and antimicrobial resistance genes following the procedures outlined below.

Antimicrobial Analysis of Runoff and Manure Samples

All samples were analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS) Zhu et al. (2001; Snow et al. (2003). Run-off water samples, collected in 1-liter amber glass bottles, were held on ice until delivery to the Water Sciences Laboratory for filtration and extraction. Swine manure samples were collected in 250 milliliter amber jars and frozen after delivery to the laboratory. Sediment cores were collected in acrylic tubes and stored frozen until processing for measurement of antibiotic residues and resistance genes. All runoff water samples were logged in and extracted within 24 hours of collection using off-line solid phase extraction (SPE) on 200 mg Oasis HLB cartridges. Briefly, each sample container was weighed and then extracted by suction through a Gellman A/E binderless glass fiber filter (0.5 um) and a labeled and precondition SPE cartridge. After filtration and extraction of approximately 500 milliliters of each water sample, the container was reweighed and the extracted volume determined by difference for calculation of antibiotic concentrations. Suspended material accumulating on the filter was saved and stored frozen for subsequent analysis. SPE cartridges were stored at -20^{O}C until they could be further processed for analysis by liquid chromatography tandem mass spectrometry.

SPE cartridges were eluted into borosilicate test tubes using 3 mL of a solution of 130 mM ammonium citrate in methanol, containing oleandomycin at 16 nanograms to be used as a surrogate. The solvent was reduced in volume to approximately 200 µL under a stream of dry nitrogen, and transferred quantitatively to an autosampler vial and silane treated insert and mixed with 200 µL of reagent water. Internal standards (roxithromycin and doxycycline @ 40ng) were added by pipette during the concentration step. Recovery of chlortetracycline, bactracin A, tylosin, and fenbendazole was determined from extraction and analysis of fortified reagent water during the elution stage. Fortified blanks and method blanks were analyzed at a frequency of 1 in 20 samples (5%). Method detection limits were determined by extraction and analysis of 10 replicates of 500 milliliter reagent water fortified with 0.5 µg/L.

Antibiotics were extracted from solid samples using solvent extraction followed by SPE for cleanup. Well-mixed samples of sediment (10 grams) and manure (0.2 grams mixed with 5 gram clean sand) were spiked with surrogate (16 ng) mixed with 14 milliliters of 5 mM ammonium citrate mixed with 6 milliliters of methanol in 50 milliliter Teflon centrifuge tubes. Mixtures were shaken briefly by hand and then on a Burrell Wrist-action shaker for 30 minutes. Solids and solvent were separated by centrifugation, with the supernatant decanted into a glass evaporation tube (RapidVap, Labconco Corporation). Solid material was extracted again using 20 mL ammonium citrate and methanol followed by centrifuging with supernate combined with the first extract. Solid material was extracted a third time using acetone (20 mL) and combined with previous extracts. Internal standards (doxycycline and roxithromycin, 40 ng each) were mixed with methanol/acetone/citrate mixture which was slowly concentrated using a RapidVap N_2 sample concentrator at 25°C (90% rotation speed) until the volume was reduced by half. Ammonium citrate solution (5 mM, pH=6.0) was added to bring the volume up to 100 mL and the resulting solution extracted using 200mg Oasis HLB SPE cartridges. Cartridges were then processed in a manner identical to the water samples. Method detection limits were determined by extraction and analysis of 10 replicates of 10 grams clean sand fortified at 0.5 ng/g of all analytes. Recovery of chlortetracycline, bactracin A, tylosin, and fenbendazole were monitored from extraction and analysis of clean sand fortified with 2 ng/g of all analytes.

All sample extracts were analyzed on a Waters Quattro Micro triple quadrupole mass spectrometer and 2695 high pressure liquid chromatograph (HPLC) and autosampler. Analytes were separated on a reverse phase (HyPurity C18, 250 mm x 2.1 mm, 5 µm particle size) column at 50°C with a 50 µL injection volume. For the water and sediment samples, a gradient mobile phase (0.2 mL/min) was used consisting of A) 97:3 aqueous

citric acid (1 mM):methanol and B) 97:3 methanol:aqueous citric acid (1 mM). Initial gradient conditions (95% A) were held for 2.0 minutes then ramped to 5% A, held at 5% A until 18 min., then to 95% A to equilibrate the column. Total run time was 23 min. For manure samples, the same gradient was used with the addition of a constant 4% component of 10% aqueous ammonium hydroxide with adjustments to the gradient to replace the aqueous component of mobile phase B.
Analytes were detected using Multiple Reaction Monitoring (MRM) mode with positive electrospray ionization (ESI). The most intense MS/MS transitions were determined by infusion and monitored for each analyte (Table 1) and linear calibration curves were generated for all analytes and surrogates with r2 values > 0.995. Because bacitricin A is rapidly hydrolyzed in water at near neutral pH, a standard for bacitracin F (one of its degradation products) was synthesized and used to quantify this compound in the last group of run-off samples (Pavli and Kmetec 2006).

Table 1. Molecular weight, retention times, and MRM transition of antibiotics measured, internal standards, and surrogate compound.

Analyte	Molecular weight	Retention time (min)	MRM Transition (m/z)
Bactracin A	1422.7	9.82	712.10->86.20
Bactracin F	1419.64	10.05	710.19->281.26
Chlortetracycline	478.88	8.71	478.90->444.00
Fenbendazole	299.35	10.63	300.20->268.20
Tylosin	916.10	10.40	916.9->174.2
Surrogate (S) and Internal Standards (IS)			
Doxycycline (IS)	444.4	8.63	445.05->428.05
Oleandomycin (S)	687.86	10.51	688.35->544.10
Roxythromycin (IS)	837.05	11.58	837.55->679.50

Antimicrobial Resistance Genes Analysis of Runoff and Manure Samples

For runoff samples, each 500 mL of well-mixed sample was centrifuged for 5 min at 10,000×g in sterile 50mL centrifuge tubes. Supernatants were decanted and pellets were stored at -20ºC till DNA extraction. Soil cores were extruded from plastic sleeves and separated into top, middle, and bottom sections. The soil cores varied from 6-10 inches in length. The top two inches of soil and the bottom two inches of soil were analyzed separately for antimicrobial resistance genes.

DNA from the runoff solids and soil samples was extracted using the MoBio UltraClean® Soil DNA Isolation Kit according to manual except that a 40-second bead beating was used to lyse the cells. DNA extracts were quantified using a NanoDrop spectrometer.
All DNA samples evaluated for chlorotetracycline (CTC) resistance genes were diluted to 4 ng/uL with sigma water. qPCR protocol for *tetQ* and *tetX* was followed the ones in published studies (Aminov *et al.* 2001; Ghosh *et al.* 2009)

All samples evaluated for tylosin (TYL) resistance genes were diluted to 10 ng/µL with sigma water as directed in (Koike *et al.* 2010). Six resistance genes, *ermA*, *ermB*, *ermC*, *ermF*, *ermG* and *ermQ*, were evaluated through running PCR (Takara PCR kit) and gel electrophoresis. Two resistance genes were present in our samples: *ermB* and *ermF*. These PCR products were purified using a QIAquick PCR Purification Kit, cloned and transformed using the TOPO® TA Cloning® Kit for Sequencing with One Shot® TOP10. Plasmids were extracted from the transformed E. coli cells using Qiagen's Plasmid Mini Kit. The plasmid extracts containing target ARG regions were quantified using the NanoDrop spectrometer and were diluted accordingly with sigma water to form a standard series. Quantitative PCR reactions were conducted according to Koike, et al. (2010).

All samples evaluated for bacitracin (BAC) resistance genes were diluted to 4 ng/uL and PCR was run according to Yoshida et al. (2011) for *bceA* and *bceR*. Regular PCR was also run on manure samples for resistance genes *bcrA*, *bcrB*, and *bcrC* according to Murphy et al. (2008). All qPCR results on ARGs were normalized to the 16S rRNA gene, which was quantified using the qPCR protocol from Suzuki et al. (2000).

