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Influence of the Method and Timing of the Land Application of Manure on the Fate and Transport of Manure Constituents in Soil

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RESEARCH REPORT
ENVIRONMENT

Title: Influence of the Method and Timing of the Land Application of Manure on the Fate and Transport of Manure Constituents in Soil, N**PB # 15-045**

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Industry Summary
Through land application multiple manure constituents are introduced to soil. These constituents include nutrients, organic matter, and contaminants such as antimicrobials and antimicrobial resistance genes (AMR genes). Many factors could affect the persistence and retention of these constituents in soil. Some manure constituents are more prone to degradation in soil than other constituents. Some constituents tend to accumulate in soil, while others tend to be removed from soil by runoff. The method of land application and the timing of land application in relation to rainfall events could also affect the persistence and retention of these constituents in soil. As a supplementary project to NPB 14-121, this project has two specific objectives: (1) determine how the method of manure land application affects the persistence and retention of multiple manure constituents, including nutrients, antimicrobials, and AMR genes, in soil; and (2) determine how the timing of manure land application in relation to rainfall events affects the persistence and retention of multiple manure constituents in soil. Swine manure slurry was land applied to plots in the field in the summer of 2014, and rainfall simulation tests were conducted 1 day (referred to as 0 week in this report), 1 week, 2 weeks, and 3 weeks after manure application.

Manure land application methods had noticeable impacts on the concentrations of nitrate and antimicrobials in soil, but not on the soil concentrations of ammonium, phosphorus, or AMR genes. Broadcast resulted in higher nitrate and antimicrobial concentrations in top soils than did injection. For plots receiving swine manure slurry through broadcast, the length of the time period between manure application and rainfall events had no impacts on nitrate, water soluble phosphorus, or Bray 1 phosphorus concentrations in top soils, while longer time period led to lower ammonium in top soils. Similarly, the length of the time period had no significant impacts on chlortetracycline or tiamulin concentration in top soil, while longer time period resulted in lower lincomycin concentrations in soil. Finally, three of the four AMR genes tested were not affected by the length of the time period between manure application and rainfall events, while the concentration of one AMR gene *tet*(Q) in top soils increased towards the end of the three week testing period.

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Keywords
Manure constituents, land application, rainfall simulation, soil, nutrients, antimicrobials, and antimicrobial resistance genes (AMR genes).

Scientific Abstract
Swine manure has been used as a soil amendment for crop production because it can provide nutrients, increase soil productivity, improve water infiltration, and reduce the potential for soil erosion. It is important to understand how different land application strategies may affect the fate and transport of various manure constituents in the environment. The objective of this project was to determine how the method and timing of swine manure application may impact the levels of multiple manure constituents in soil. The manure constituents included nutrients, antimicrobials, and antimicrobial resistance genes (AMR genes).

A series of plot-scale field experiments were performed. In these experiments, swine manure slurry from a commercial farm was either broadcast or injected into test plots by a commercial manure applicator. A set of three 30-min simulated rainfall events, 24 hour apart, were initiated on the manure amended plots 1 day (and referred as 0 week thereafter), 1 week, 2 weeks, or 3 weeks after the manure application. Soil cores were collected before and after the rainfall simulation tests and analyzed for nutrients using standard methods, for antimicrobials using liquid chromatography tandem mass spectroscopy, and for AMR genes using quantitative polymerase chain reactions (qPCR).

Broadcast resulted in higher nitrate concentrations in soil than did injection. In terms of application timing, three of the four nutrient compounds tested (i.e., nitrate, water soluble phosphorus, and Bray 1 phosphorus) did not show significant decrease in broadcast plots during the three weeks following manure application. Ammonium concentration dropped significantly in the third week. Simulated rainfall events lowered the concentrations of the nitrogen species in top soils significantly, and showed minor impacts on the phosphorus species in the top soils.

For top soils the antimicrobial concentrations in broadcast plots were higher than those in injection plots. For broadcast plots, chlortetracycline was detected in both the top and bottom of the soil cores before and after rainfall events. Lincomycin and tiamulin were only detected in the top of the soil cores. The antimicrobial concentrations in top soils decreased with time after manure application, although the trend is significant only for lincomycin.

AMR gene levels in top soil were not affected by land application methods. For broadcast plots, the length of the time between manure application and rainfall events had no significant effects on the abundance of three of the four AMR genes tested. The exception was *tet*(Q), which increased in Week 3. The abundance of the AMR genes in soil were not affected by the simulated rainfall events. The only treatment factor that showed significant effect was soil position: the AMR genes were more abundant in the top portion than the bottom portion of the soil cores collected.

Introduction
Pork production is an important agricultural enterprise in the U.S. with the greatest concentration of swine operations occurring in the Midwest and North Carolina. Swine manure provides a valuable source of nutrients, including nitrogen and phosphorus, and has been historically used as a soil amendment for crop production. A sustained elevation in inorganic fertilizer costs will likely dictate continued integration of swine manure into crop fertility programs as a valuable nutrient component.

There has been a steady increase in the numbers of livestock being raised in confinement housing over the past thirty years. Many of these systems are defined by regulatory standards as concentrated animal feeding

operations (CAFOs) based upon a site capacity greater than 1000 "animal units" (e.g., 2500 swine weighing 55 lb or more). The benefits of CAFOs to swine producers include economies of scale and enhanced production quality controls. Current swine industry practice is to house animals in confinement facilities with capture and storage of liquid or semi-liquid manure in pits or lagoons. At CAFOs, antimicrobials and other pharmaceuticals are often used for disease treatment, prophylaxis, and in some production settings, for growth promotion (Gaskins *et al.* 2002). The antimicrobials added in animal feed are often not completely absorbed in the animal gut; therefore, antimicrobial residues may be excreted in the animals' wastes. The antimicrobial residues in the animal gut and in animal manure may contribute to the emergence of antimicrobial resistance among commensal and pathogenic bacteria (Salyers *et al.* 2004).

The benefits of manure application to agricultural fields include addition of valuable nutrients and organic matter, increased soil productivity, improved water infiltration, and reduced soil erosion potential. However, the presence of antimicrobial compounds and AMR genes in manure introduces the potential for these constituents to enter the environment when manure is land applied. Recent studies have attempted to relate the environmental occurrence of antimicrobial compounds and associated AMR genes to the distribution of livestock production in watersheds. Antimicrobial residues and AMR genes, the genetic material that confers antimicrobial resistance to bacteria, have been documented in water bodies adjacent to CAFO sites, although the links between sources and occurrence have not yet been fully established (Koike *et al.* 2007; Dolliver and Gupta 2008; Chee-Sanford *et al.* 2009).

The persistence of antimicrobials in the environment is compound specific. For example, the half-life of sulfamethazine in soil is 32 days (Stoob *et al.* 2007), while the half-lives of chlortetracycline and tiamulin could be seven to nine months under similar environmental conditions (Hamscher *et al.* 2002; Schlusener and Bester 2006). Soil temperature also has a significant impact on the persistence of antimicrobials after manure application. For example, lincomycin is relatively persistent in winter but only has a half-life of about 18 days when soil temperatures increase in spring (Kuchta *et al.* 2009).

As with nutrients, the fate and transport of antimicrobials and AMR gene in soil and runoff can be quantified on test-plots with simulated rainfall events. Information collected on test-plots provides insight into the important transport mechanisms occurring at the field scale. Many factors may influence the fate and transport of nutrients, antimicrobials, and AMR genes from land applied manures, including manure management (application method and timing) and source management (applied versus soil nutrients).

Significant gaps remain regarding our knowledge about the fate and transport of manure constituents in the environment. The research discussed in this report was designed to provide key information on the quantity of nutrients, antimicrobials, and AMR genes in soil following land application of swine manure slurry. This information is critical in developing on-farm manure management practices that will reduce the potential for the accumulation of these manure constituents in soil after land application.

Objectives

The objectives of this project were to (1) determine how the method of manure land application affects the persistence and retention of multiple manure constituents, including nutrients, antimicrobials, and AMR genes, in soil; and (2) determine how the timing of manure land application in relation to rainfall events affects the persistence and retention of multiple manure constituents in soil.

Materials and Methods

Study Site Characteristics

This field study was conducted from June through August 2014 at the University of Nebraska Rogers Memorial Farm, located 18 km east of Lincoln, NE. The Aksarben silty clay loam soil at the site (fine, smectitic, mesic Typic Argiudoll) contained 16% sand, 48% silt, 36% clay, 4.0% organic matter, 1.8% total carbon, and had a

mean slope of 9.8% (Kettler *et al.* 2001). This soil developed in loess deposits under prairie vegetation and is considered a bench mark soil within the Corn Belt. The study site has been cropped using a no-till management system under a corn (*Zea Mays L.*), grain sorghum (*Sorghum bicolor* (L.) Moench), soybean (*Glycine max* (L.) Merr.), and winter wheat (*Triticum aestivum* L. cv. Pastiche) rotation. The site where field tests were conducted had not had a manure application since 1966. Total cumulative precipitation during the study period was 0.19 inches.

Slurry Collection and Plot Preparation

Swine slurry was collected from a deep pit of a commercial 8,000-head wean-to-finish swine operation in north central Nebraska just prior to field application. Samples of the swine slurry were collected at the time of application for solids and nutrient analyses, which were performed at a commercial laboratory. Antimicrobial administration information was obtained from the facility operator. A commercial manure applicator was hired to broadcast and inject slurry at the experimental site. The slurry was applied at a rate of approximately 46,800 L/ha (5,000 gal/ac). For broadcasting, the applicator was lifted above the soil while maintaining a steady speed and flow rate to ensure uniform slurry distribution. For injection, a v-shaped chisel (horizontal sweep) implement was used on an 8-row applicator for manure placement (Figure 1).

Figure 1. Injection of swine manure slurry in the field.

Thirty-two plots (0.75-m wide × 2-m long each) were established along the slope (2 application methods × 4 application timing relative to rainfall × 4 replicate plots per treatment combination, Figure 2). Eight plots were examined during each of the weekly testing periods. Each plot was examined only once throughout the course of the study.

