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 Runoff, **NPB #14-121**

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Industry Summary

The objective of this study was to determine how the method and timing of swine manure application may impact the movement of multiple manure constituents in agricultural runoff. The manure constituents included nutrients, antimicrobials, antimicrobial resistant bacteria, and antimicrobial resistance genes. To achieve the objective, a series of field rainfall simulation tests were conducted. In the tests, swine manure slurry from a commercial farm was broadcast on or injected into plots by a commercial manure applicator. A set of 3 simulated rainfall events were initiated on the manure amended plots 1 day, 1 week, 2 weeks, or 3 weeks after the manure application. Such experimental design allowed us to systematically study the impacts of both the application method and the timing of application relative to rainfall on the movement of manure constituents in runoff. Results show that manure application method had no significant impact on the transport of nitrogen, but had significant impact on dissolved and total phosphorus in runoff. The levels of three antimicrobials, chlortetracycline, lincomycin, and tiamulin, were higher in runoff from broadcast plots than from injected plots. Other than *E. coli*, the two application methods did not yield significantly different levels of microbial constituents in runoff. On the other hand, the timing of land application relative to rainfall events exhibited significant impacts on the levels of nearly all manure constituents in runoff. Longer intervals led to less load in runoff. The only noticible exception was the antimicrobial resistance gene *tet*(X), whose relative abundance in runoff increased with longer time between application and rainfall. These findings show how the movement of different manure constituents in runoff may be affected by different manure land application strategies, highlighting the complexity in designing best management practice for manure application.

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Keywords

Manure constituents, land application, runoff, nutrients, antimicrobials, antimicrobial resistant bacteria, and antimicrobial resistance genes.

Scientific Abstract

Swine manure has been used a soil amendment for crop production because it can provide a source of nutrients, increase soil productivity, improve water infiltration, and reduce the potential of soil erosion. It is important to understand how different manure 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 fate and transport of multiple constituents in surface runoff. The manure constituents included nutrients, antimicrobials, antimicrobial resistant bacteria, and antimicrobial resistance genes.

A series of field rainfall simulation experiments were performed. In these tests, swine manure slurry from a commercial farm was either broadcast on 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, 1 week, 2 weeks, or 3 weeks after the manure application. Runoff samples were collected and analyzed for nutrients using standard methods, for antimicrobials using liquid chromatography tandem mass spectroscopy, for antimicrobial resistant bacteria using culture-based methods, and antimicrobial resistance genes using quantitative polymerase chain reactions.

Results show that manure application methods had no significant impact on the transport of NH_3-N, NO_3-N, or total nitrogen in runoff, but had significant impact on dissolved and total phosphorus in runoff. The levels of three antimicrobials, chlortetracycline, lincomycin, and tiamulin, were higher in runoff from broadcast plots than from injected plots. Broadcast also caused higher *E. coli* level in runoff than did injection; however, the two application methods did not yield significantly different levels of other microbial constituents in runoff, such as the relative abundance of tetracycline resistant bacteria, and the relative abundance of bacteria carrying tetracycline resistance genes *tet*(Q) and *tet*(X).

On the other hand, the timing of land application relative to rainfall events exhibited significant impacts on the levels of nearly all manure constituents in runoff. Longer intervals typically led to less load in runoff. The only noticeable exception was the antimicrobial resistance gene *tet*(X), whose relative abundance in runoff increased with longer interval between application and rainfall. This finding suggests that either manure bacteria carrying *tet*(X) could survive better in amended soil than bacteria that didn't carry this resistance gene, or the *tet*(X) resistance gene proliferated to other bacteria during the test period.

The findings from this project show how the movement of different manure constituents in the environment was affected by the method and timing of manure land application. The differences in the effects highlight the complexity in designing best management practice for manure application.

Introduction

Pork production is a significant 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 antimicrobial resistant bacteria (AMR bacteria) in manure introduces the potential for these constituents to enter the environment when manure is land applied. Recent studies have attempted to relate the environmental occurrence of antimicrobial compounds and associated AMR bacteria to the distribution of livestock production in watersheds. Antimicrobial residues and antimicrobial resistance genes (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).

Soil temperature 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). In addition, the persistence of antimicrobials is also 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).

As with nutrients, antimicrobials and AMR bacteria/gene movement in 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 bacteria/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 bacteria/genes in swine manure, as well as on the environmental fate of these constituents following land application of swine manure slurry. This information is critical in developing on-farm manure management practices that will reduce the potential for transport of these manure constituents to water after land application.

Objectives

The objective of this project was to determine how the method and timing of swine manure application may impact the fate and transport of multiple constituents including nutrients, antimicrobials, and AMR bacteria/genes in surface runoff.

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, Nebraska in Lancaster County. 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 also obtained from the facility operator. A commercial manure applicator was hired to inject and broadcast slurry at the experimental site. The slurry was applied at a rate of approximately 46,800 L/ha (5,000 gal/ac). For injection, a v-shaped chisel (horizontal sweep) implement was used on an 8-row applicator for manure placement. For broadcasting, the applicator was lifted above the soil while maintaining a steady speed and flow rate to ensure uniform slurry distribution.