Statistical Analysis

The effects of manure application method, antibiotic in manure, and rainfall simulation run on antimicrobials and antimicrobial resistance genes in runoff were determined using analysis of variance (ANOVA) (SAS Institute, 2003). By using ANOVA it was possible to test for significant differences among experimental variables. If a significant difference was identified, the least significant difference test (LSD) was used to identify differences among experimental treatments. A probability level < 0.05 was considered significant.

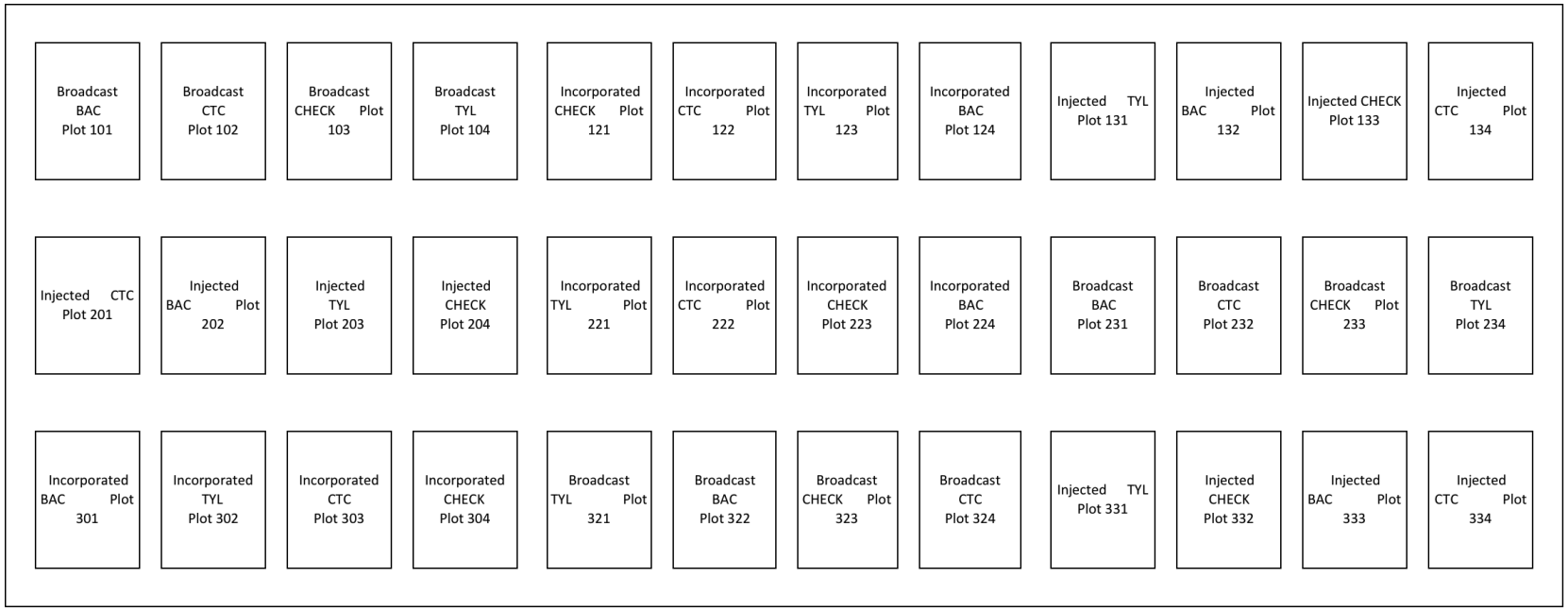

Figure 1. Schematic showing plot layout, manure application method and antibiotic.

Results

Objective 1) Measure the presence of specific commonly-used antimicrobials and AMR bacteria/genes in swine wastes as a function of antimicrobial administration.

The concentration of antimicrobials detected in the swine manure slurry that was used in the field rainfall simulation experiments is given in Table 1. Degradation of CTC and corresponding resistance genes (*tetQ* and *tetX*) in laboratory storage experiments is given in Figures 2 and 3. Degradation of tylosin and corresponding resistance genes (*ermB* and *ermF*) is given in Figures 4 and 5. Bacitracin detected in laboratory storage experiments is given in Figure 6. No bacitracin resistance genes were detected in manure samples.

Table 2. Initial antimicrobial concentrations in swine manure slurry.

Antimicrobial Administered	Antimicrobial in manure		
	CTC (ng/g)	TYL (ng/g)	BAC (ng/g)
	Ave ± SD	Ave ± SD	Ave ± SD
CTC	3323.8 ± 3487.57	102.1 ± 89.40	16.3 ± 21.15
TYL	2 ± 1.83	287.4 ± 277.29	123.6 ± 152.13
BAC	171.9 ± 367.61	6.6 ± 14.70	777.4 ± 1683.89

All concentrations expressed on a wet weight basis.

Antimicrobial resistance genes were tested for their corresponding antibiotics. Three chlortetracycline related ARGs (i.e., *tetD*, *tetQ*, and *tetX*) were tested in all CTC manure samples. Because *tetD* was not detected in any of the samples, only *tetQ* and *tetX* were further quantified for all TYL manure samples. Six tylosin related ARGs (i.e., *ermA*, *ermB*, *ermC*, *ermF*, *ermG*, and *ermQ*) were tested on all TYL manure samples. Two ARGs, *ermB* and *ermF,* were detected in all TYL manure samples, and therefore were selected for further quantification. Five bacitracin resistance genes were tested for the BAC manure samples, *bceA*, *bceR*, *bcrA*, *bcrB* and *bcrC*, using PCR. None of these ARGs were amplified in PCR reactions, suggesting no detectable BAC resistance genes in the BAC manure samples.

Each primer set had a slightly different linear range, the lower bounds are listed: 100 copies/µL for the *tetQ* primer set (regression coefficient 0.996, 102% efficiency); 10-100 copies/µL for the *tetX* primer set (regression coefficient 0.996, 87.9% efficiency); 10-1000 copies/µL for the *ermB* primer set (regression coefficient 0.982, 109% efficiency); and 10-100 copies/µL for the *ermF* primer set (regression coefficient 0.978, 89.9% efficiency). The relative abundance of the ARGs in manure, soil, and runoff samples were calculated by normalizing the abundance of ARGs with the abundance of the 16S rRNA gene in each sample (i.e., ARG abundance/16S abundance). Overall, the relative abundance of *tetQ* and *ermB* were more often present than *tetX* and *erm*F. The average relative abundance of each resistance gene in the manure samples were:1.33±0.57 for *tetQ*, $(7.83\pm 0.04)\times10^{-2}$ for *tetX,*: $(1.19\pm0.05)\times10^{-1}$ for *ermB,* and $(2.20\pm0.00)\times10^{-3}$ for *ermF*.

Over the 40-day storage experiments, the relative abundance of ARGs followed a generally decreasing trend. The relative abundance of ARG *tetQ* dropped by an order of

magnitude over the course of the experiment (Figure 3). During the same period, the relative abundance of *tetX* decreased by less than one order of magnitude. The relative abundance of *tetQ* was consistently higher than that of *tetX* in the experiment (Figure 3). For the TYL resistance genes, the relative abundance of *ermB* dropped by about one order of magnitude within the first 48 hours of the experiment and then remained at the same level for the remainder of the 40-day experiment (Figure 5). The relative abundance of *ermF* was more complicated. It dropped nearly three orders of magnitude in the first 48 hours, but then increased about 1.5 orders of magnitude over the next 15 days before it leveled off (Figure 5). The relative abundance of *ermB* was consistently higher than that of *ermF* by one order of magnitude.