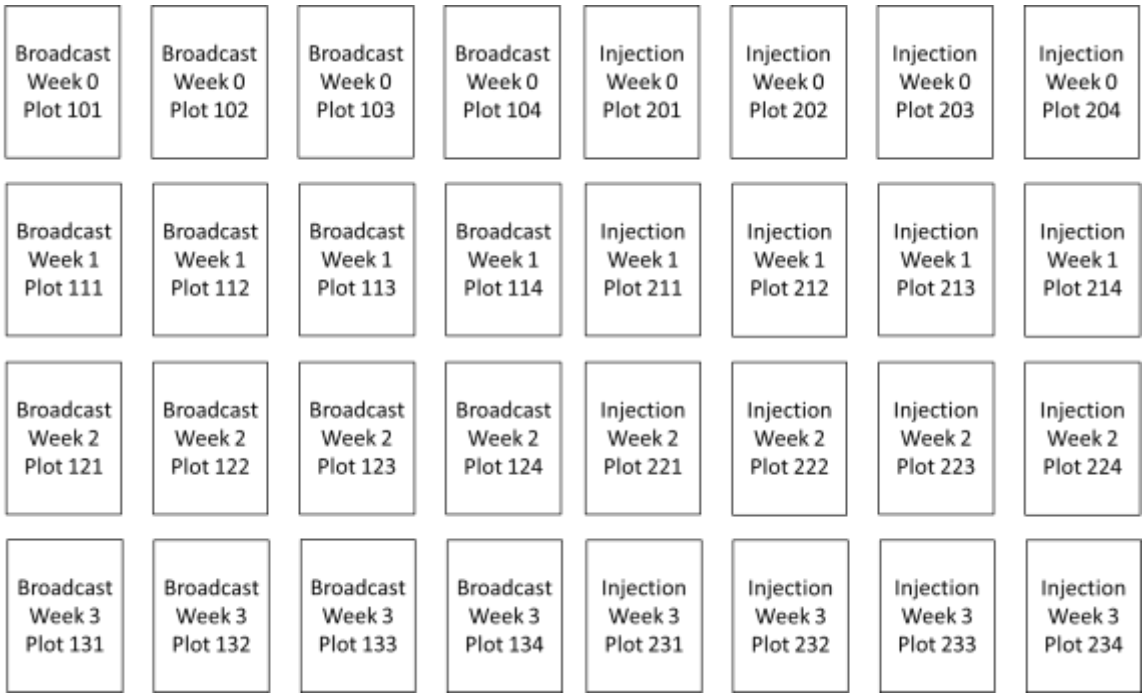


Figure 2. Schematic showing the plot layout and rainfall simulation period for the broadcast and injected plots. The eight plots in each row were subject to rainfall simulation test each week. "Week 0" means that rainfall simulation occurred 1 day after the land application of swine manure slurry. "Week 1" means that rainfall simulation occurred 1 week after land application, and so forth.

Rainfall Simulation Procedures

The rainfall simulation procedures used in the study were adopted from the National Phosphorous Research Project (Sharpley and Kleinman 2003). Well water was applied to paired plots at an intensity of approximately 70 mm/h (2.75 in/h) for 30 minutes using a portable rainfall simulator, based on the design by Humphry *et al*. (2002). Two additional rainfall simulation tests were conducted on the same plots at approximately 24-hour intervals. Precipitation application rates were measured with two rain gauges placed along the outer edge of the plots and one placed in the center between the plots.

Field rainfall simulations were initiated 1 day (and referred to 0 week), 1 week, 2 weeks, and 3 weeks following slurry application. Water used in the study was obtained from an on-site irrigation well. Reported nutrient contents represent the difference between nutrient measurements in the runoff and those in the irrigation water. Measured mean concentrations of dissolved phosphorus (DP), total phosphorus (TP), NO_3-N, NH_4-N, and total nitrogen (TN) in the irrigation water were 0.19, 0.19, 15.3, 0.04, and 15.3 mg/L, respectively. The irrigation water had a mean electrical conductivity (EC) of 0.75 dS m^{-1} and a pH of 7.4.

Plot borders channeled runoff into a sheet metal lip that emptied into a trough that extended along the bottom edge of the plots. The trough then diverted the runoff into small plastic buckets, where it was transferred by a sump pump into larger plastic buckets. Following each rainfall simulation event, the containers were weighed to determine the mass of the runoff.

Soil Core Collection

Soil cores were obtained from 4 plots just before the application of slurry to identify the background levels of antimicrobials and AMR genes. All thirty two plots receiving swine manure slurry on Day 0, half through broadcast and half through injection application. Each week soil cores were collected from 8 plots before rainfall simulation tests (4 broadcast plots and 4 injection plots, Table 1, lightly shaded cells), and later were collected again from the same 8 plots after three simulated rainfall events (Table 1, heavily shaded cells). Plots that were amended using the same land application method and sampled at the same time served as replicates (n=4).

Table 1. Soil core sampling scheme. The numbers in the table are the numbers of soil cores collected.

	Designed Application Timing			
	0 week	**1 week**	**2 weeks**	**3 weeks**
Day 0	4			
Day 0	Manure Application			
Day 1	8			
Day 1	Rain			
Day 2	Rain			
Day 3	Rain			
Day 3	8			
Day 8		8		
Day 8		Rain		
Day 9		Rain		
Day 10		Rain		
Day 10		8		
Day 15			8	
Day 15			Rain	
Day 16			Rain	
Day 17			Rain	
Day 17			8	
Day 22				8
Day 22				Rain
Day 23				Rain
Day 24				Rain
Day 24				8

To collect soil core samples, a 5 cm wide × 30 cm long core sampler manufactured by AMS Inc. was used to collect soil cores enclosed in plastic liners (Figure 3). For injection plot, the soil cores were collected from the space between the injection slots. The plastic liners were placed in coolers containing ice packs soon after collection. The soil cores were then transferred to the University of Nebraska-Lincoln and stored in a -20°C freezer until processing.

Figure 3. A soil core sampler and a plastic liner.

During processing, frozen soil cores were pushed out of the plastic liners after thawing and then divided into three segments: top (0-3 inch from soil surface), middle (3-6 inch), and bottom (6-9 inch). Each soil segment was then homogenized in a Ziploc bog by hand. Homogenized soil samples were placed in sterile 50-mL centrifuge tubes for AMR gene analyses and in 250-mL amber glass jars for antimicrobial analyses. Remaining soil samples in Ziploc bags were used for nutrient analyses.

Soil Moisture Content

The moisture contents in the soil samples were determined using the gravimetric method (APHA-AWWA-WEF 2005).

Nutrient Analyses

The soil subsamples allocated for nutrient analyses were air dried, ground, and analyzed for water soluble phosphorus, Bray and Kurtz No.1 phosphorus (BKP), NO_3^--N, NH_4^+-N, EC and pH. The Murphy and Riley method (1962), which involved shaking 2 g of soil for 5 min with 20 mL of deionized water, was used to measure water soluble phosphorus. As an index of phosphorus availability, the BKP procedure provides a relative estimate of phosphorus concentration in the soil solution that limits the growth of plants (Bray and Kurtz, 1945). The concentrations of soil NO_3^--N and NH_4^+-N, which were extracted using a 2 molar KCl solution, were measured with a flow injection analyzer using spectrophotometry (SEALAutoAnalyzer3 from SEAL Analytical Ltd, Southampton, UK).

Antimicrobial Analyses

Antimicrobial were analyzed in manure slurry samples. Target compounds, chosen based on the usage data from the facility operator and expected persistence or chemical properties of the parent compounds, were chlortetracycline, lincomycin, tiamulin, penicillin G and its metabolite penicillic acid. Methods were validated for each matrix using standard protocols for recovery and method detection limits.

Soil samples (5-10 g) were mixed with sodium EDTA, 100 mM ammonium citrate (pH 6), and acetonitrile. The mixture was thoroughly mixed, spiked with 100 ng surrogate, and shaken for 30 minutes on a wrist action shaker. After centrifuging, the supernatant was transferred to a glass evaporation tube and the solids shaken and extracted for a second time with 100 mM ammonium acetate and acetonitrile. The supernatant was combined with the first aliquot, spiked with internal standards, and evaporated under nitrogen. The remaining aqueous extract was mixed with 80 mL of reagent water and extracted using the Oasis HLB cartridges.

All extracts were analyzed on a Waters Quattro Micro triple quadrupole mass spectrometer coupled with a Waters 2695 high pressure liquid chromatograph (HPLC) and an autosampler. Compounds were separated with a HyPurity C18 column (250 mm × 2.1 mm, 5 µm particle size) at 50°C using a gradient (0.2 mL/min) that consisted of A) 24:16:58:2 acetonitrile:methanol:water:formic acid, B) 97:3 aqueous ammonium citrate (1 mM, pH4): methanol, and C) 97:3 methanol:aqueous ammonium citrate (1 mM, pH4). The gradient was initialized at 95% B / 5% C, then ramped to 100% A for 2.0 min and switched back to 40% B until 4 min, then held at 5% B until 17 min. Column was rinsed with 5% formic acid in acetonitrile until 22 min, then set at 95% B / 5% C to equilibrate the column. Total run time was 30 min. Analytes were detected using multiple reaction monitoring (MRM) mode with positive electrospray ionization (ESI). The most intense MS/MS transitions were monitored for each analyte (Table 2) and linear calibration curves were generated for all analytes and surrogates with R^2 values > 0.995. Method detection limits were determined by 8-10 replicates of a low level fortified blank (Table 2).

Table 2. The MRM transition, method detection limit, and recovery rate of antimicrobial compounds, surrogate, and internal standards.

Compound	MRM Transition (m/z)	Method Detection Limits (µg/L)	Recovery (%)
Analytes			
Chlortetracycline	478.90>444.00	0.005	87.5
Lincomycin	407>126	0.008	34.0
Penicillin G	335>160	0.010	58.1
Penicillic acid	171.2>125.2	0.090	58.1
Tiamulin	493.9>191.9	0.014	49.0
Surrogate			
Oleandomycin	688.35->544.10	--	
Internal Standard			
Roxythromycin	837.55->679.50	--	
Doxycycline	445.05->428.05	--	
Penicillin V	351>160	--	

Antimicrobial Resistance Gene Analyses

The DNA in soil samples was extracted using the MoBio PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) according to the manual. DNA extracts were quantified using a NanoDrop spectrometer (Thermo Scientific, Wilmington, DE). All DNA extracts were purified using the ZYMO OneStep™ PCR Inhibitor Removal Kit (Irvine, CA). Polymerase chain reaction (PCR) was used to determine what AMR genes occurred in the manure samples. Nine tetracycline resistance genes, *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(O), *tet*(Q), *tet*(W), and *tet*(X), and five lincomycin resistance genes, *erm*(A), *erm*(B), and *erm*(C), *erm*(F), and *erm*(G), were tested according to published protocols (Aminov *et al.* 2001; Ng *et al.* 2001; Koike *et al.* 2010). Gel electrophoresis results show that four AMR genes, *tet*(Q), *tet*(X), *erm*(A), and *erm*(B), occurred consistently in manure. While there four genes were further quantified using published quantitative PCR (qPCR) protocols (Aminov *et al.* 2001; Ghosh *et al.* 2009; Koike *et al.* 2010), the *erm*(B) primer set could not yield satisfactory results with the samples. The soil samples were re-examined using five qPCR primer sets for the aforementioned *erm* genes. *erm*(C) emerged as an AMR gene that also constantly occur in manure amended soils. As a result, *erm*(C) along with *tet*(Q), *tet*(X), and *erm*(A) were focused on in this study. In addition to AMR genes, the 16S rRNA gene in each sample was also quantified using qPCR (Suzuki *et al.* 2000).