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 confirmed 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, 1 week, 2 weeks, and 3 weeks following slurry application. Thirty-two plots (0.75-m wide x 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 1). Eight plots were examined during each of the weekly test periods. Each plot was examined only once throughout the course of the study. In addition, eight additional plots were used to study nutrients only. Rainfall simulation tests were conducted on these eight plots six weeks following slurry application (Figure 1).

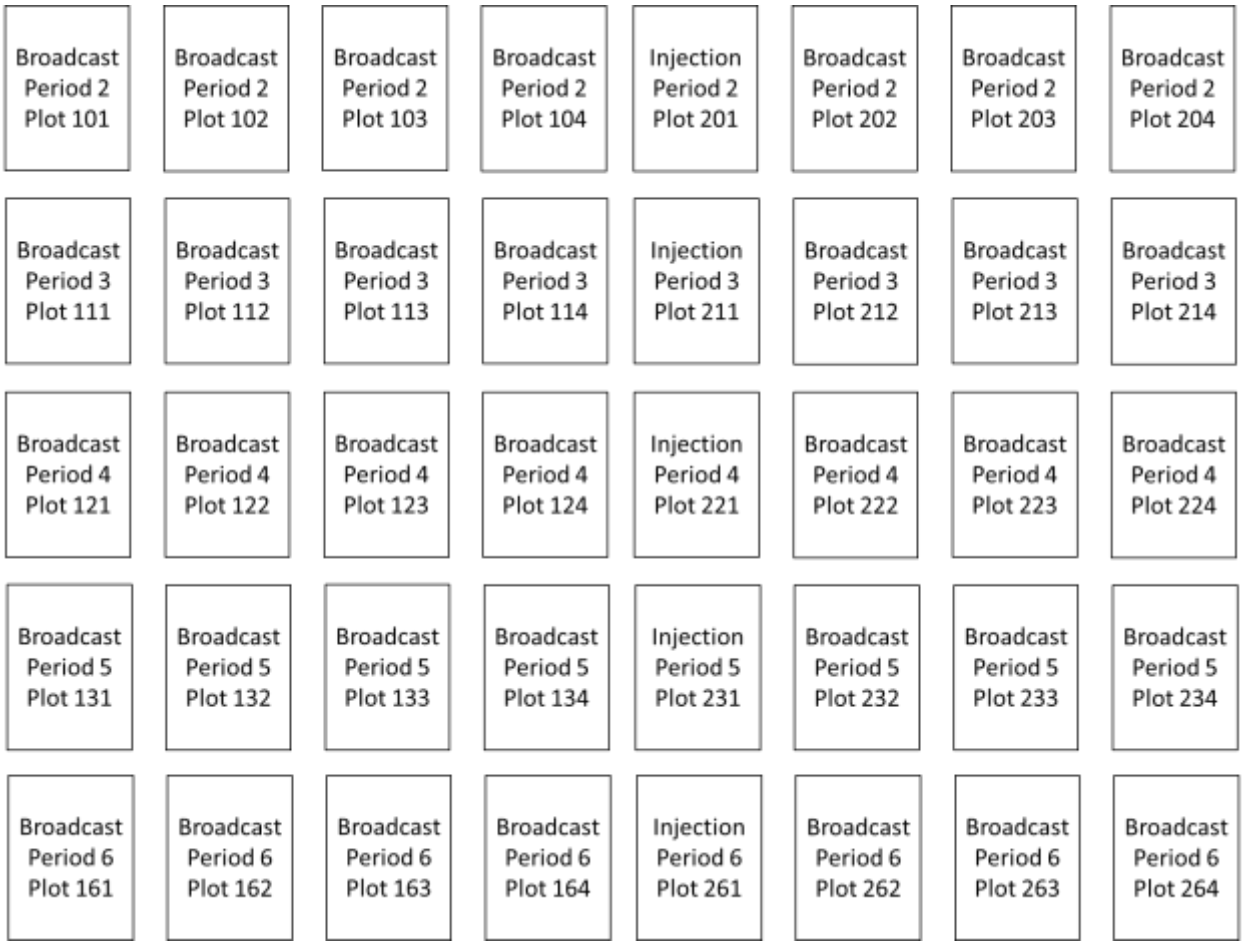


Figure 1. Schematic showing the plot layout and rainfall simulation period for the broadcast and injected plots. The eight plots in each row were subject to rainfall simulation test each week. The plots in the last row were used for nutrient analyses only, while those in the other rows were also used for the analyses of other manure constituents.

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.

The accumulated runoff was agitated immediately before sample collection to maintain suspension of solids. Runoff samples collected for water quality and sediment analyses were obtained within a few minutes following completion of the rainfall simulation tests.

Composite runoff samples were collected from each rainfall event container and stored in three 1-L sterile plastic bottles for nutrients, AMR bacteria, and AMR gene analyses, as well as one 250-mL

amber glass jar for antimicrobial analysis. All the containers were kept on ice until delivery to the laboratories at UNL.