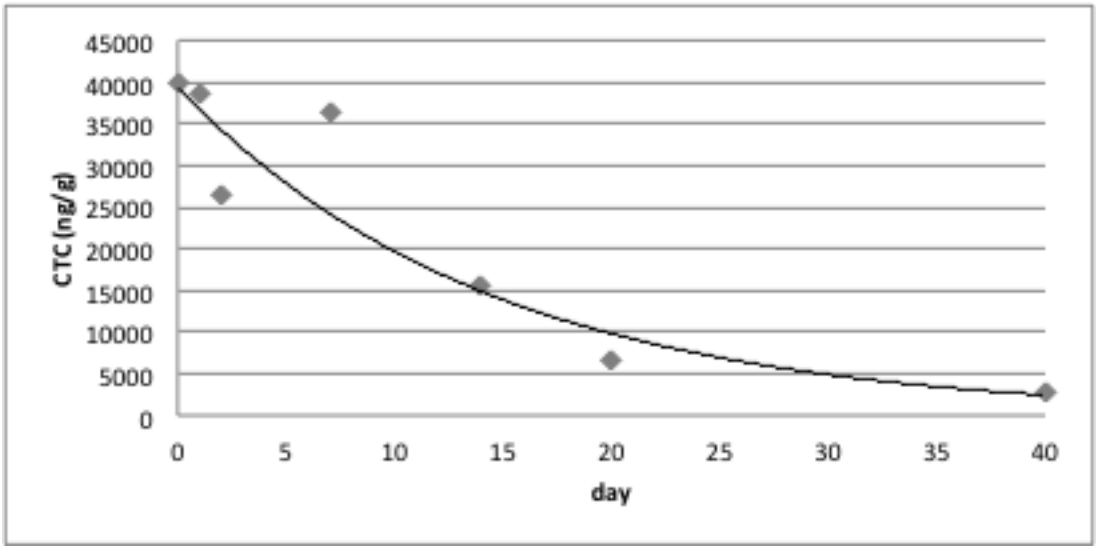

Figure 2. Degradation of chlortetracycline in manure.

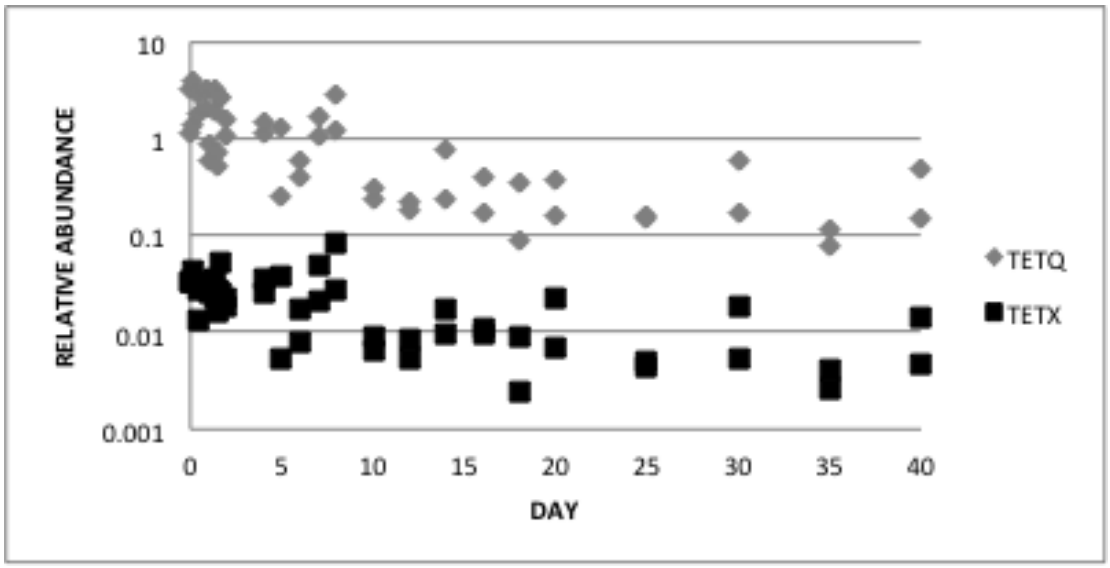

Figure 3. Degradation of *tet*Q and *tet*X in manure.

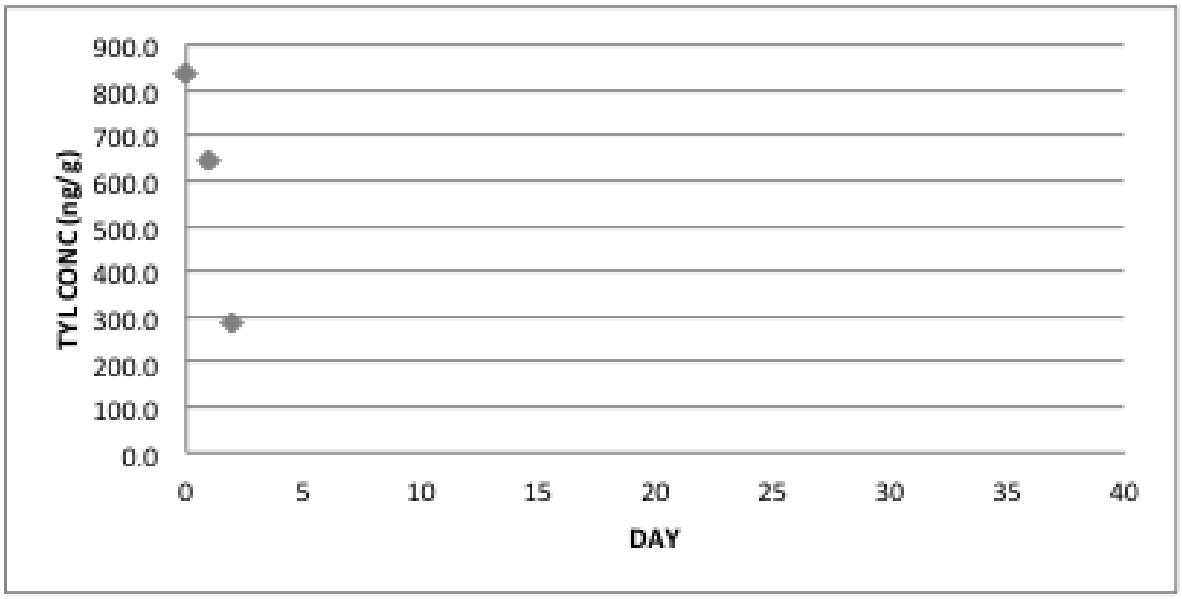

Figure 4. Degradation of tylosin in manure.

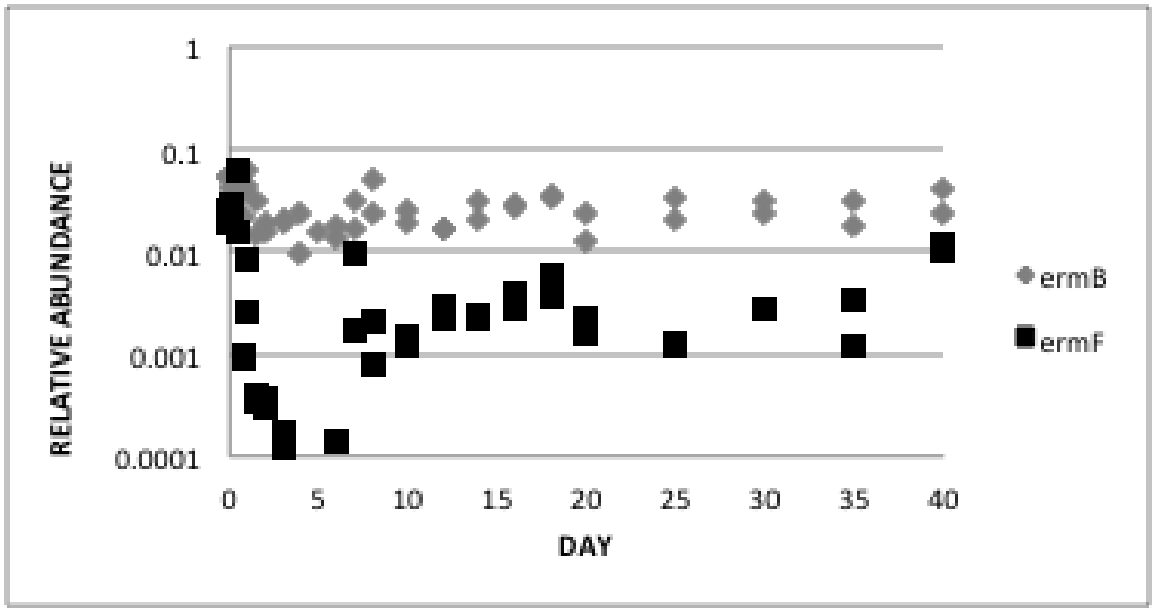

Figure 5. Degradation of *erm*B and *erm*F in manure.