I. Results and Discussion

The results and discussion are organized according to the type of manure constituents: nutrients, antimicrobials, and AMR genes. Within the section for each constituent, the effects of the method and timing of manure application were described separately. It is worth pointing out that some caution is needed in interpreting the results from the injection plots. As mentioned in the Materials and Methods section, soil cores were collected from the space between the injection slots. This sampling design was used because had soil cores been collected right on the injection slots, the cores would be primarily composed of manure materials. Measurements of manure constituents from such cores would overestimate the levels of manure constituents of the plots. However, core samples collected from the space between injection slots, on the other hand, could potentially underestimate the actual concentrations in the field. This sampling challenge was a result of the heterogeneity of the injection plots.

Nutrients

Soil nitrate concentrations for plots receiving manure through broadcast and injection are reported in Figures 4A and 5A, respectively. For top soils, the nitrate concentration in broadcast plots ranged between 45 and 51 µg/g in the three weeks following the land application of swine manure slurry (Figure 4A), while the nitrate concentration in injection plots fell in a similar range (between 43 and 60 µg/g, Figure 5A). Under both manure

application methods, the nitrate concentration in top soil decreased substantially after simulated rainfalls, likely due to surface runoff and infiltration. Compared to the broadcast plots, the injection plot retained less nitrate after rainfalls. For both application methods, the nitrate concentration in the bottom soil were between 10 and 20 µg/g and was hardly impacted by rainfall events (Figures 4A and 5A).

During the 3-week period after manure application, irrespective of the manure land application method, the nitrate concentrations in top soils remained steady both before and after simulated rainfalls (Figures 4A and 5A). This finding suggests that the timing of rainfall in relation to manure application has little impacts on the fate of nitrate in top soil when rainfalls occur within three weeks of manure application. For samples from the bottom of the soil cores, the nitrate concentrations before simulated rainfall events ranged between 12 and 21 µg/g in broadcast plots and between 10 and 15 µg/g in injection plots. Due to the large standard deviations, the differences among the three time points were not significant. Similarly, the timing of rainfalls in relation to manure application did not appear to affect the trend of nitrate concentration in the bottom segments of the soil cores.

Soil ammonium concentrations for plots receiving manure slurry through broadcast and injection are reported in Figure 4B and Figure 5B, respectively. For top soils, the ammonium concentration in broadcast plots was at around 40 µg/g in the first two weeks after manure application. The ammonium concentration then dropped to about 8 µg/g in Week 3. The ammonium concentration in injection plots was exceptionally high in Week 1 (i.e., 299.60±32.81 mg/L based on 4 replicates, Figure 5B) and then continued to drop. These abnormally high ammonium concentrations were likely result from accidental placing of soil samplers too close to the injection slots. After rainfall events, for both land application methods, the ammonium concentration in rainfall events dropped to below 10 mg/L.

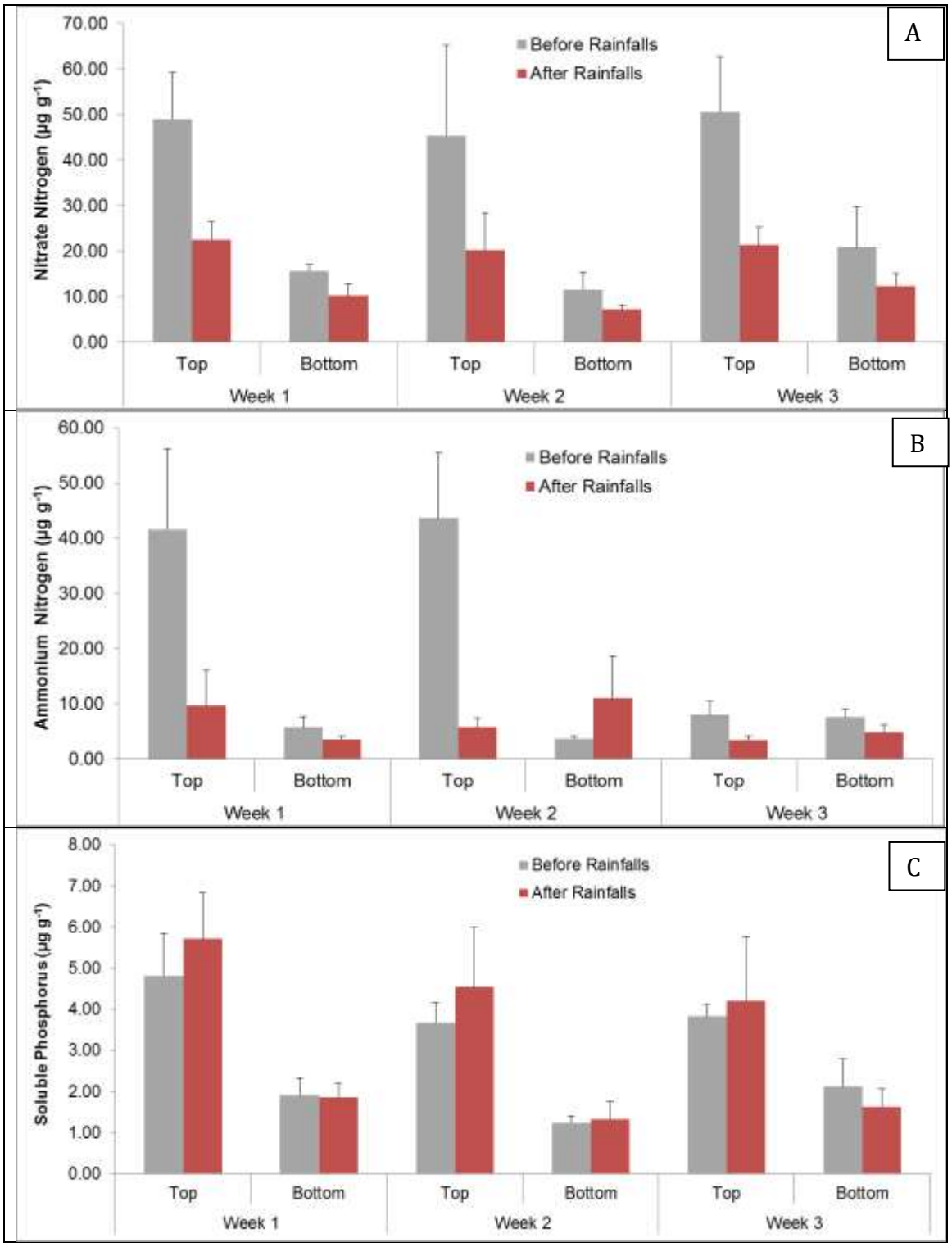
The gradual deceases of ammonium concentration in top soils, from both land application methods, indicate that ammonium nitrogen was converted to other nitrogen species in top soils in the weeks following manure application. Nitrification, the process in which ammonium is oxidized to nitrite and/or nitrate, could be a potential mechanism responsible for the conversion. Similar to nitrate, ammonium concentration in top soils decreased sharply after the simulated rainfall events, likely due to loss to surface runoff and infiltration. For the bottom segment of the soil cores, neither the timing of rainfalls in relation to manure application nor the rainfall event themselves appeared to have significant impacts on ammonium concentrations.

Water soluble phosphorus values are reported for broadcast plots in Figure 4C and for injection plots in Figure 5C. For top soils, the concentrations of soluble phosphorus were similar for both land application methods. One exception was the pre-rainfall concentration in the injection plots one week after manure application. This distinctively high concentration was likely due to the same reason as the distinctively high ammonium concentration in Figure 5B: the soil cores were probably placed too close to the injection slots. Excluding this exception, the concentration of soluble phosphorus in soil were not significantly affected by rainfall events (Figures 4C and 5C). For bottom soils, the soluble phosphorus concentration were ranged between 1 and 2 µg/g and were not affected by rainfall events.

For top soils, the water soluble phosphorus concentration slowly decreased in broadcast plots over the course of the three weeks following manure application. No such tend was observed in injection plots, partially due to the abnormally high soluble phosphorus concentration in Week 1. For the soil samples from the bottom of the soil cores, water soluble phosphorus remained at a steady level during the three weeks after manure application and simulated rainfall events appear to have little impact on water soluble phosphorus levels.

Bray 1 phosphorus concentrations were much higher than water soluble phosphorus measured for these soil samples, however, the trends of the Bray 1 phosphorus concentration were nearly identical to those of the

soluble phosphorus concentrations (Figures 4D and 5D). Therefore, conclusions drawn on the effects of manure application method and timing for soluble phosphorus also apply to Bray 1 phosphorus.


A
Nitrate Nitrogen (µg g⁻¹)
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Before Rainfalls
After Rainfalls
Top
Bottom
Week 1
Week 2
Week 3
B
Ammonium Nitrogen (µg g⁻¹)
60.00
50.00
40.00
30.00
20.00
10.00
0.00
C
Soluble Phosphorus (µg g⁻¹)
8.00
7.00
6.00
5.00
4.00
3.00
2.00
1.00
0.00

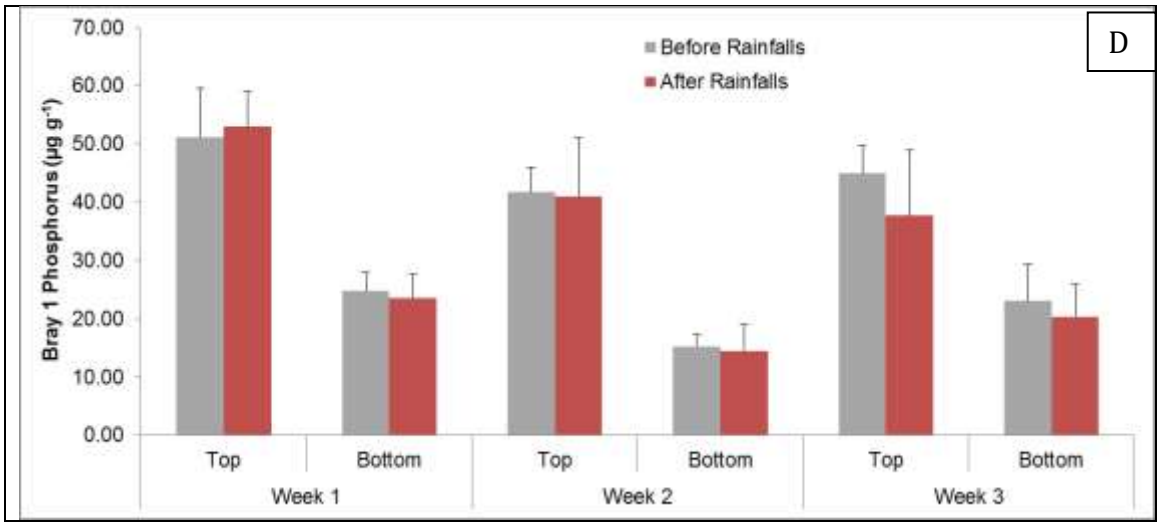
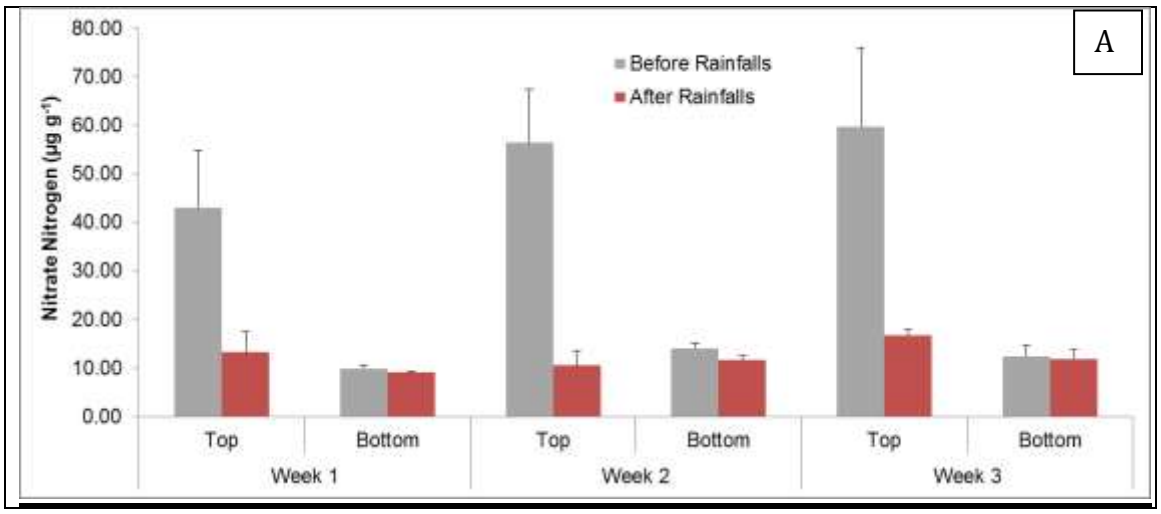