Addition of Inflow

After the completion of the experimental schedule outlined in Figure 1, the effects of runoff rates on the transport of nutrients in runoff were also examined. Increased runoff rates resulting from larger upslope contributing areas were simulated in this test using well-established experimental procedures (Monke *et al.* 1977; Laflen *et al.* 1991; Misra *et al.* 1996). Simulated overland flow was applied at the up gradient end of each plot after the third rainfall simulation to examine the influence of varying runoff rates on the transport of nutrients. Rainfall continued during the simulated overland flow tests. Inflow was added in four successive increments to produce average runoff rates of 2.3, 6.0, 9.5, and 12.6 L min^{-1}.

A mat, made of a green synthetic material and often used as an outdoor carpet, was placed beneath the inflow device at the up gradient end of the plot to prevent scouring and create a more uniform runoff distribution over the plot. Runoff from this test was channeled into a flume with a stage recorder to measure flow rate. Overland flow rate increments were only increased once the previous rate had achieved a steady runoff value (determined with the flume and stage recorder) and samples for that rate had been collected. Each inflow increment had a duration of approximately 8 min, which was the period of time generally required for steady-state flow conditions to become established and for nutrient and sediment analyses samples to be collected.

Solid and Nutrient Analyses

The total solids in runoff samples were determined using the gravimetric method (APHA-AWWA-WEF 2005), and were expressed as solid weight divided by water weight. Extracts from centrifuged and filtered runoff samples were used to determine nutrient concentrations. A Lachat system (Zellweger Analytics, Milwaukee, WI) was employed to analyze all samples for dissolved phosphorous (DP), NO_3-N, and NH_4-N. Non-filtered samples were stored in a cooler at 2°C and then analyzed at a commercial laboratory for total phosphorous and total nitrogen.

Antimicrobial Analyses

Antimicrobial were analyzed in runoff and 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, penicillin G and its metabolite penicillic acid, lincomycin, and tiamulin. Methods were validated for each matrix using standard protocols for recovery and method detection limits. Briefly, runoff samples were collected in pre-cleaned, 250 mL amber jars and stored on ice or refrigerated for up to 48 hours after collection. Prior to extraction, a 100 mL aliquot of each sample was weighed, mixed with 0.1 grams of sodium EDTA and 100 ng of surrogate (oleandomycin). After equilibration, the sample was passed through a glass microfiber filter and, preconditioned Oasis HLB solid phase extraction cartridge to concentrate the antimicrobials. Each cartridge was rinsed with 5 mL reagent water, dried and stored for 1-3 months prior to elution. Cartridges were eluted with 6 mL 0.1% formic acid in acetonitrile, spiked with 100 ng internal standards (roxithromycin, doxycycline, and penicillin V), and evaporated under nitrogen to approximately 100 µL. Concentrated extracts were mixed with 100 µL regent water and 250 µL 2 mM ammonium acetate and transferred to an autosampler vial insert.

Manure slurry samples (0.5 g) were mixed with 5 g clean silica sand, 0.1 g sodium EDTA, 4 mL 100 mM ammonium citrate (pH 6), and 16 mL of 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 4 mL of 100 mM ammonium acetate and 16 mL of acetonitrile. The supernatant was combined with the first aliquot, spiked with internal standards, and evaporated under nitrogen to approximately 18 mL. 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 x 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 1) 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 1).

Table 1. 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 (%)
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 Resistant Bacteria

Diluted runoff samples were spiral plated using the Eddy Jet System (IUL, S.A., Barcelona, Spain) on MacConkey agar (MAC) and MacConkey amended with 16 mg/L tetracycline (TMAC) and incubated at 37°C for 24 hours. Colony counts were determined using the Flash & Go automatic colony counter (IUL, S.A., Barcelona, Spain). Up to 2 pink colonies from each sample and each media type were streaked for isolation on MacConkey MUG agar (Sigma, St. Loius, MO) or MacConkey MUG agar amended with 16 mg/L tetracycline (MACmug or TMACmug, respectively). After an overnight incubation at 37°C, streaked isolates were examined under ultraviolet light to test for fluorescence that is characteristic of *Escherichia coli*. If present, *E. coli* isolates were frozen in glycerol at -80°C for future screening for antibiotic resistance using the Clinical and Laboratory Standards Institute (CLSI, Wayne, PA) antibiotic disc diffusion methods for 12 antibiotics. Zones of clearing were measured and isolates were labeled as susceptible or resistant according to the CLSI standards for each of the 12 antibiotics.

Selected samples were cultured for *Salmonella* using the Hydrophobic Grid Membrane Filtration (HGMF) Spreadfilter Technique (Filtaflex, LTD. Ontario, Canada). Filters were placed on Xylose-Lysine-Deoxycholate medium (Oxoid, Remel) with the addition of 4.6 ml/L tergitol, 15 mg/L novobiocin and 10 mg/L cefsulodin (XLD_{tnc}) and incubated at 37°C for 18-20 hours. Typical H_2S producing *Salmonella* appear as black colonies with a clear, pink outer ring. Plates were incubated for an additional 18-20 hours at room temperature. Black colonies were isolated on XLD_{tnc} and confirmed using InvA PCR (Rahn *et al.* 1992) on the isolated colonies and a 1:100 dilution of the isolated colony. Additional analyses included the EPA-approved Quantitray system (IDEXX Laboratories, Westbrook, ME) combined with Enterolert and Colilert for Most Probable Number enumeration of *Enterococcus* and a combined test for Total Coliforms and *E. coli* bacteria, respectively.