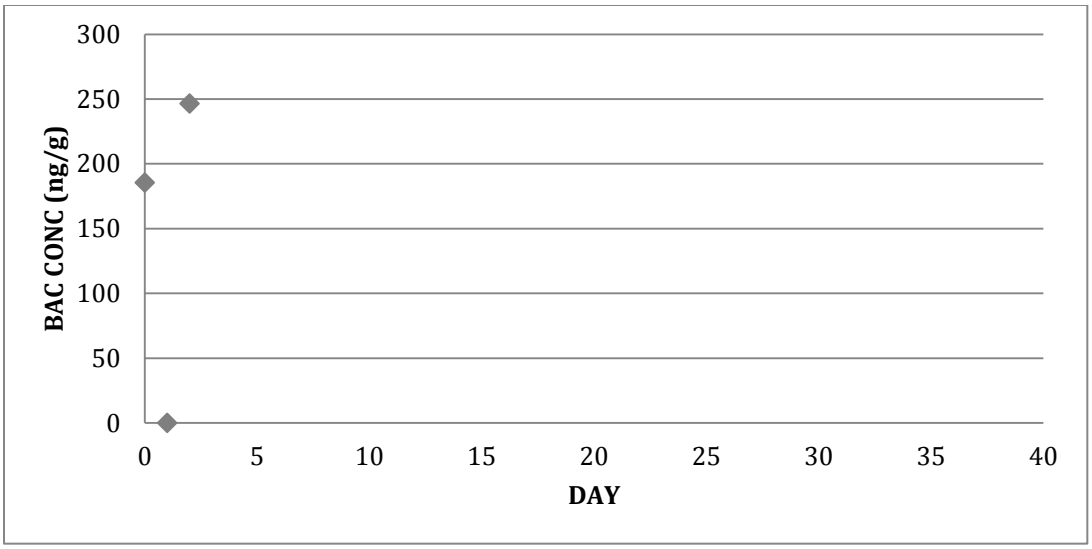

Figure 6. Degradation of bacitracin in manure.

Objective 2) Quantify the fate and transport of these antimicrobials, antimicrobial resistance genes and antimicrobial resistant bacteria in soil and surface runoff after land applications of surface applied, incorporated and injected swine manure.

The relative abundance of antimicrobial resistance genes in soil before and after rainfall simulation experiments are given in Figures 7 and 8. The concentration of antimicrobials detected in field rainfall simulation experiments is given in Figure 9. The relative abundance of antimicrobial resistance genes for CTC and TYL in runoff are given in Figures 10 and 11, respectively. The results from ANOVA testing are provided in Table 3.

Soil

In general, the relative abundance of ARM genes decreased between top and bottom soil by at least order of magnitude. The broadcast plots showed an overall increase of ARGs in the bottom soil after rainfall simulation, whereas many incorporated plots show a distinct difference in topsoil abundances before and after rainfall simulation.

The relative abundance of *tetQ* in CTC manure slurry was 1.33±0.57. After the land application of the manure slurry to soil, the relative abundance of ARGs decreased slightly after both broadcast and incorporation (Figure 7). The top soils of the broadcast plots show generally the same relative abundance before and after three simulated rainfall events (Figure 7, top panel A), whereas the top soils of the incorporated plots show a slightly decreased relative abundance after the three rainfall events (Figure 7, top panel B). The relative abundance of *tetQ* in the bottom soils in the treated plots increased slightly for both application methods after rainfall events (Figure 7, top panels A and B), while the increase in the broadcast plots is more pronounced than the increase in the incorporation plots. Most check plots did not have any detectable *tetQ* except for the bottom soil sample in the broadcast plots, which exhibited low tetQ abundance accompanied with low 16S rRNA gene abundance.

The relative abundance for *tetX* genes in manure samples was 0.078±0.040. For both broadcast and incorporated plots, the relative abundance of *tetX* in top soils was higher than that in manure (Figure 7, bottom panels A and B). The relative abundance of *tetX* in bottom soils was 1-2 orders of magnitude lower that that in bottom soil. Similar to the trend in the *tetQ* broadcast plots (Figure 7, top panel A), the relative abundance of *tetX* in bottom soil increased slightly after the three rainfall events (Figure 7, bottom panel A). In comparision, no increase of *tetX* was observed in the bottom soil of the incorporated plots (Figure 7, bottom panel B). No *tetX* was detected in check plots.

The relative abundance of *ermB* was 0.12±0.054 in TYL manure samples. In the top soils of broadcast plots, the relative abundance of *ermB* was not affected by the three rainfall events (Figure 8, top panel A). In contrast, the top soil in incorporated plots showed a large decrease in the relative abundance of *ermB* after the rainfall events (Figure 8, top panel B). The difference in *ermB* relative abundance between top and bottom soils was about one order of magnitude (Figure 8, top panels).

The relative abundance of *ermF* in TYL manure samples was 0.0022±0.001, which was the lowest among all ARM genes tested. Similar to *ermB*, the *ermF* in the topsoil in broadcast plots was not affected by the rainfall events, while the *ermF* in the topsoil in

incorporated plots showed a decrease (Figure 8). No clear trend was detected in the bottom soil before and after rainfall events. The ARG *ermF* was not detected in most of the check plots, except for the top soil sample from a check plot. The reason for this result is being evaluated, but it may be due to cross-contamination.

Runoff

Application methods affected the relative abundance of *tetQ* in runoffs from each rainfall events in three consecutive days. The relative abundance of *tetQ* decreased by about one order of magnitude in the broadcast and injected plots, whereas the that remained constant in the incorporated plots (Figure 10). The relative abundance of *tetX* exhibited a similar trend to that of *tetQ*. For example, a general decrease in abundance in broadcast and injected plots can be seen along with a constant level in incorporated plots. For both *tetQ* and *tetX*, the difference between treated and untreated plots was 1-2 orders of magnitude, with only one interaction showed. The relative abundance of *tetQ* and *tetX* in the initial runoff from broadcast plots was generally higher than those from incorporate and inject plots. Untreated (check) plots show relative abundance 1-2 orders of magnitude less than the treated plots.

For all three manure application methods, the relative abundance of *ermB* in runoff followed a decreasing trend across the three rainfall events (Figure 11). The relative abundance of *ermF* decrease in the runoff from the incorporated and injected plots after three rainfall events (Figure 11). In comparison, the relative abundance of *ermF* in broadcast runoff remained at a consistent level. Overall, the relative abundance of *ermF* was less that of *ermB* (Figure 11). The relative abundance of *ermB* and *ermF* in the runoff from check plots were 1-2 orders of magnitude lower than that from treated plots.

Chlortetracycline and tylosin were detected in runoff from the experimental plots from all three manure application treatments, however, the concentrations of chlortetracycline and tylosin in runoff from the plots with broadcast manure were higher than those from plots that had injected or incorporated manure. Bacitracin was not detected in runoff for the broadcast manure, and was detected after days 2 and 3 for incorporated manure plots, and day 3 for injected manure plots. In general, the concentration of antimicrobials in runoff was highest after the first day of runoff simulation and decreased on days 2 and 3.