Figure 4. The levels of nitrate nitrogen (A), ammonium nitrogen (B), water soluble phosphorus (C), and Bray 1 phosphorus (D) in the top and the bottom portion of the soil cores. The soil cores were collected at different time points after the land application of swine manure slurry using broadcast. Error bars represent standard errors from four replicate plots.



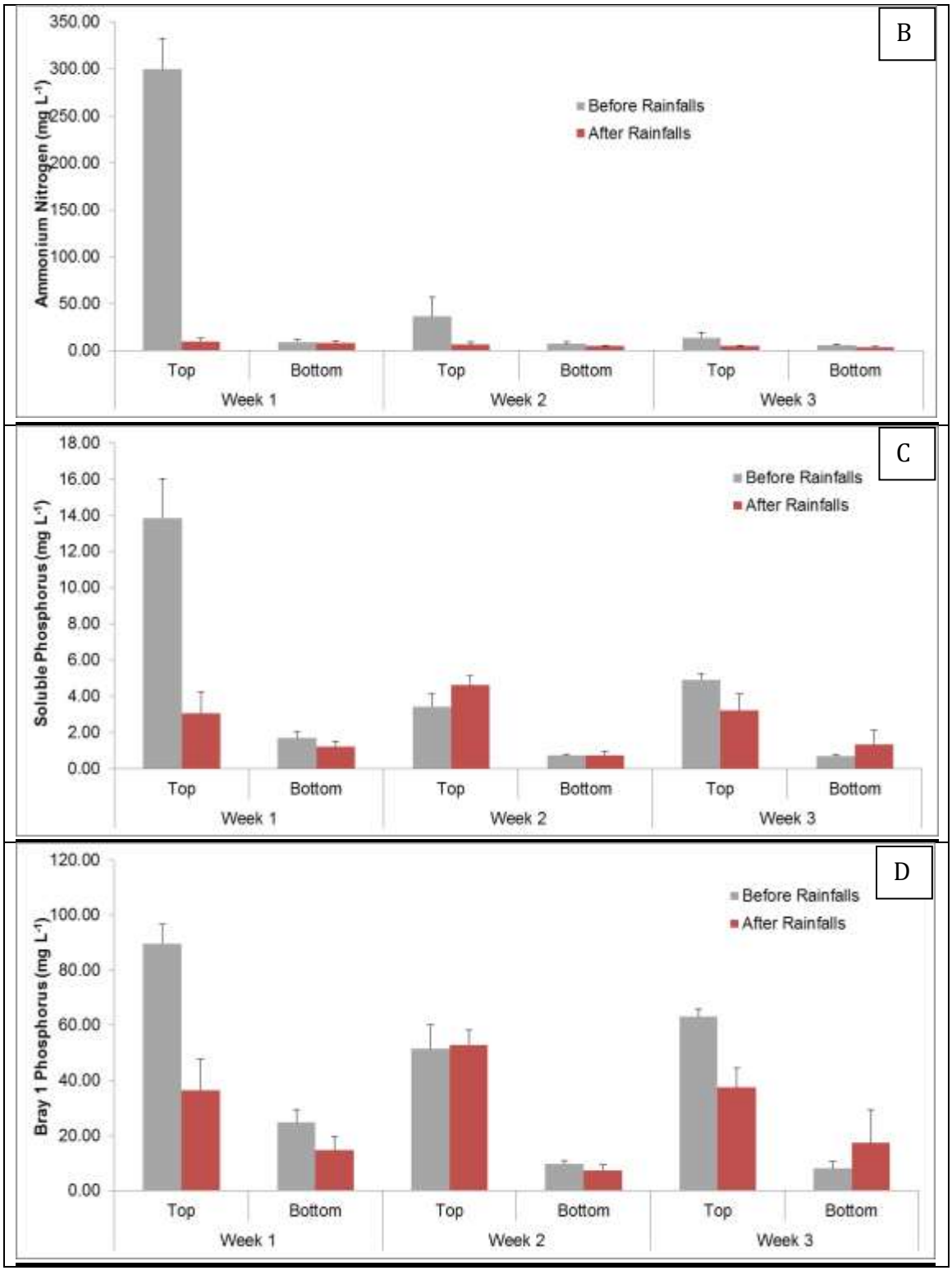


Figure 5. The levels of nitrate nitrogen (A), ammonium nitrogen (B), water soluble phosphorus (C), and Bray 1 phosphorus (D) in the top and the bottom portion of the soil cores. The soil cores were collected at different time points after the land application of

swine manure slurry using injection. Error bars represent standard errors from four replicate plots.

Antimicrobials

Three antimicrobials, chlortetracycline (CTC), lincomycin (LCM), and tiamulin (TML), were included in the project. All three antimicrobials were used in the swine facility where manure slurry was collected for the field tests. In addition to these three antimicrobials, penicillin was also used at the facility. However, due to fast degradation, penicillin was not detected in manure slurry samples. Hence, penicillin was not included in the soil analyses. The concentrations of chlortetracycline, lincomycin, and tiamulin are summarized in Table A1 of the Appendix for soil core samples collected in broadcast plots, while the antimicrobial concentrations in the top soils of the injection plots were reported in Table A2.

The means and the standard deviations of the antimicrobial concentrations calculated from replicate plots are summarized in Table 3 for broadcast plots. Chlortetracycline was the only antimicrobial that were consistently detected in both the top and the bottom segments of the soil cores. The concentrations of the bottom segments were much lower than those of the corresponding top segments. With a couple of exceptions, both lincomycin and tiamulin were only detected in the top segment of the soil cores. Even the few positive results of lincomycin for the bottom segment of the soil cores had relatively large standard deviations (Table 3).

The means and the standard deviations of the antimicrobial concentrations in top soils are summarized in Table 4 for injection plots. Similar to the antimicrobial concentrations in the broadcast plots, chlortetracycline had higher concentrations than lincomycin and tiamulin. Also, simulated rainfall events significantly reduce the antimicrobial concentrations in top soils. Comparing the top soils between the two land application methods (Tables 3 and 4), it is noticed that in general the concentrations of antimicrobials were lower in the injection plots than in the broadcast plots. One noticeable exception was the values for samples collected in Week 1. Similar phenomenon was observed for nutrient concentrations. This was likely because the soil samplers for Week 1 were accidentally placed too close to the injection slots.

The concentrations of all three antimicrobials in top soils exhibited overall decreasing trends during the three weeks following manure application in broadcast plots (Table 3). Chlortetracycline and tiamulin concentrations decreased by more than 50%, while the decrease of lincomycin concentration was even more substantial. The three rainfall simulations had significant impacts on the antimicrobial concentrations in top soils. Comparing the antimicrobial concentrations before and after simulated rainfall events for each week, it is clear that a significant amount of the antimicrobial compounds were lost from soil due to runoff and/or infiltration. For manure applied by injection (Table 4), although the effects of rainfall events on antimicrobial concentration in top soils are evident, no trend was observed for these antimicrobial concentrations as a function of the timing of the rainfalls.

It is worth noting that many of the mean values reported in Tables 3 and 4 are accompanied with large standard deviations. Large standard deviations are often observed in field testing at the scale conducted in this project. Because of the large standard deviations among replicate plots, some of the trends described in the previous paragraph cannot be considered statistically significant (See below).

Table 3. The means and the standard deviations (in parentheses) of antimicrobial concentrations in soil before and after simulated rainfall events for broadcast manure application. The upper and the lower halves of the table summarize the results from the top and the bottom of the soil cores, respectively. CTC: chlortetracycline; LCM: lincomycin; and TML: tiamulin.

	Before Rainfall Simulations (ng/g dry soil)			After Rainfall Simulations (ng/g dry soil)		
	CTC	LCM	TML	CTC	LCM	TML
Top of Soil Core						
Week 0	66.6 (46.4)	7.15 (5.3)	15.5 (11.7)	7.0 (10.5)	0.4 (0.2)	1.4 (1.4)
Week 1	36.0 (23.9)	6.1 (4.5)	5.4 (1.8)	24.3 (35.4)	2.0 (2.9)	6.8 (8.7)
Week 2	45.9 (74.4)	0.5 (0.6)	7.0 (8.7)	3.2 (3.7)	0.2 (0.2)	0.4 (0.2)
Week 3	29.1 (36.5)	0.8 (0.7)	3.1 (2.9)	4.9 (7.6)	0.1 (0.2)	0.4 (0.2)
Bottom of Soil Core						
Week 0	1.0 (1.2)	0.0	0.0	1.0 (1.9)	0.0	0.0
Week 1	1.3 (1.7)	0.1 (0.1)	0.0	0.9 (1.8)	0.0	0.0
Week 2	3.3 (6.5)	0.1 (0.1)	0.0	2.7 (5.4)	0.0	0.0
Week 3	3.8 (5.8)	0.0	0.0	0.1 (0.1)	0.0	0.0

Table 4. The means and the standard deviations (in parentheses) of antimicrobial concentrations in the top segment of the soil cores before and after simulated rainfall events for injection manure application. CTC: chlortetracycline; LCM: lincomycin; and TML: tiamulin.