Antimicrobial Resistance Gene Analyses

Well mixed runoff and manure slurry samples were centrifuged at 15,000×g in 50 mL centrifuge tubes for 10 min. For each sample, the pellet collected from 300 mL runoff or 50 mL manure slurry was stored at -80°C until DNA extraction. DNA from manure and runoff 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). 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 and runoff samples, so they were further quantified using published quantitative PCR (qPCR) protocols (Aminov *et al.* 2001; Ghosh *et al.* 2009; Koike *et al.* 2010). In addition to AMR genes, the 16S rRNA gene in each sample was also quantified using qPCR (Suzuki *et al.* 2000).

Results

Nutrients

Nutrient contents in the swine slurry samples were determined to be at 2.4 mg kg^{-1} NO_3-N, 3912 mg kg^{-1} NH_4-N, 5490 mg kg^{-1} TN, 580 mg kg^{-1} TP, 3.43% total solids, 42.4 dS m^{-1} EC, and 8.0 pH. Nutrient analyses were also performed on runoff samples obtained from plastic buckets containing cumulative rainfall from the 30 minute rainfall simulation events. The effects of slurry application method and rainfall time relative to manure application on nutrient loads in runoff were shown in Figure 2. Figure 2 shows that the broadcast method led to higher phosphorus loadings in runoff than did the injection method. The two methods did not cause much difference in the nitrogen loadings in runoff. Results also show that the timing of manure application relative to rainfall events had impacts on nutrient loadings in runoff. In order to determine if the effects observed in Figure 2 were statistically significant, analysis of variance (ANOVA) tests were conducted and their results were reported in Table 2.

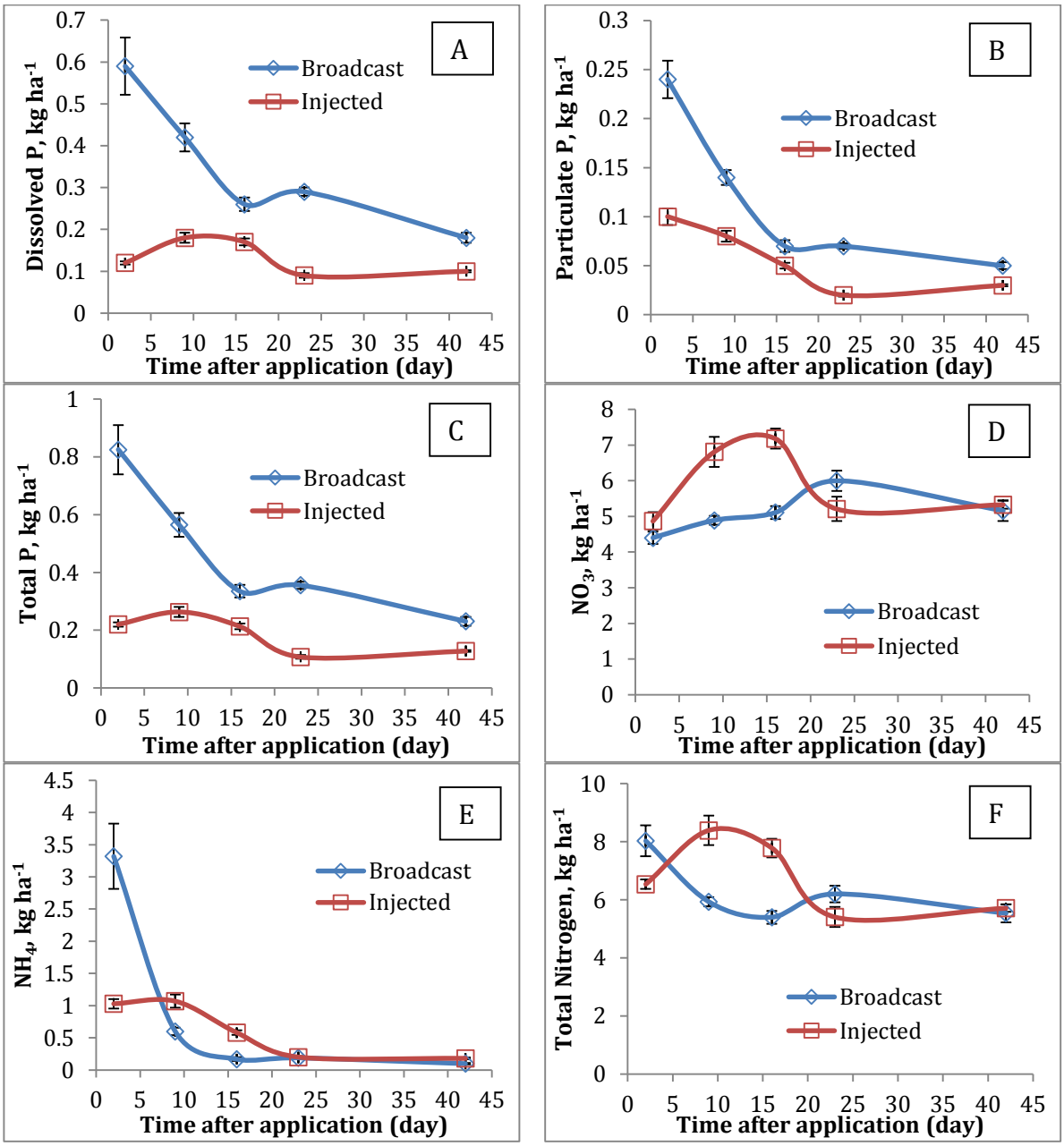


Figure 2. Transport of dissolved phosphorus (A), particulate phosphorus (B), total phosphorus (C), NO_3-N (D), NH_4-N (E), and total nitrogen (F) after manure application using broadcast and injection methods. Vertical bars represent the standard errors from replicate plots.