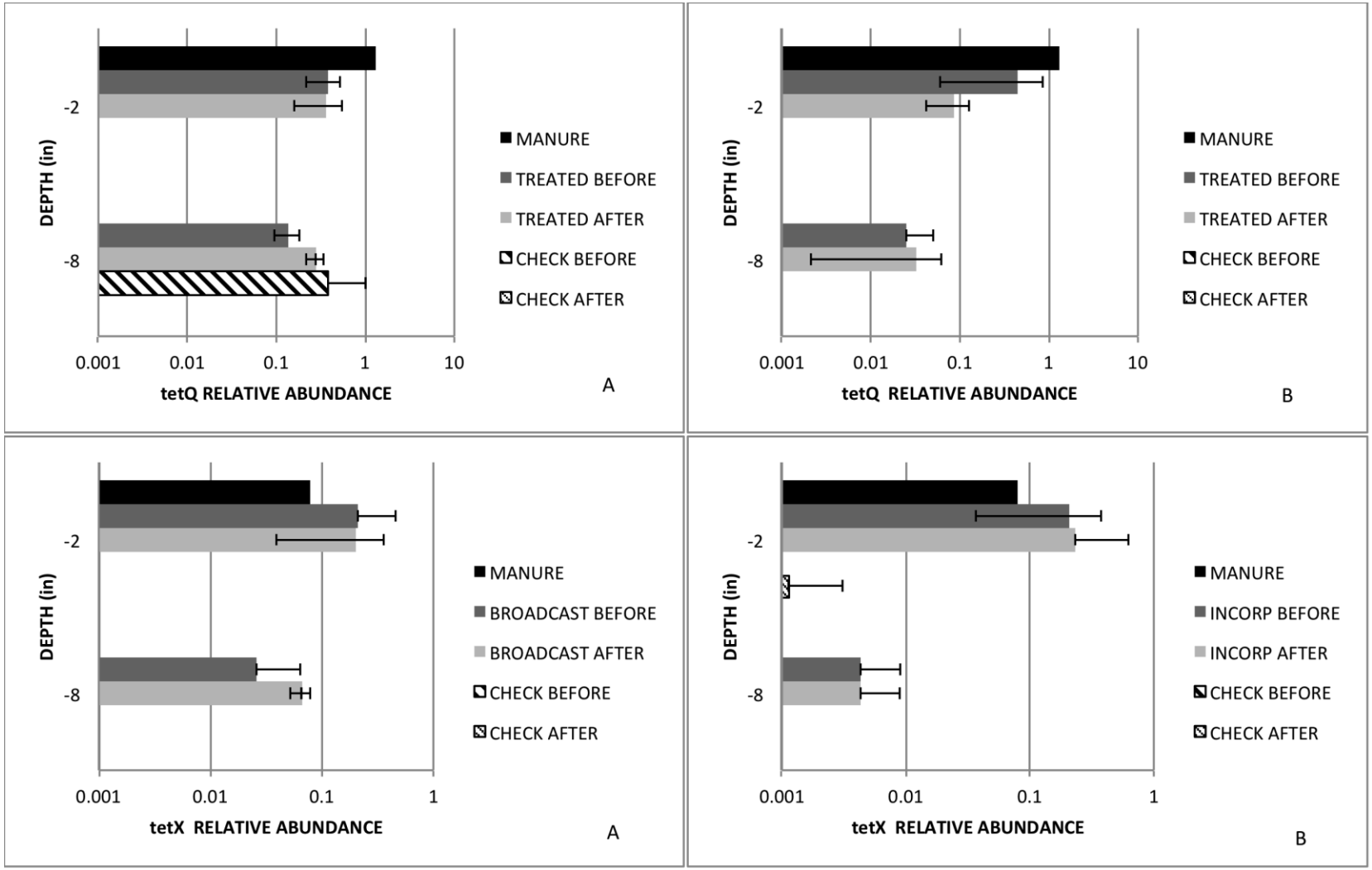

Figure 7. The relative abundances for CTC resistance genes *tetQ* (top) and *tetX* (bottom) in soil at two depths for check and treated plots using two manure application methods broadcast (A) and incorporated (B) before and after three simulated rainfall events.

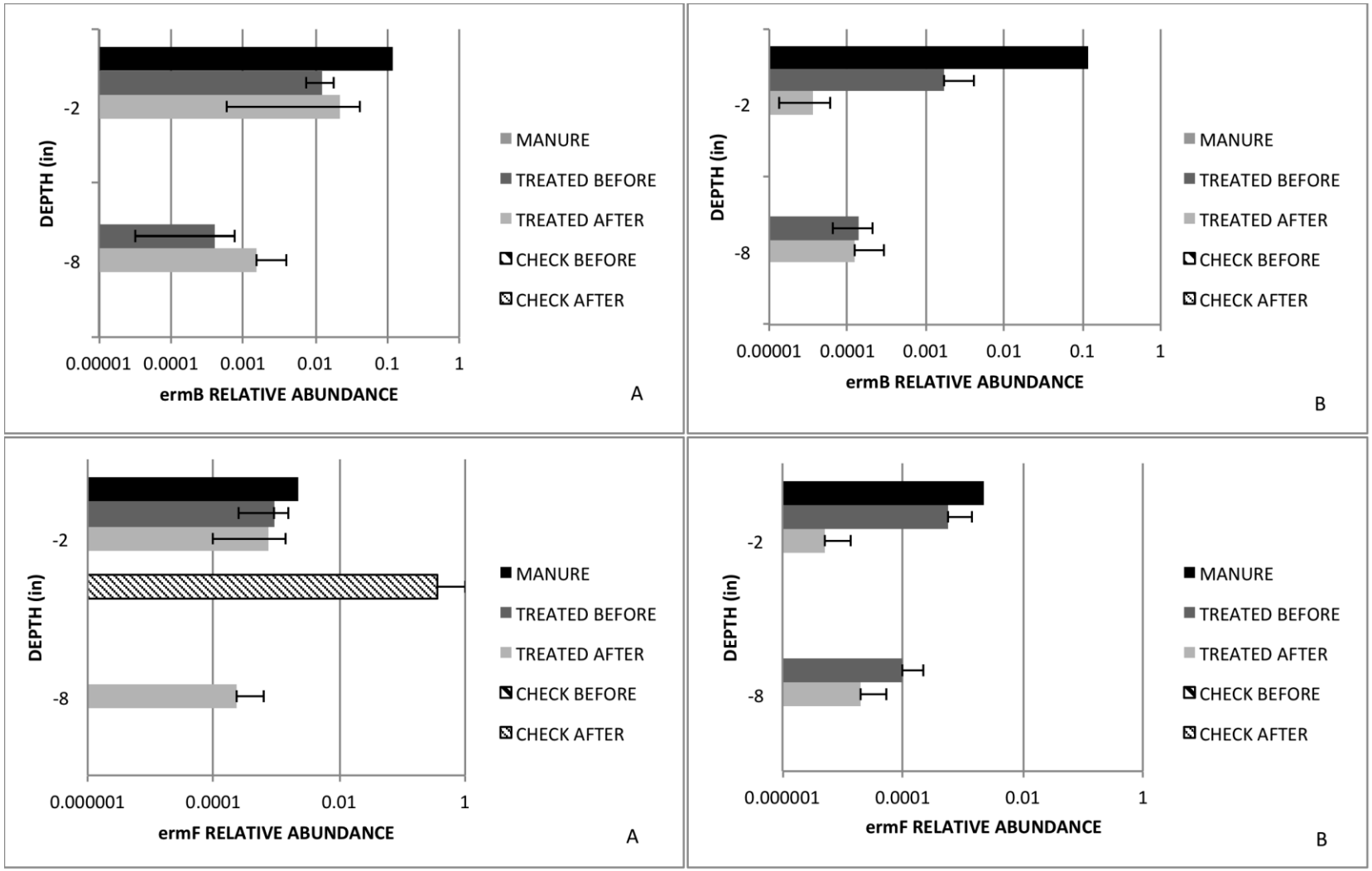

Figure 8. The relative abundances for TYL resistance genes *ermB* (top) and *ermF* (bottom) in soil at two depths for check and treated plots using two manure application methods broadcast (A) and incorporated (B) before and after three simulated rainfall events.