	Before Rainfall Simulations (ng/g dry soil)			After Rainfall Simulations (ng/g dry soil)		
	CTC	LCM	TML	CTC	LCM	TML
Week 0	2.0 (3.9)	0.2 (0.4)	0.6 (1.1)	0.0	0.0 (0.1)	0.1 (0.2)
Week 1	15.6 (20.5)	6.5 (10.9)	21.3 (6.2)	3.4 (3.4)	0.3 (0.3)	0.8 (0.8)
Week 2	10.3 (7.9)	1.0 (1.0)	3.4 (4.1)	0.0	0.1 (0.0)	0
Week 3	27.2 (21.6)	2.2 (2.5)	3.9 (3.1)	0.0	0.0	0.1 (0.1)

In addition to calculating the means and the standard deviations, the antimicrobial results for broadcast plots were also analyzed using rANOVA. Results from the rANOVA analyses are summarized in Table 5. Consistent with the findings from Table 3, rANOVA analyses confirmed that soil position (top vs. bottom) and rainfall event (before vs. after simulated rainfall events) had statistically significant impacts on all antimicrobials. This is supported by the fact that the *p* values for these two treatment

factors are smaller than 0.05 (lower part of Table 5). It is worth noticing that the timing of land application in relation to rainfall events had significant impact only to lincomycin, but not to chlortetracycline or tiamulin (Table 5).

Table 5. rANOVA tests on the effects of manure application timing, soil position, and rainfall event on the concentrations of antimicrobials in soil receiving broadcast treatment (ng/g dry soil).

	Chlortetracycline	Lincomycin	Tiamulin
Application Timing[a,b]			
0	0.294	0.012a	0.029
1	1.270	0.039b	0.045
2	0.379	0.004c	0.022
3	0.239	0.002d	0.014
Soil Position			
Top	17.828a	0.218a	2.413a
Bottom	0.010b	0.000b	0.000b
Rainfall Event			
Before	4.465a	0.024a	0.070a
After	0.041b	0.003b	0.009b
rANOVA value for[c]			
Application timing	0.9270	0.0354	0.7129
Soil position	0.0001	<0.001	<0.001
Rainfall event	0.0251	0.0056	0.0160
Timing × Position	0.9268	0.0363	0.4867
Timing × Rainfall	0.9547	0.9198	0.7340
Position × Rainfall	0.1133	0.0470	0.0220
Timing × Position × Rainfall	0.9014	0.3736	0.9505

[a] Values reported under "Application Timing", "Soil Position", and "Rainfall Event" are treatment averages, which were calculated based on all the data for one particular treatment level. For example, 0.294 ng/g soil dry weight was calculated using chlortetracycline concentrations of all soil samples from all plots that had 1 day (or 0 week) between land application and rainfall simulations, regardless whether they were collected from top vs. bottom soil or before vs. after rainfall simulations.

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

[c] rANOVA values are displayed as *p* values.

Because the rANOVA tests on antimicrobial concentrations pointed out that soil position and rainfall simulation had significant impacts on the concentration of antimicrobials in soil, Figures 6 and 7 are prepared to better illustrate the effects of these two treatment factors. Figure 6 shows the effects of soil depth on antimicrobial concentrations in soil, while Figure 7 demonstrates that simulated rainfall events significantly lowered the antimicrobial concentrations in soil.

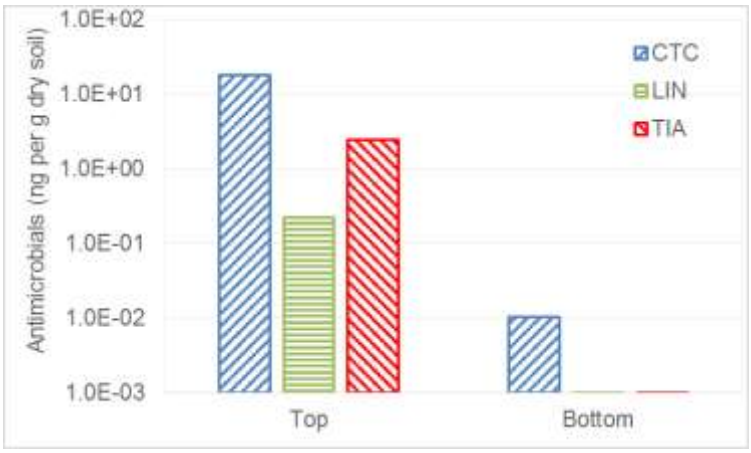


Figure 6. The least squares mean values of antimicrobial concentrations in the top and the bottom portions of soil cores.

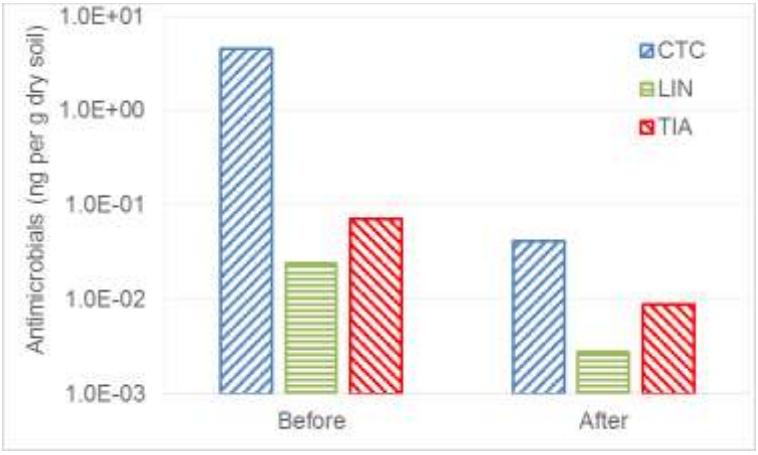


Figure 7. The least squares mean values of antimicrobial concentrations in soils before and after simulated rainfall events.

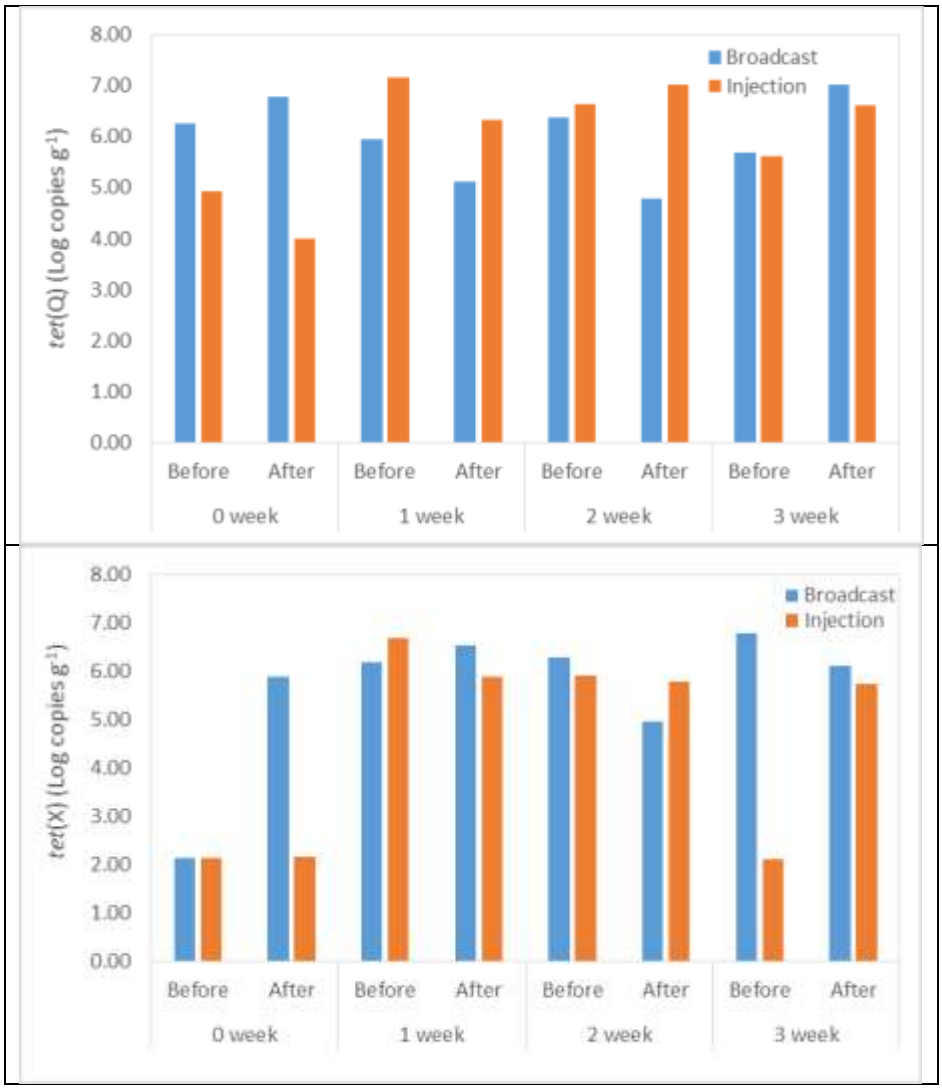
Antimicrobial Resistance Genes

Two tetracycline resistance genes *tet*(Q) and *tet*(X) and two lincomycin resistance genes *erm*(A) and *erm*(C) were selected for this study. The 16S rRNA gene was also included, because this gene is often used as a biomarker to identify and quantify bacterial and archaeal species in an environmental sample. In other words, the number of the 16S rRNA gene copy can serve as an estimate of the total number of bacteria and archaea in soil.

The concentrations of the AMR genes and the 16S rRNA genes in soil are summarized in Tables A3 and A4 of the Appendix for broadcast and injection plots, respectively. The absolute abundances of AMR genes (Log copies per gram of soil dry weight) are also plotted in Figure 8. When rainfall simulations occurred 1 day after manure application (i.e., the "0 week" in Figure 8), the AMR gene levels in top soil were always higher for broadcast plots than for injection plots, regardless whether the soil samples were taken before or after rainfall events. This trend indicates that before manure-born AMR gene-

carrying bacteria proliferate or attenuate in soil, broadcast resulted in higher AMR gene levels in top soil than the injection method.

This trend did not hold for three of the four AMR genes tested (i.e., *tet*(Q), *tet*(X), and *erm*(A)) when the simulated rainfall events occurred 1, 2, or 3 weeks after manure application (Figure 8). This finding suggests that there were multiple processes that could affect the fate of AMR gene-carrying bacteria in soil in the weeks following manure application, some resulting in proliferation of these bacteria and other resulting in killing of these bacteria. As a result, neither broadcast nor injection appeared to consistently result in lower AMR gene levels in top soils than the other. The AMR gene *erm*(C) was an exception, for which the broadcast method consistently resulted in higher *erm*(C) concentrations in soil than the injection method.