Runoff water quality characteristics were determined as affected by manure application method, time since manure application, and runoff rate using ANOVA (Table 2). No significant differences in mean NO_3-N transport rates were found based on slurry application method. Additionally, time since manure application did not have a significant effect on transport rates of NO_3-N. However, overland flow rate had a significant impact on the transport rate of NO_3-N with values consistently increasing from 314 to 1341 g ha^{-1} min^{-1}, as runoff rate increased from 2.3 to 12.6 L min^{-1}.

No significant differences in transport rates of NH_4-N were found between the broadcast and injection treatments (Table 2). Mean NH_4-N transport rates were significantly affected by time since slurry application, with values decreasing from 39.1 to 6.6 g ha^{-1} min^{-1} as time after slurry application increased from 2 to 44 days. Additionally, runoff rate had a significant impact on NH_4-N transport

rates, which increased from 13.9 to 25.1 g ha^{-1} min^{-1} as runoff rate increased from 2.3 to 12.6 L min^{-1}. The runoff transport rates for NH_4-N were much smaller than those measured for NO_3-N.

Runoff transport rates for TN were not significantly affected by manure application method or the time that had elapsed since slurry application. However, overland flow rate significantly impacted TN transport rates with values consistently increasing from 346 to 1460 g ha^{-1} min^{-1} as runoff rates increased from 2.3 to 12.6 L min^{-1}.

Table 2. Selected water quality parameters as affected by slurry application method, time since application, and runoff rate[a].

	DP (g ha^{-1} min^{-1})	PP (g ha^{-1} min^{-1})	TP (g ha^{-1} min^{-1})	NO_3-N (g ha^{-1} min^{-1})	NH_4-N (g ha^{-1} min^{-1})	TN (g ha^{-1} min^{-1})
Application method [a]						
Broadcast	24.6a	5.1	29.7a	818	19.6	902
Injected	16.6b	3.9	20.5b	869	22.3	949
Time (days)						
2	21.3	4.7	25.9	811	39.1a	938
9	25.2	7.2	32.3	979	31.3a	1140
16	19.9	4.7	24.5	834	14.9b	849
23	20.9	3.7	24.6	870	12.8b	884
44	16.0	2.4	18.3	722	6.6b	819
Runoff rate (L min^{-1})						
2.3	10.1d	2.8b	12.9d	314d	13.9c	346d
6	18.5c	5.2a	23.7c	699c	21.3b	770c
9.5	24.2b	4.2ab	28.4b	1020b	23.4a	1130b
12.6	29.8a	5.8a	35.5a	1341a	25.1a	1460a
ANOVA					Pr > F	
Application	0.01	0.27	0.01	0.53	0.57	0.61
Time	0.24	0.08	0.11	0.39	0.01	0.21
Runoff rate	0.01	0.01	0.01	0.01	0.01	0.01
Application x time	0.55	0.22	0.42	0.88	0.01	0.89
Application x runoff rate	0.01	0.21	0.01	0.60	0.13	0.48
Time x runoff rate	0.01	0.23	0.01	0.12	0.01	0.02
Application x time x runoff rate	0.24	0.60	0.38	0.71	0.01	0.71

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

Manure application method significantly affected DP and TP (Table 2). Time since slurry application did not significantly affect DP or TP measurements, while runoff rate has a significant impact. It is noticed that Application x Runoff Rate interactions were found for DP and TP (Table 2). For both broadcast and injection treatments, the transport rates of DP and TP in runoff increased with elevated runoff rates (Figure 3). Mean transport rates for DP and TP were significantly greater for the broadcasted plots than for the injection plots. Transport rates of 24.6 and 16.6 kg ha^{-1} min^{-1} were

obtained for DP and 29.7 and 20.5 kg ha^{-1} min^{-1} for TP for the broadcast and injection treatments, respectively.