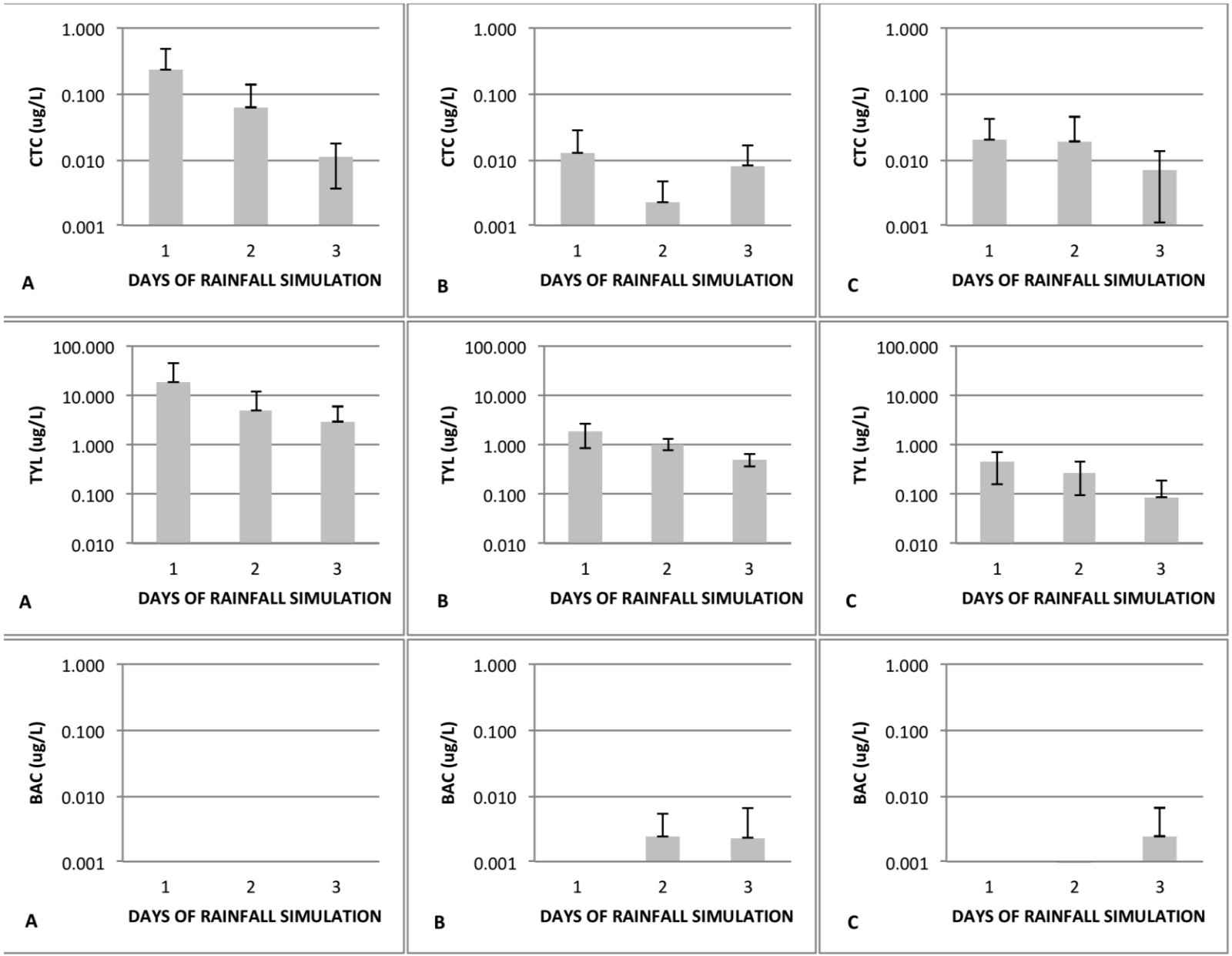

Figure 9. Concentrations of CTC, TYL and BAC in runoff from field plots with broadcast (A); incorporated (B); and injected (C) manure.

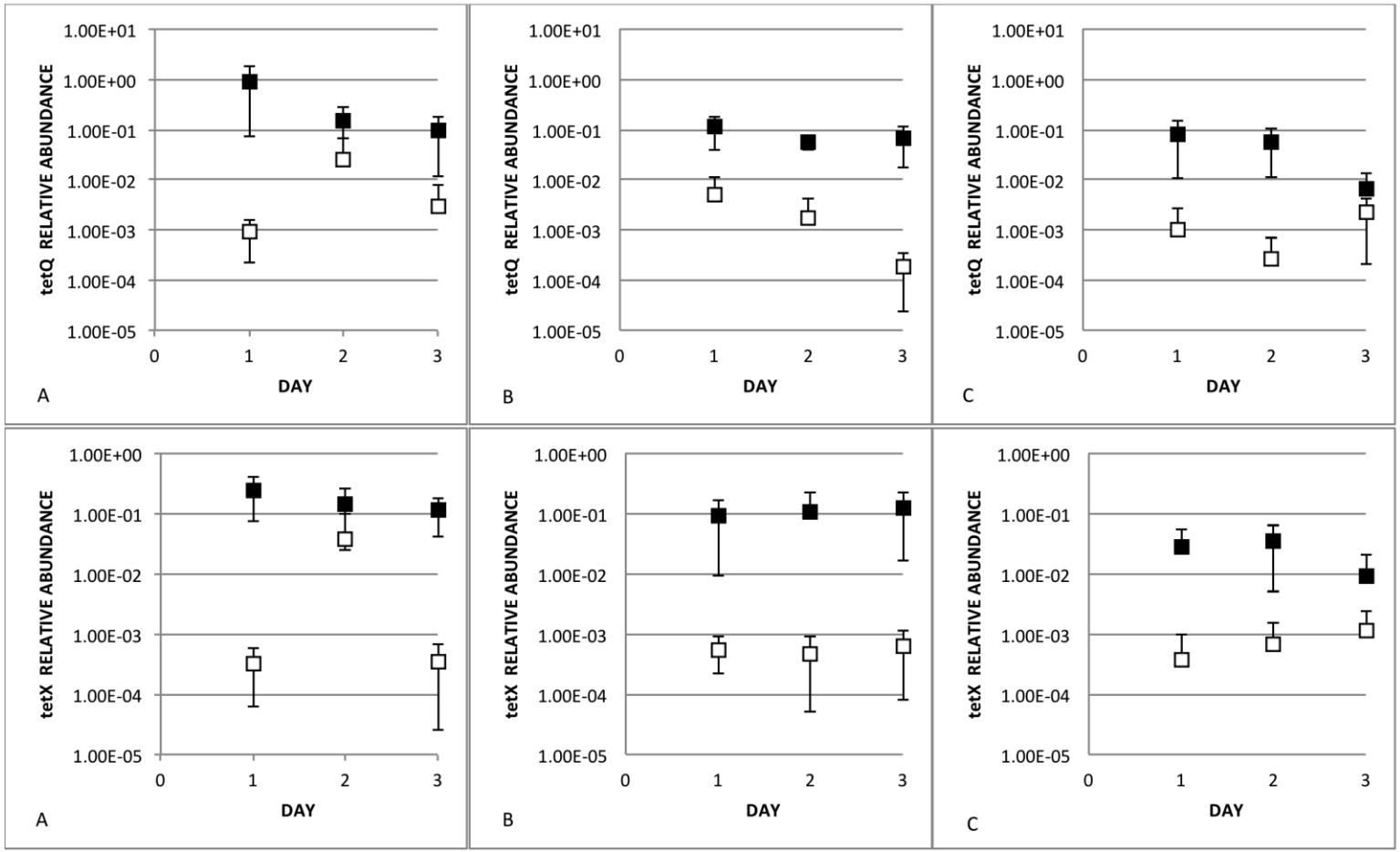

Figure 10: The relative abundances for CTC resistance genes tetQ (top) and tetX (bottom) in runoff for treated (black) and check (white) plots using three manure application methods broadcast (A), incorporated (B), and injected (C) over three days of rainfall simulation. Error bars represent the standard deviations from replicate plots.