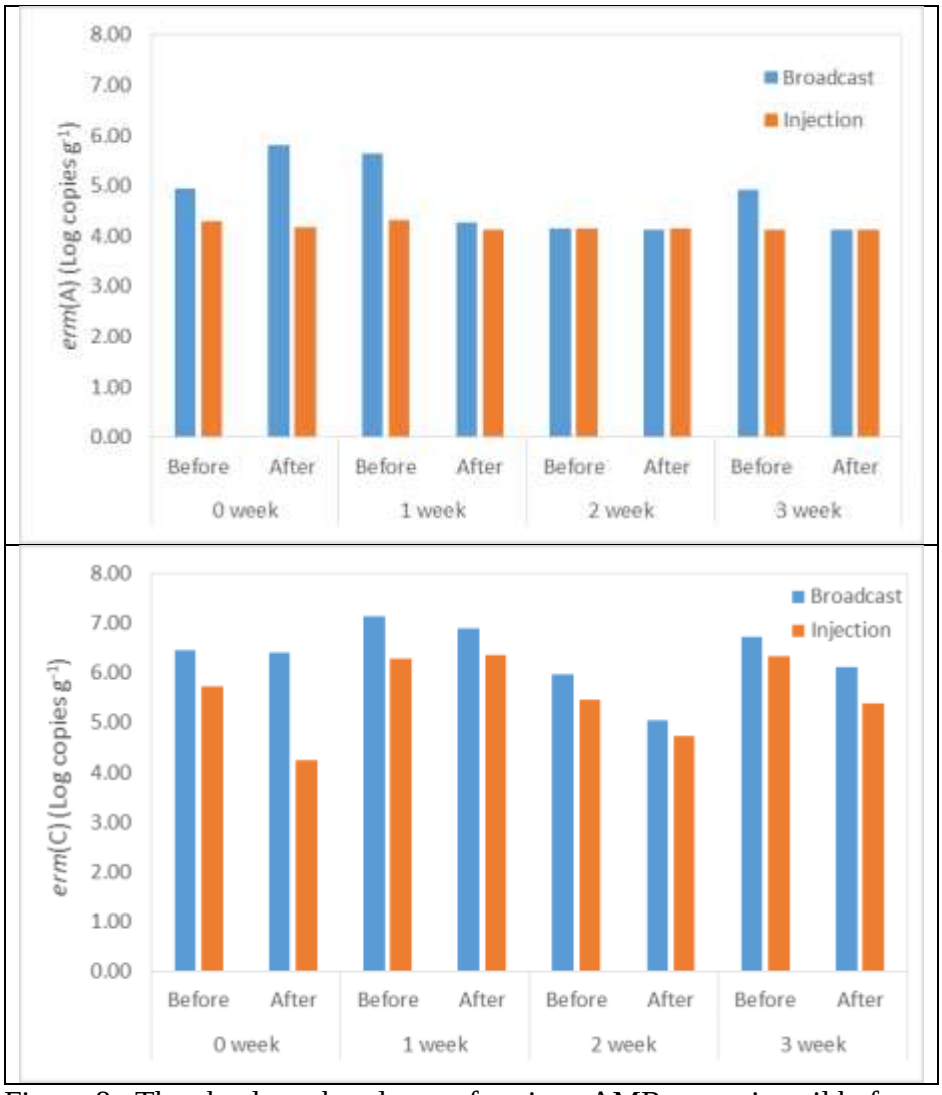


Figure 8. The absolute abundance of various AMR genes in soil before and after simulated rainfall events. The rainfall events were simulated 1 day (or 0 week), 1, 2, and 3 weeks after manure was land applied through either broadcast or injection.

The gene concentrations from the broadcast plots were further analyzed using rANOVA and the results are reported in Table 6. Three treatment effects were included in the analyses: application timing (i.e., rainfall simulation occurred 0, 1, 2, and 3 weeks after manure application), soil position (i.e., top and bottom portion of the 9-inch soil cores collected from the plots), and rainfall events (i.e., before and after three simulated rainfall events).

As shown in Table 6, none of the interaction terms from the ANOVA analyses is significant (i.e., $p > 0.05$). The length the time period between manure application and rainfall events had no significant effects on the level of AMR genes in soil for three of the four AMR genes tested (Table 6). Application timing exhibited significant impacts only on AMR gene *tet*(Q) and the 16S rRNA gene. Soil position clearly had a significant impact on the level of AMR genes and the 16S rRNA gene in soil (Table 6). For all four

AMR genes and the 16S rRNA gene, the absolute abundance was higher in the top portion than in the bottom portion of the soil cores. Rainfall event did not have significant impacts on the level of AMR genes in soil.

Table 6. rANOVA tests on the effects of manure application timing, soil position, and rainfall event on the concentrations of AMR genes and the 16S rRNA gene in soil receiving broadcast treatment (copies per g dry soil).

	tet(Q)	*tet*(X)	*erm*(A)	*erm*(C)	16S rRNA gene
***Application Timing*[a,b]**					
0	3.20×10^5	1.15×10^3	3.53×10^4	2.06×10^5	1.32×10^9
1	7.30×10^4	4.39×10^3	2.66×10^4	3.76×10^5	4.31×10^9
2	7.90×10^4	5.69×10^3	1.36×10^4	1.22×10^5	1.33×10^9
3	1.01×10^6	9.06×10^3	1.80×10^4	4.41×10^5	5.22×10^9
Soil Position					
Top	4.43×10^5 a	1.99×10^4 a	3.31×10^4 a	9.03×10^5 a	4.46×10^9 a
Bottom	9.74×10^4 b	8.10×10^2 b	1.45×10^4 b	7.15×10^4 b	1.41×10^9 b
Rainfall Event					
Before	2.11×10^5	3.83×10^3	2.40×10^4	3.42×10^5	1.72×10^9
After	2.05×10^5	4.20×10^3	2.00×10^4	1.89×10^5	2.96×10^9
***rANOVA value for*[c]**					
Application timing	0.0251	0.6936	0.1871	0.2665	0.0257
Soil position	0.0087	0.0051	0.0058	<0.0001	0.0009
Rainfall event	0.9604	0.9378	0.4858	0.2412	0.3285
Timing × Position	0.1070	0.6504	0.2087	0.0092	0.8062
Timing × Rainfall	0.1500	0.1628	0.0771	0.5855	0.6963
Position × Rainfall	0.2463	0.3629	0.0712	0.0921	0.2604
Timing × Position × Rainfall	0.2307	0.0945	0.0568	0.9244	0.8373

[a] Values reported under "Application Timing", "Soil Position", and "Rainfall Event" are treatment averages, which were calculated based on all the data for one particular treatment level. For example, 3.20×10^5 copy/g soil dry weight was calculated using *tet*(Q) concentrations of all runoff samples from all plots that had 1 day between land application and rainfall simulations, regardless whether they were collected from top vs. bottom soil or before vs. after rainfall simulations.

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

[c] rANOVA values are displayed as *p* values.

Because soil position is the only treatment factor that exhibited significant impact on all AMR genes tested, this factor was plotted to better illustrate its impacts on AMR genes. The four AMR genes, grouped based on resistance gene families, are plotted in Figure 9. Figure 9 clearly demonstrates that the AMR genes were higher in top soil than in deeper soil during the three week time period we tested.

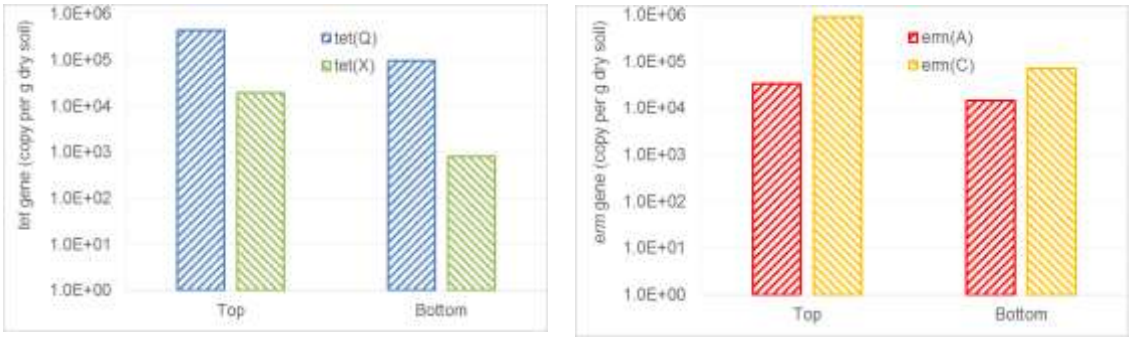


Figure 9. The absolute abundance of *tet*(Q), *tet*(X), *erm*(A), and *erm*(C) in the top and bottom portion of the soil cores from plots receiving manure through broadcast.

Similar to the case for antimicrobials, the concentrations of AMR genes in soil were not significantly affected by the length of time between manure application and rainfall events (Table 6). This is in part due to the large standard deviations exhibited in the dataset. In addition, unlike chemical compounds, AMR genes could increase in abundance if the cells carrying the AMR genes increase in number. Some bacteria species carrying an AMR gene may replicate in soil, while other bacterial species carrying the same AMR gene may die in soil. Together, the net results of the AMR genes can be dynamic in the field.

The effects of soil depth on the concentration of AMR genes and the 16S rRNA gene are not surprising. Because surface soil often contains higher levels of substrates and nutrients than does subsoil, microbial level is often the highest in surface soil and then decreases as the soil gets deeper. The abundance of the AMR genes was lower in the bottom than in the top of the soil cores, likely because manure-borne AMR genes did not transport much in the vertical direction from the top soil over the testing period of this study (i.e., 3 weeks).

One major difference from the antimicrobial results is that the rANOVA analyses showed no significant impacts of rainfall events on the levels of AMR genes in soil (Tables 5 and 6). During rainfall events, the soluble portion of the antimicrobial compounds were lost to either runoff or infiltration. Cells carrying AMR genes are usually associated with solids such as manure or soil particles. It is plausible to expect that solids were lost to runoff and infiltration to a lesser extent than soluble compounds. Finally, increased soil moisture resulting from rainfall events could also benefit the growth of cells carrying AMR genes.

References Cited

Aminov, R. I., N. Garrigues-Jeanjean and R. I. Mackie (2001). Molecular ecology of tetracycline resistance: Development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection proteins. Appl. Environ. Microbiol. **67**(1): 22-32.

APHA-AWWA-WEF (2005). Standard Methods for the Examination of Water and Wastewater. Washington, DC, American Public Health Association.

Chee-Sanford, J. C., R. I. Mackie, S. Koike, I. G. Krapac, Y. F. Lin, A. C. Yannarell, S. Maxwell and R. I. Aminov (2009). Fate and Transport of Antibiotic Residues and Antibiotic Resistance Genes following Land Application of Manure Waste. Journal of Environmental Quality **38**(3): 1086-1108.

Dolliver, H. and S. Gupta (2008). Antibiotic losses in leaching and surface runoff from manure-amended agricultural land. Journal of Environmental Quality **37**(3): 1227-1237.

Gaskins, H. R., C. T. Collier and D. B. Anderson (2002). Antibiotics as growth promotants: Mode of action. Anim. Biotechnol. **13**(1): 29-42.

Ghosh, S., S. J. Ramsden and T. M. LaPara (2009). 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. **84**(4): 791-796.

Hamscher, G., S. Sczesny, H. Hoper and H. Nau (2002). Determination of Persistent Tetracycline Residues in Soil Fertilized with Liquid Manure by High-Performance Liquid Chromatography with Electrospray Ionization Tandem Mass Spectrometry. Anal. Chem. **74**(7): 1509-1518.

Humphry, J. B., T. C. Daniel, D. R. Edwards and A. N. Sharpley (2002). A portable rainfall simulator for plot-scale runoff studies. Appl. Eng. Agric. **18**(2): 199-204.