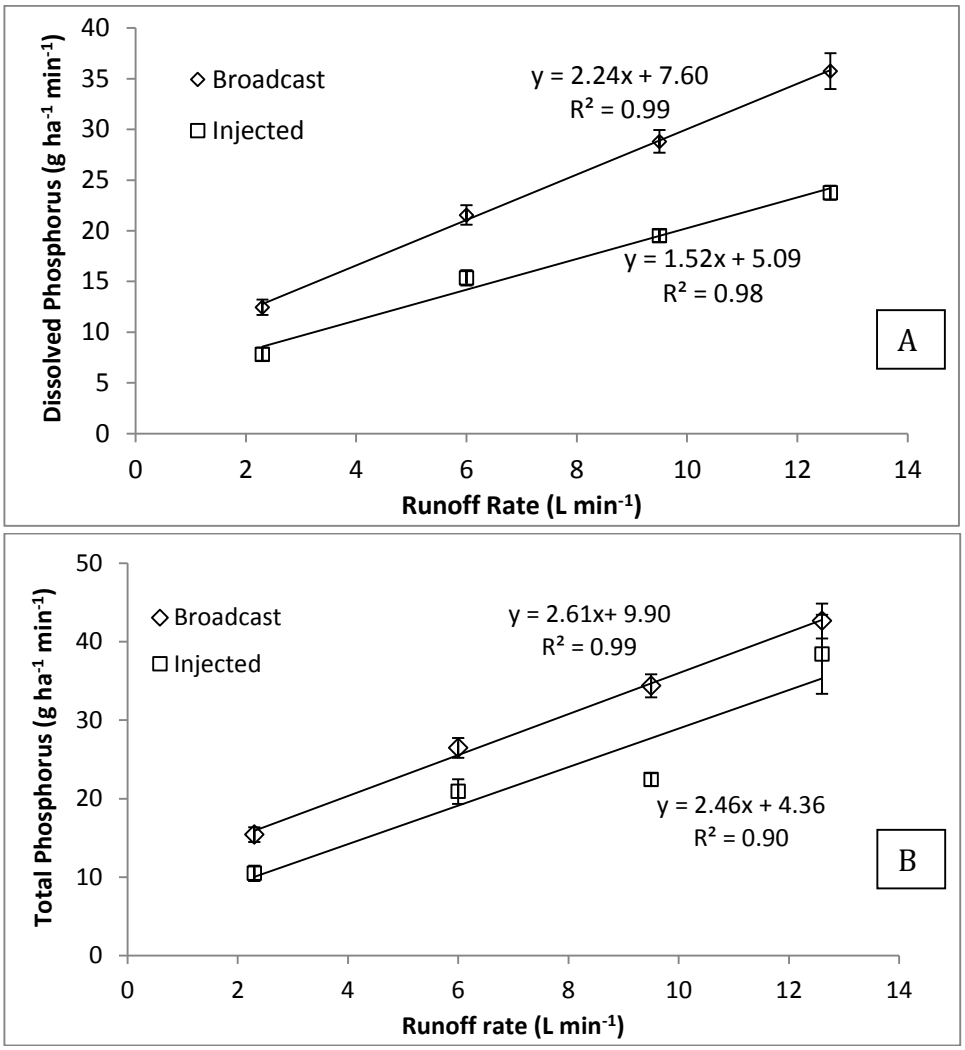


Figure 3. Transport rate of DP (A) and TP (B) as affected by runoff rate for the broadcast and injected plots. Vertical bars represent the standard errors from replicate plots.

Antimicrobials

The concentrations of the antimicrobials in the swine manure slurry that was land applied was determined in this study (Table 3). Table 3 provides results of antimicrobials detected in the fresh swine slurry (ng/g wet slurry). Chlortetracycline had the highest concentration in the slurry, while tiamulin and lincomycin were less abundant. No penicillin G was detected in the slurry sample. Hence, chlortetracycline, lincomycin, and tiamulin were focused in the antimicrobial analyses of the runoff samples.

Table 3. Concentration of antimicrobials detected in fresh swine manure slurry.

Compound	**Concentration (ng/g wet slurry)**
Chlortetracycline	3953.3
Lincomycin	49.4
Tiamulin	502.6
Penicillin G	Not detected

The concentrations of antimicrobials were higher in runoff from broadcast plots than from injected plots, and longer time between manure application and rainfall events led to lower concentrations of antimicrobials in runoff (Figure 4). Chlortetracycline was detected only during the first two weeks in runoff from broadcast plots at a maximum concentration of 0.14 μg/L, but only during the first week in runoff from injected plots. Furthermore, the concentrations of chlortetracycline in runoff from the injected plots were much lower than those from the broadcast plots. Lincomycin was detected in runoff from week 0 to week 3 in both broadcast and injected plots at maximum concentrations of 3.8 μg/L and 1.5 μg/L, respectively. Tiamulin was detected in runoff from plots with broadcast method from week 0 to week 3. However, this antibiotic was detected only during the first two weeks in runoff from injected plots. Overall, the concentration of these three antimicrobials was higher in runoff from plots with broadcast method than that found with injected method (Figure 4).

Concentrations of antimicrobials in runoff decreased with time between application and rainfall, with the lowest concentrations of antimicrobials occurring in runoff events conducted 3 weeks after manure application. This indicates that the timing of manure application relative to rainfall may be an important factor controlling antimicrobials occurrence in runoff.