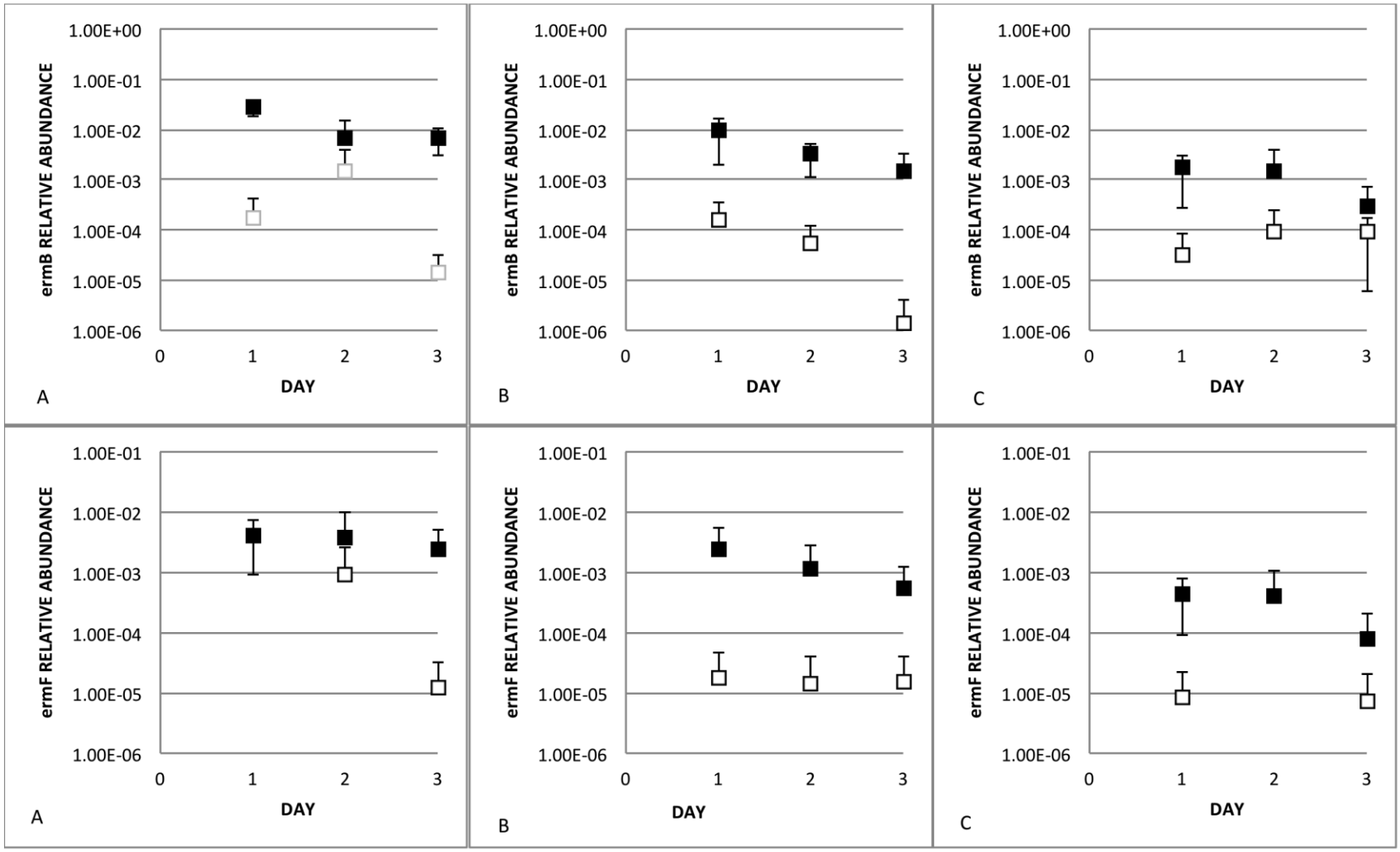

Figure 11. The relative abundances for TYL resistance genes ermB (top) and ermF (bottom) in runoff for treated (black) and check (white) plots using three manure application methods broadcast (A), incorporated (B), and injected (C) over three days of rainfall simulation. Error bars represent the standard deviations from replicate plots.

Table 3. Antimicrobial and AMR genes as affected by manure application method, type of antimicrobial in manure, and rainfall simulation run.

	CTC (ug/L)	*tetQ* (rel abundance)	*tetX* (rel abundance)	TYL (ug/L)	*ermB* (rel abundance)	*ermF* (rel abundance)
Application method[a]						
Broadcast	0.051	0.200	0.089a	0.070	0.0070	0.0026
Disc	0.006	0.040	0.053ab	0.064	0.0024	0.0007
Injected	0.008	0.025	0.012b	0.007	0.0060	0.0002
Antibiotic in manure						
Check	0.002	0.004	0.005b	0.050	0.0002b	0.0001b
Chlortetracycline	0.042	0.172	0.098a			
Tylosin				0.045	0.0064a	0.0022a
Run						
1	0.045	0.188a	0.060a	0.056	0.0064a	0.0016
2	0.016	0.049b	0.053ab	0.080	0.0021b	0.0011
3	0.005	0.028b	0.041b	0.006	0.0014b	0.0008
ANOVA			Pr > F			
Application	0.25	0.19	0.02	0.52	0.06	0.09
Antibiotic	0.10	0.05	0.02	0.91	0.01	0.02
Run	0.08	0.03	0.18	0.39	0.01	0.60
Application x Antibiotic	0.19	0.17	0.27	0.31	0.04	0.10
Application x Run	0.09	0.04	0.04	0.35	0.01	0.99
Antibiotic x Run	0.07	0.02	0.11	0.31	0.01	0.53
Application x Antibiotic x Run	0.09	0.03	0.01	0.49	0.01	0.94

[a] Values followed by different letters are significantly different at the 0.05 probability level based on the LSD test.

Objective 3) Evaluate the potential for transport of these antimicrobials and antimicrobial resistant bacteria at the watershed scale using current nutrient-based best management practices (BMPs), and identify practices to minimize transport of these constituents and their risk to the environment.

Table 4 provides the mass loadings in ug/m^2 for each antimicrobial determined from the rainfall simulation experiments.

Table 4. Calculated mass loading of antimicrobials in runoff after land application of swine manure slurry.

CTC Plots	Run	CTC (ug/m^2) Ave ± SD	TYL (ug/m^2) Ave ± SD	BAC (ug/m^2) Ave ± SD
Broadcast	1	4.54±4.95	0.55±0.41	0.00±0.00
	2	1.11±1.49	0.29±0.21	0.00±0.00
	3	0.15±0.08	0.18±0.13	0.00±0.00
Incorporated	1	0.23±0.34	2.07±3.20	0.00±0.00
	2	0.06±0.06	1.94±3.35	0.00±0.00
	3	0.14±0.12	0.37±0.59	0.00±0.00
Injected	1	0.30±0.41	0.05±0.08	0.00±0.00
	2	0.27±0.41	0.07±0.06	0.00±0.00
	3	0.09±0.10	0.02±0.03	0.02±0.03

TYL Plots	Run	CTC (ug/m^2) Ave ± SD	TYL (ug/m^2) Ave ± SD	BAC (ug/m^2) Ave ± SD
Broadcast	1	0.06±0.11	280.51±370.07	0.14±0.24
	2	0.04±0.07	89.02±121.73	0.01±0.02
	3	0.03±0.06	56.37±59.09	0.08±0.13
Incorporated	1	0.03±0.05	33.56±38.89	0.00±0.00
	2	0.00±0.00	11.55±2.85	0.00±0.00
	3	0.00±0.00	4.50±0.98	0.00±0.00
Injected	1	0.00±0.00	5.23±3.94	0.00±0.00
	2	0.00±0.00	4.59±3.19	0.00±0.00
	3	0.00±0.00	1.73±2.17	0.00±0.00

BAC Plots	Run	CTC (ug/m^2) Ave ± SD	TYL (ug/m^2) Ave ± SD	BAC (ug/m^2) Ave ± SD
Broadcast	1	0.00±0.00	61.81±29.37	0.00±0.00
	2	0.00±0.00	25.60±11.80	0.00±0.00
	3	0.02±0.03	15.33±11.07	0.00±0.00
Incorporated	1	0.00±0.00	52.57±51.40	0.00±0.00
	2	0.00±0.00	16.37±12.80	0.03±0.05
	3	0.00±0.00	5.75±3.60	0.02±0.03
Injected	1	0.00±0.00	24.34±38.32	0.00±0.00
	2	0.00±0.00	3.44±3.18	0.01±0.01
	3	0.00±0.00	3.35±4.24	0.01±0.03

Discussion

Objective 1: All three antimicrobials were detected in freshly-collected swine manure slurry. Although animals were only administered a single antibiotic at a time, all three antimicrobials were detected in each of the manures. In all cases, the antimicrobial that was administered was detected in manure at the highest concentrations (Table 2). Detection of the non-administered antimicrobials is likely due to the method of manure collection as outlined in the methods and materials section. Although the manure collection system was isolated as much as possible, the likely source of the non-administered antimicrobials may be that manure containing these antimicrobials was present in the common collection system at the time of manure collection. For animals administered CTC, the average CTC concentration detected in manure was 3324 ng/g. TYL and BAC were also detected in this manure at average concentrations of 102 ng/g and 16 ng/g, respectively. Manure generated from animals administered TYL was found to contain TYL at an average concentration of 288 ng/g. The concentration of CTC in this manure was 2 ng/g and the concentration of bacitracin was 124 ng/g. The manure from animals administered bacitracin contained BAC at a concentration of 777 ng/g, with 172 ng/g CTC and 6.6 ng/g TYL.