Kettler, T. A., J. W. Doran and T. L. Gilbert (2001). Simplifed method for soil particle-size determination to accompany soil-quality analyses. Soil Sci. Soc. Am. J. **65**(3): 849-852.

Koike, S., R. I. Aminov, A. C. Yannarell, H. D. Gans, I. G. Krapac, J. C. Chee-Sanford and R. I. Mackie (2010). Molecular ecology of Macrolide-Lincosamide-Streptogramin B methylases in waste lagoons and subsurface waters associated with swine production. Microb. Ecol. **59**(3): 487-498.

Koike, S., I. G. Krapac, H. D. Oliver, A. C. Yannarell, J. C. Chee-Sanford, R. I. Aminov and R. I. Mackie (2007). Monitoring and source tracking of tetracycline resistance genes in lagoons and groundwater adjacent to swine production facilities over a 3-year period. Appl. Environ. Microbiol. **73**(15): 4813-4823.

Kuchta, S. L., A. J. Cessna, J. A. Elliott, K. M. Peru and J. V. Headley (2009). Transport of lincomycin to surface and ground water from manure-amended cropland. J. Environ. Qual. **38**(4): 1719-1727.

Murphy, J. and J. P. Riley (1962). A modified single solution method for the determination of phosphate in natural waters. Anal. Chim. Acta **27**: 31-36.

Ng, L. K., I. Martin, M. Alfa and M. Mulvey (2001). Multiplex PCR for the detection of tetracycline resistant genes. Mol. Cell. Probes **15**(4): 209-215.

Salyers, A. A., A. Gupta and Y. P. Wang (2004). Human intestinal bacteria as reservoirs for antibiotic resistance genes. Trends Microbiol. **12**(9): 412-416.

Schlusener, M. P. and K. Bester (2006). Persistence of antibiotics such as macrolides, tiamulin and salinomycin in soil. Environ. Pollut. **143**(3): 565-571.

Sharpley, A. and P. Kleinman (2003). Effect of rainfall simulator and plot scale on overland flow and phosphorus transport. Journal of Environmental Quality **32**(6): 2172-2179.

Stoob, K., H. P. Singer, S. R. Mueller, R. P. Schwarzenbach and C. H. Stamm (2007). Dissipation and transport of veterinary sulfonamide antibiotics after manure application to grassland in a small catchment. Environmental Science & Technology **41**(21): 7349-7355.

Suzuki, M. T., L. T. Taylor and E. F. DeLong (2000). Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5 '-nuclease assays. Appl. Environ. Microbiol. **66**(11): 4605-4614.

APPENDIX

Table A1. Sumary of the concentrations of antimicrobial compounds in the soil samples collected from plots receiving manure through broadcast. The antimicrobial concentrations are in the unit of "ng/g dry soil". Cells highlighted in yellow indicate values below method detection limit (i.e., 0.05 ng/g dry soil). Half of the method detection limit (i.e., 0.025 ng/g dry soil) was used for these cells for data log transformation necessary for rANOVA analyses.

Sample No	Plot	Time[a]	Rain	Position	Chlortetracycline	Lincomycin	Tiamulin
1	101	0	Bef.	Top	80.738	8.974	21.257
2	101	0	Aft.	Top	0.025	0.305	0.523
3	102	0	Bef.	Top	0.025	0.025	0.025
4	102	0	Aft.	Top	5.703	0.690	1.677
5	103	0	Bef.	Top	107.795	12.557	27.032
6	103	0	Aft.	Top	22.327	0.320	3.184
7	104	0	Bef.	Top	77.860	6.740	13.578
8	104	0	Aft.	Top	0.025	0.194	0.123
9	111	1	Bef.	Top	43.905	1.980	3.414
10	111	1	Aft.	Top	21.139	0.727	6.301
11	112	1	Bef.	Top	3.454	9.340	6.452
12	112	1	Aft.	Top	0.634	0.733	1.675
13	113	1	Bef.	Top	36.414	2.464	4.367
14	113	1	Aft.	Top	0.025	0.093	0.025
15	114	1	Bef.	Top	60.280	10.459	7.333
16	114	1	Aft.	Top	75.341	6.318	19.297
17	121	2	Bef.	Top	11.997	0.173	2.757
18	121	2	Aft.	Top	0.521	0.088	0.173
19	122	2	Bef.	Top	10.376	0.425	2.805
20	122	2	Aft.	Top	7.902	0.471	0.496
21	123	2	Bef.	Top	157.487	1.450	20.084
22	123	2	Aft.	Top	4.364	0.225	0.632
23	124	2	Bef.	Top	3.913	0.060	2.423
24	124	2	Aft.	Top	0.025	0.164	0.185
25	131	3	Bef.	Top	5.044	0.237	0.914
26	131	3	Aft.	Top	3.408	0.025	0.189
27	132	3	Bef.	Top	1.545	0.237	0.364
28	132	3	Aft.	Top	16.075	0.396	0.409
29	133	3	Bef.	Top	29.025	0.994	5.420
30	133	3	Aft.	Top	0.025	0.072	0.728
31	134	3	Bef.	Top	80.663	1.749	5.843

32	134	3	Aft.	Top	0.025	0.025	0.383
3365	101	1	Bef.	Bottom	2.250	0.026	0.025
34	101	1	Aft.	Bottom	0.025	0.025	0.025
35	102	1	Bef.	Bottom	0.025	0.025	0.025
36	102	1	Aft.	Bottom	3.726	0.025	0.025
37	103	1	Bef.	Bottom	0.025	0.033	0.025
38	103	1	Aft.	Bottom	0.025	0.025	0.025
39	104	1	Bef.	Bottom	1.833	0.025	0.046
40	104	1	Aft.	Bottom	0.025	0.025	0.025
41	111	2	Bef.	Bottom	0.025	0.025	0.025
42	111	2	Aft.	Bottom	0.025	0.029	0.025
43	112	2	Bef.	Bottom	3.763	0.025	0.017
44	112	2	Aft.	Bottom	0.025	0.025	0.025
45	113	2	Bef.	Bottom	0.083	0.025	0.025
46	113	2	Aft.	Bottom	0.025	0.030	0.025
47	114	2	Bef.	Bottom	1.427	0.133	0.031
48	114	2	Aft.	Bottom	3.587	0.025	0.025
49	121	3	Bef.	Bottom	0.025	0.232	0.119
50	121	3	Aft.	Bottom	0.025	0.025	0.025
51	122	3	Bef.	Bottom	0.025	0.025	0.025
52	122	3	Aft.	Bottom	0.025	0.025	0.025
53	123	3	Bef.	Bottom	12.983	0.031	0.025
54	123	3	Aft.	Bottom	0.025	0.025	0.025
55	124	3	Bef.	Bottom	0.025	0.068	0.025
56	124	3	Aft.	Bottom	10.750	0.025	0.025
57	131	4	Bef.	Bottom	0.025	0.025	0.025
58	131	4	Aft.	Bottom	0.025	0.025	0.025
59	132	4	Bef.	Bottom	0.025	0.025	0.025
60	132	4	Aft.	Bottom	0.212	0.025	0.025
61	133	4	Bef.	Bottom	3.034	0.025	0.025
62	133	4	Aft.	Bottom	0.025	0.025	0.025
63	134	4	Bef.	Bottom	12.216	0.025	0.025
64	134	4	Aft.	Bottom	0.025	0.025	0.076

[a.] The numbers represent the number of weeks between manure land application and the initial rainfall simulation event. 0 week means that the intial rainfall simulation event occurred 1 day after the land application of manure slurry.

Table A2. Sumary of the concentrations of antimicrobial compounds in the soil samples collected from plots receiving manure through injection. The antimicrobial concentrations are in the unit of "ng/g dry soil". Cells highlighted in yellow indicate values below method detection limit (i.e., 0.05 ng/g dry soil). Half of the method detection limit (i.e., 0.025 ng/g dry soil) was used for these cells for data log transformation necessary for rANOVA analyses.

Sample No	Plot	Time[a]	Rain	Position	Chlortetracycline	Lincomycin	Tiamulin
65	201	0	Bef.	Top	7.765	0.713	2.197
66	201	0	Aft.	Top	0.025	0.025	0.025
67	202	0	Bef.	Top	0.025	0.000	0.025
68	202	0	Aft.	Top	0.025	0.000	0.378
69	203	0	Bef.	Top	0.025	0.000	0.025
70	203	0	Aft.	Top	0.025	0.000	0.025
71	204	0	Bef.	Top	0.025	0.000	0.015
72	204	0	Aft.	Top	0.025	0.126	0.025
73	211	1	Bef.	Top	46.169	0.090	30.280
74	211	1	Aft.	Top	6.641	0.684	1.733
75	212	1	Bef.	Top	5.169	22.831	19.439
76	212	1	Aft.	Top	0.025	0.203	0.117
77	213	1	Bef.	Top	8.460	0.957	19.382
78	213	1	Aft.	Top	6.125	0.133	0.025
79	214	1	Bef.	Top	2.542	1.969	16.131
80	214	1	Aft.	Top	0.977	0.129	1.209
81	221	2	Bef.	Top	4.683	0.184	0.599
82	221	2	Aft.	Top	0.025	0.128	0.025
83	222	2	Bef.	Top	20.634	1.358	8.979
84	222	2	Aft.	Top	0.025	0.035	0.025
85	223	2	Bef.	Top	3.424	0.074	0.025
86	223	2	Aft.	Top	0.025	0.068	0.025
87	224	2	Bef.	Top	12.695	2.213	4.003
88	224	2	Aft.	Top	0.025	0.025	0.025
89	231	3	Bef.	Top	51.981	1.905	7.509
90	231	3	Aft.	Top	0.025	0.025	0.025
91	232	3	Bef.	Top	8.295	0.025	0.025
92	232	3	Aft.	Top	0.025	0.025	0.025
93	233	3	Bef.	Top	9.946	1.029	3.554
94	233	3	Aft.	Top	0.025	0.025	0.025
95	234	3	Bef.	Top	38.945	5.695	4.479

96	234	3	Aft.	Top	0.025	0.037	0.277

[a.] The numbers represent the number of weeks between manure land application and the initial rainfall simulation event. 0 week means that the intial rainfall simulation event occurred 1 day after the land application of manure slurry.

Table A3. Sumary of the abundance of AMR genes and the 16S rRNA gene in the soil samples collected from plots receiving manure through broadcast. The gene abundance in the table is in the unit of "log copy/g dry soil".