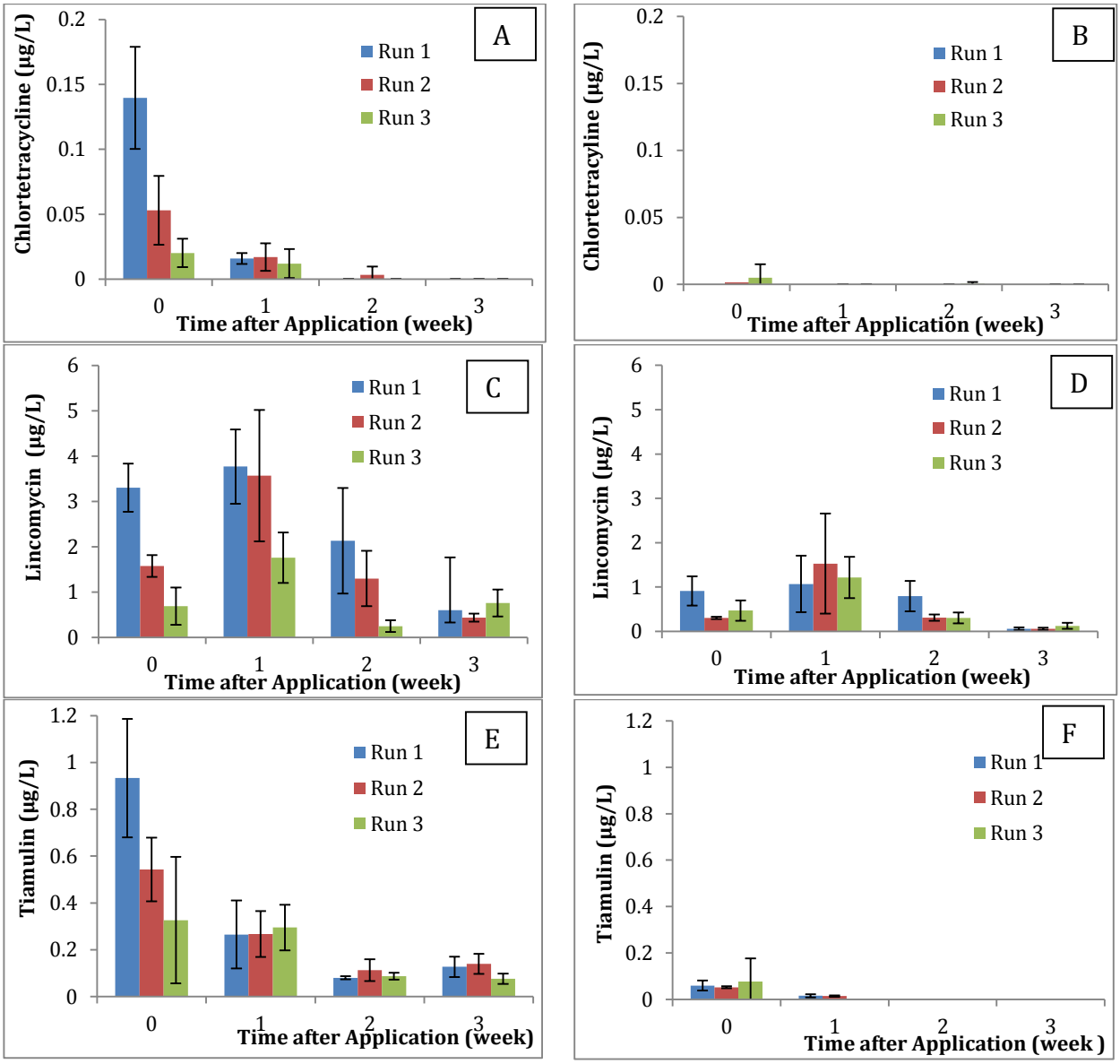


Figure 4. Concentrations of chlortetracycline, lincomycin and tiamulin in runoff from broadcast (A, C, E) and injected (B, D, F) plots when three consecutive rainfall events occurred 1 day (0 week), 1 week, 2 weeks, and 3 weeks after manure application. Error bars represent the standard deviations from replicate plots.

Antimicrobial Resistant Bacteria

A total of 131 samples were collected and analyzed for this project, including 1 source manure, 5 clean water, 43 pre-application soils, 3 pre-application runoff and 79 runoff samples. Selected samples were cultured for *Salmonella*, and quantified for fecal indicator bacteria, including Total Coliforms, *E. coli,* and *Enterococcus*. All samples were quantified for tetracycline resistant fecal bacteria. *E. coli* isolates, if present, were screened for resistance to 12 additional drugs.

Salmonella was not detected in the source manure, and did not grow after manure application to the soil. *E. coli* numbers were significantly lower (approximately two log reduction) in the runoff coming off of injected plots, compared to runoff from broadcast plots. Data show a steady decline in *E. coli* numbers over the course of the experiment for both application methods (Table 4).

Table 4. Fecal indicator counts.

Sample type (number of samples*)	Total Coliforms (MPN/mL)	*E. coli* (MPN/mL)	*Enterococcus* (MPN/mL)
Source Manure (n=1)	5.48E+03	4.04E+03	1.28E+04
Clean Water (n=5)	N/A**	N/A	N/A
Pre-application Soil (n=3)	N/A	N/A	N/A
Pre-application Runoff (n=3)	2.40E+04	7.15E-01	1.51E+03
Broadcast Runoff (n=6)	2.29E+04	1.22E+03	7.56E+02
Injected Runoff (n=6)	3.39E+04	3.16E+01	1.09E+03
Runoff 1 week post-application (n=6)	4.36E+04	1.24E+03	1.48E+03
Runoff 6 weeks post application (n=6)	1.31E+04	6.66E+00	3.65E+02