Antimicrobial concentrations were found to decrease in laboratory storage experiments. In these laboratory storage experiments, manure was collected directly from the facility floor, and the flushing system described in the materials and methods was not used. Due to the differences in manure collection, the initial antimicrobial concentration in the manure was much higher. For animals administered CTC, the initial CTC concentration in the manure used in storage experiments was 40 mg/g (40,000 ng/g). Over the 40-day storage experiment, the CTC concentration in the manure decreased to <5000 ng/g, which was approximately 10% of the starting concentration (Figure 2). Using a first-order decay equation, a half-life for CTC in manure was calculated to be approximately 10 days.

Information on degradation of TYL and BAC in laboratory manure storage experiments is available for the first 24 hours of storage. TYL concentrations in manure decreased from an initial concentration of 800 ng/g to 300 ng/g by 24 hours (Figure 4). The initial BAC concentration in the storage experiments was 250 ng/g, and the concentration detected at 24 hours was 250 ng/g, indicating that BAC was not significantly degraded over a 24-hour period (Figure 6). Samples for TYL and BAC degradation in our laboratory storage reactors through day 40 are currently being processed.

As pointed in a recent review paper, there is very limited information in the literature on how storage may affect the quantity of antibiotic resistant bacteria or AMR genes in livestock manure (Chee-Sanford *et al.* 2009). In our experiment, the four AMR genes monitored generally followed a decreasing trend over the 40-day experimental period. The four AMR genes tested in this study followed a general decreasing trend, although some variations were observed (Figures 3 and 5). The phenomenon of different AMR genes exhibiting different behaviors is not uncommon in manure/soil systems. In a study, Alexander and co-worker used qPCR to monitor the abundance of AMR genes in cattle fecal deposits (Alexander *et al.* 2011). Their results showed that some AMR genes (*tetB*, *tetC*, *sul1*, *sul2*, and *ermA*) increased first and then declined, while other AMR genes (*tetM* and *tetW*) gradually decreased over 175 days.

Oxygen may affect the fate of AMR genes during swine manure storage. Diehl and LaPara tested the effects of oxygen and temperature on the degradation of AMR genes in the biosolids of a wastewater treatment plant (Diehl and LaPara 2010). They observed decease in AMR genes in anaerobic digesters under high temperature, while detecting no evident decrease in aerobic digesters regardless the temperatures tested. Another study reported increase in AMR genes during manure storage under aerobic environment (Heuer *et al.* 2008). In that study, both sulfonamide resistance genes tested *sul1* and *sul2* increased exponentially during the first 60 days of storage. In this study, we demonstrated that anaerobic storage could lower the relative abundance of AMR genes by about one order of magnitude over a 40-day period.

One interesting observation is that the sharp decreases in CTC and TYL concentrations during manure storage were not observed for AMR gene. Instead, the decreases in AMR genes were more moderate. There could be multiple explanations for the difference in the chemical and microbial profiles. In this study, the metabolites of antimicrobials were not analyzed. Because some metabolites may still have antimicrobial effects, the total selective pressure could remain high during the storage experiment. Also, because qPCR analyses could not differentiate DNA from live cells from that from dead cells, the AMR gene profiles do not necessarily represent the live populations in the manure.

Objective 2:

Rainfall events would increase the relative abundance of AMR genes in deep soils. In Figures 7 and 8, the relative abundance of AMR genes in deep soil increased after rainfall events. This is likely due to the infiltration of rainfall water which transported AMR genes from top soil to bottom soil. One recent study reported increased levels of tetracycline resistance in groundwater near livestock lagoons (Koike *et al.* 2007), suggesting antibiotic resistant bacteria may transport vertically in soil. Because each of our three simulated rainfall events only lasted one hour, the amount of resistant bacteria transported downwards was likely not substantial.

For the top soils, most of the AMR genes remained at the same levels after rainfall events. This was likely due to the relative short timeframe used in our experiments (i.e., three days). Different from other scenarios, the two tylosin resistance genes *ermB* and *ermF* decreased after the rainfall events in the incorporated plots (Figure 8, B). It is well documented that microorganisms could survive the transition from effluent pit or lagoon into soil (Boes *et al.* 2005). However, it is debatable whether antibiotic resistance can persist after the transition. One study showed that AMR genes *sul1* and *sul2* in soil amended with swine manure would decrease over 175-day period (Heuer *et al.* 2008). However, repeated application of manure containing antimicrobials could cause accumulation of the corresponding antimicrobial resistance genes in soil (Heuer *et al.* 2011).

It was expected that the AMR gene profiles in incorporated plots would be more homogenous than that in broadcast plots. However, neither the tetracycline resistance genes nor the tylosin resistance genes exhibited this trend. Because of the size of the experimental plot used in this study, it is speculated that the heterogeneity of the plot may account for the lack of the expected trend.

To our knowledge, this is the first study to investigate how different manure land application methods affect the ARM gene levels in agricultural runoff. There are studies comparing how different manure land application methods affect nutrient loss to agricultural runoffs. Some studies argue that compared to broadcasting, incorporation of manure into soil can reduce suspended phosphorus loss in runoffs (Daverede *et al.* 2004). In contrast, some people believe that incorporation could increase particulate loss and cause erosion (Withers *et al.* 2000). Our results showed despite of some difference in the runoff profiles, the ARM gene runoff profiles were similar for the three application methods. Because the experiment only lasted three days, it is likely that the indigenous soil microbes had not developed substantial levels of resistance and the resistant bacteria in the runoffs were largely from the original manure. The concentrations of antimicrobials in the runoff indicate that manure injection may reduce transport of antimicrobials compared with broadcast and incorporation. The transport of antimicrobials is also affected by the timing of rainfall, as lower antimicrobial concentrations were observed in runoff after the third runoff event.

As observed in Table 3, ANOVA analysis indicated that the application method was significant for CTC, *tetQ,* TYL, *ermB,* and *ermF* in runoff. The observed differences between the manure applied (treated) and control (check) plots was also found to be significant for the antibiotics (CTC and TYL), but not for the resistance genes, with the exception of *tetQ*. The run, or timing of rainfall application (1, 2, or 3 days after manure application) was determined to be significant for CTC, *tetX*, TYL, and *ermF.* The two way interaction of antibiotic x application was significant for all antimicrobials and AMR genes except *ermB.* The application x run was only significant for CTC, TYL, and *ermF.* The two-way interaction of antibiotic x run was significant for CTC, *tetX*, TYL and *ermF.* The three way interaction (application x antibiotic x run) was significant for CTC, TYL and *ermF.* In ANOVA analysis, significant differences were considered at the 0.05 probability level.

Objective 3:

Using information collected on runoff concentrations and total runoff volumes collected, we determined mass loading rates for each antibiotic as a function of manure application method and timing of rainfall. The highest mass loading rates were associated with broadcast manure application, and loading rates decreased with subsequent rainfall events after manure application, with the lowest loading rates after the third rainfall simulation. The largest mass loading rates were determined for TYL under the broadcast manure application method, followed by CTC under the broadcast manure application method. Essentially no bacitracin was exported from the field plots under any manure application strategy. These loading rates could be used in a watershed model to antimicrobial export in runoff after land application of swine manure. To our knowledge, this type of information has not previously been determined.

Other results: A study investigating decay of odorous VOCs after swine manure application was conducted on the rainfall simulation plots funded by this study. A publication detailing the results of this study was published in 2012: Parker, D.B.; Gilley, J.; Woodbury, B; Kim, K.-H.; Galvine, G.; Bartelt-Hunt, S.L.; Li, X.; Snow, D.D. (2012). Odorous VOC emission decay following land application of swine manure slurry. Atmospheric Environment, DOI 10.1016/j.atmosenv.2012.01.001.

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