Sample No	Plot	Time[a]	Rain	Position	*tet*(Q)	*tet*(X)	*erm*(A)	*erm*(C)	16S rRNA gene
1	101	0	Bef.	Top	6.04	2.14	4.48	6.55	9.79
2	101	0	Aft.	Top	6.81	5.92	4.14	5.09	9.07
3	102	0	Bef.	Top	4.98	2.14	4.14	4.71	7.98
4	102	0	Aft.	Top	6.68	5.77	6.16	6.67	9.67
5	103	0	Bef.	Top	6.52	2.13	5.47	6.66	9.62
6	103	0	Aft.	Top	7.12	6.19	6.03	6.73	9.99
7	104	0	Bef.	Top	6.46	2.17	4.17	6.51	9.25
8	104	0	Aft.	Top	4.71	2.14	4.14	4.94	9.54
9	111	1	Bef.	Top	6.12	6.62	4.12	7.51	10.46
10	111	1	Aft.	Top	4.63	2.16	4.16	5.44	9.74
11	112	1	Bef.	Top	5.93	6.15	5.27	6.54	9.78
12	112	1	Aft.	Top	5.62	7.08	4.15	7.28	10.10
13	113	1	Bef.	Top	5.66	2.11	6.14	7.07	10.11
14	113	1	Aft.	Top	4.52	2.13	4.13	5.44	10.27
15	114	1	Bef.	Top	5.97	5.78	5.29	6.87	9.74
16	114	1	Aft.	Top	4.34	6.18	4.49	7.04	9.58
17	121	2	Bef.	Top	5.12	5.16	4.14	6.08	8.16
18	121	2	Aft.	Top	4.57	2.14	4.14	5.42	9.62
19	122	2	Bef.	Top	6.36	5.79	4.14	4.93	9.54
20	122	2	Aft.	Top	5.13	5.56	4.13	4.81	9.04
21	123	2	Bef.	Top	6.83	6.81	4.14	6.35	10.08
22	123	2	Aft.	Top	4.87	2.13	4.13	5.05	9.86
23	124	2	Bef.	Top	5.46	5.59	4.15	5.44	9.35
24	124	2	Aft.	Top	3.14	2.14	4.14	4.14	9.26
25	131	3	Bef.	Top	4.43	2.12	4.12	5.82	8.45
26	131	3	Aft.	Top	7.38	6.52	4.10	6.27	10.01
27	132	3	Bef.	Top	4.78	2.15	4.15	5.01	10.07
28	132	3	Aft.	Top	6.74	2.16	4.16	4.96	10.39
29	133	3	Bef.	Top	6.15	6.84	5.36	7.25	10.04
30	133	3	Aft.	Top	7.07	6.22	4.11	6.51	10.77
31	134	3	Bef.	Top	5.70	7.23	4.90	6.47	9.85
32	134	3	Aft.	Top	4.86	2.15	4.15	5.04	9.59
33	101	0	Bef.	Bottom	3.52	2.12	4.12	4.12	9.18
34	101	0	Aft.	Bottom	4.88	2.14	4.14	4.73	8.76

35	102	0	Bef.	Bottom	4.65	2.14	4.14	4.14	8.28
36	102	0	Aft.	Bottom	6.56	5.44	5.13	5.62	9.09
37	103	0	Bef.	Bottom	5.76	2.12	4.12	4.12	8.45
38	103	0	Aft.	Bottom	3.61	2.13	4.13	4.57	8.59
39	104	0	Bef.	Bottom	5.00	2.12	4.12	4.85	9.27
40	104	0	Aft.	Bottom	4.78	2.13	4.13	5.01	9.37
41	111	1	Bef.	Bottom	6.29	5.13	4.12	5.65	9.27
42	111	1	Aft.	Bottom	4.26	2.13	4.13	4.13	8.99
43	112	1	Bef.	Bottom	4.04	2.13	4.13	4.13	9.46
44	112	1	Aft.	Bottom	5.43	2.14	4.14	5.58	9.85
45	113	1	Bef.	Bottom	3.76	2.13	4.13	4.13	9.18
46	113	1	Aft.	Bottom	4.94	2.13	4.13	4.13	8.62
47	114	1	Bef.	Bottom	3.00	2.14	4.14	4.14	9.49
48	114	1	Aft.	Bottom	3.29	2.14	4.14	4.14	9.49
49	121	2	Bef.	Bottom	4.62	2.12	4.12	5.25	8.26
50	121	2	Aft.	Bottom	4.44	2.13	4.13	4.13	9.04
51	122	2	Bef.	Bottom	4.39	2.12	4.12	4.12	8.58
52	122	2	Aft.	Bottom	6.61	6.30	4.14	6.04	9.44
53	123	2	Bef.	Bottom	4.07	2.12	4.12	5.39	9.21
54	123	2	Aft.	Bottom	4.21	2.13	4.13	4.52	9.16
55	124	2	Bef.	Bottom	5.39	5.68	4.13	5.58	9.50
56	124	2	Aft.	Bottom	3.15	2.15	4.15	4.15	7.89
57	131	3	Bef.	Bottom	6.91	5.30	4.12	5.74	9.36
58	131	3	Aft.	Bottom	7.04	6.15	4.14	5.60	9.63
59	132	3	Bef.	Bottom	5.85	2.13	4.13	5.34	9.61
60	132	3	Aft.	Bottom	5.93	2.15	4.15	4.74	9.80
61	133	3	Bef.	Bottom	5.94	2.12	4.12	4.12	9.27
62	133	3	Aft.	Bottom	6.65	5.80	4.13	5.51	9.47
63	134	3	Bef.	Bottom	4.68	2.12	4.12	6.52	9.78
64	134	3	Aft.	Bottom	5.98	2.15	4.15	5.43	9.38

[a.] The numbers represent the number of weeks between manure land application and the initial rainfall simulation event. 0 week means that the intial rainfall simulation event occurred 1 day after the land application of manure slurry.

Table A4. Sumary of the abundance of AMR genes and the 16S rRNA gene in the soil samples collected from plots receiving manure through injection. The gene abundance in the table is in the unit of "log copy/g dry soil".

Sample No	Plot	Time[a]	Rain	Position	*tet*(Q)	*tet*(X)	*erm*(A)	*erm*(C)	16S rRNA gene
1	201	0	Bef.	Top	5.51	2.13	4.55	6.31	9.75
2	201	0	Aft.	Top	3.58	2.17	4.17	4.17	9.32
3	202	0	Bef.	Top	3.15	2.15	4.15	4.15	8.55
4	202	0	Aft.	Top	4.46	2.17	4.17	4.39	9.18
5	203	0	Bef.	Top	3.13	2.13	4.13	4.13	7.03
6	203	0	Aft.	Top	3.14	2.14	4.14	4.14	9.10
7	204	0	Bef.	Top	3.38	2.14	4.14	4.14	8.16
8	204	0	Aft.	Top	3.87	2.18	4.18	4.18	9.32
9	211	1	Bef.	Top	4.87	4.19	4.13	4.13	7.01
10	211	1	Aft.	Top	4.68	2.12	4.12	4.79	10.02
11	212	1	Bef.	Top	7.62	7.16	4.44	6.66	10.49
12	212	1	Aft.	Top	5.88	2.14	4.14	5.77	9.86
13	213	1	Bef.	Top	5.41	4.98	4.12	5.84	8.74
14	213	1	Aft.	Top	6.84	6.49	4.12	6.32	10.25
15	214	1	Bef.	Top	7.17	6.65	4.45	6.38	9.86
16	214	1	Aft.	Top	5.97	2.16	4.16	6.81	10.30
17	221	2	Bef.	Top	3.08	2.14	4.14	4.54	7.51
18	221	2	Aft.	Top	7.58	6.39	4.14	4.14	9.31
19	222	2	Bef.	Top	6.97	6.50	4.15	5.36	9.89
20	222	2	Aft.	Top	6.39	2.16	4.16	5.24	9.81
21	223	2	Bef.	Top	6.89	2.14	4.14	5.56	10.29
22	223	2	Aft.	Top	3.97	2.13	4.13	4.13	10.06
23	224	2	Bef.	Top	5.41	2.14	4.14	5.70	9.17
24	224	2	Aft.	Top	4.67	2.14	4.14	4.14	10.02
25	231	3	Bef.	Top	5.61	2.10	4.10	4.62	8.79
26	231	3	Aft.	Top	7.21	6.32	4.11	5.91	10.00
27	232	3	Bef.	Top	4.93	2.16	4.16	4.16	8.35
28	232	3	Aft.	Top	5.15	2.15	4.15	4.15	9.92
29	233	3	Bef.	Top	5.98	2.09	4.09	6.89	10.41
30	233	3	Aft.	Top	4.94	2.09	4.09	5.08	10.25
31	234	3	Bef.	Top	5.27	2.14	4.14	5.93	9.55
32	234	3	Aft.	Top	3.14	2.14	4.14	4.14	9.24
33	201	0	Bef.	Bottom	4.33	2.12	4.12	4.12	8.82
34	201	0	Aft.	Bottom	3.13	2.13	4.13	4.13	8.92

35	202	0	Bef.	Bottom	3.13	2.13	4.13	4.13	9.31
36	202	0	Aft.	Bottom	4.45	2.12	4.12	4.12	8.87
37	203	0	Bef.	Bottom	4.61	2.12	4.12	4.76	9.65
38	203	0	Aft.	Bottom	3.71	2.14	4.14	4.14	8.64
39	204	0	Bef.	Bottom	4.36	2.71	4.13	4.13	6.83
40	204	0	Aft.	Bottom	3.81	2.11	4.11	4.12	9.19
41	211	1	Bef.	Bottom	3.12	2.12	4.12	4.12	7.61
42	211	1	Aft.	Bottom	4.07	2.13	4.13	4.96	9.58
43	212	1	Bef.	Bottom	4.47	2.13	4.13	4.13	8.12
44	212	1	Aft.	Bottom	6.17	4.83	4.13	5.06	7.88
45	213	1	Bef.	Bottom	4.16	2.12	4.12	4.57	9.54
46	213	1	Aft.	Bottom	6.90	6.47	4.13	6.04	9.37
47	214	1	Bef.	Bottom	3.13	2.13	4.13	4.14	6.99
48	214	1	Aft.	Bottom	6.63	5.73	4.13	5.30	9.59
49	221	2	Bef.	Bottom	3.12	2.03	4.12	4.12	8.76
50	221	2	Aft.	Bottom	3.13	2.13	4.13	4.48	8.00
51	222	2	Bef.	Bottom	7.28	6.27	4.13	5.43	9.38
52	222	2	Aft.	Bottom	4.07	3.44	4.15	4.15	6.49
53	223	2	Bef.	Bottom	7.07	5.96	4.12	4.87	9.52
54	223	2	Aft.	Bottom	5.24	2.14	4.14	5.24	9.44
55	224	2	Bef.	Bottom	3.13	2.13	4.13	4.13	8.19
56	224	2	Aft.	Bottom	3.87	2.14	4.14	4.83	6.17
57	231	3	Bef.	Bottom	5.66	2.12	4.12	4.12	8.40
58	231	3	Aft.	Bottom	6.32	5.61	4.14	5.70	6.84
59	232	3	Bef.	Bottom	4.08	3.22	4.13	4.13	6.15
60	232	3	Aft.	Bottom	3.58	2.15	4.15	4.15	8.45
61	233	3	Bef.	Bottom	4.72	3.48	4.12	4.72	7.04
62	233	3	Aft.	Bottom	6.41	4.75	4.13	4.81	8.57
63	234	3	Bef.	Bottom	6.19	5.18	4.12	5.09	7.29
64	234	3	Aft.	Bottom	3.14	2.14	4.14	4.14	8.92