*Results (displayed in light blue) are presented here in two ways: First as a comparison of broadcast vs injected. Second as a comparison of isolates from samples collected at different times after the manure was applied to the plots.
**N/A = not assayed

Tetracycline resistance is thought to be common among environmental bacteria. Our experiments were designed to specifically measure tetracycline resistance associated with fecal bacteria, and to track the tetracycline resistant fecal bacteria applied to soils (Table 5). We found 15% of the fecal bacteria in the manure to be tetracycline resistant, compared to only 0.02% tetracycline resistance in pre-application soil. Manure application method (broadcast vs injection) did not impact how many bacteria were tetracycline resistant. Over six weeks there was an overall decrease in the total number of fecal bacteria in the runoff. However a modest increase was observed in the percent of fecal bacteria that were resistant to tetracycline, with the week 0 average at 2.06%, and the week 6 average at 3.81%.

Table 5. Enumeration of tetracycline resistant bacteria.

Sample type (number of samples*)	How many fecal bacteria are resistant to tetracycline** (what percent)?
Source Manure (n=1)	15%
Clean Water (n=5)	3%
Pre-application Soil (n=43)	<1%
Pre-application Runoff (n=3)	7%
Broadcast Runoff (n=40)	2%
Injected Runoff (n=39)	2%
Period 0 Runoff (n=16)	2%
Period 1 Runoff (n=16)	1%
Period 2 Runoff (n=16)	1%
Period 3 Runoff (n=16)	3%
Period 6 Runoff (n=15)	4%

*Results (displayed in light blue) are presented here in two ways: First as a comparison of broadcast vs injected. Second as a comparison of isolates from samples collected at different times after the manure was applied to the plots.
**Phenotypic resistance, as displayed by growth of colonies on media containing 16 µg/ml of tetracycline.

All *E. coli* isolates were screened for resistance to 12 common antibiotics (Table 6). The source manure isolates both displayed multiple drug resistance (MDR). Each isolate was resistant to four drugs – with only two shared resistances (ampicillin and tetracycline).

Table 6. *E. coli* Isolate Screening.

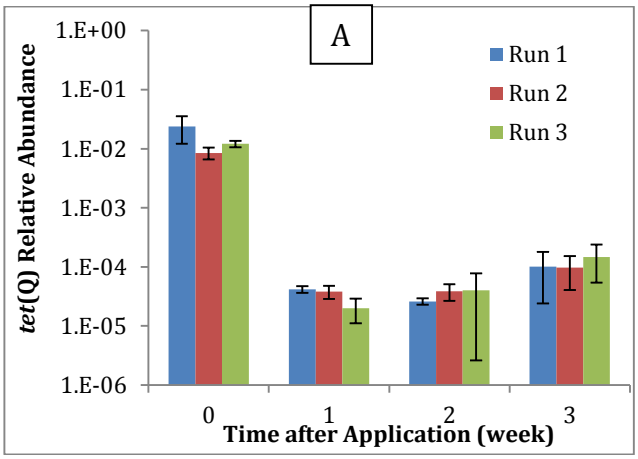
Sample type (number of samples*)	# of *E. coli* tested**	# of types of resistance (average)	# of types of resistance (range)
Source Manure (n=1)	2	4	4
Clean Water (n=5)	1	0	0
Pre-application Soil (n=43)	5	1	0-2
Pre-application Runoff (n=3)	3	2	1-2
Broadcast Runoff (n=40)	46	2	0-5
Injected Runoff (n=39)	55	2	0-10
Runoff **0 week** post-application (n=16)	26	2	0-10
Runoff **1 week** post-application (n=16)	23	2	0-5
Runoff **2 weeks** post-application (n=16)	21	2	0-5
Runoff **3 weeks** post application (n=16)	20	2	0-4
Runoff **6 weeks** post application (n=15)	11	2	0-4

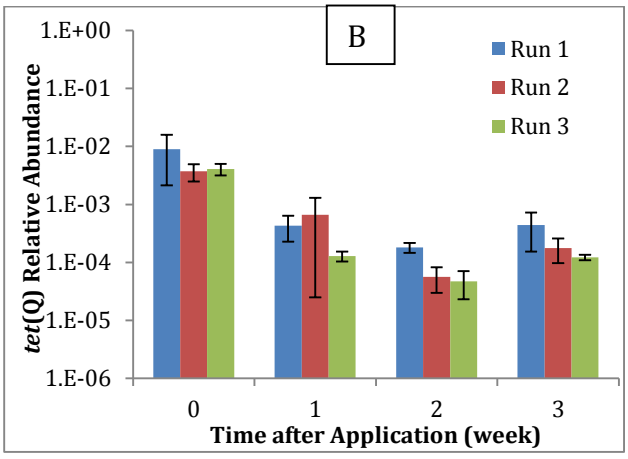
*112 total *E. coli* isolates were screened. Results (displayed in light blue) are presented here in two ways: First as a comparison of broadcast vs injected. Second as a comparison of isolates from samples collected at different times after the manure was applied to plots.

**Three isolates were picked per sample. Only those confirmed as *E. coli* were screened for multiple drug resistance.

Antimicrobial Resistance Genes

The two tetracycline resistance genes tested, *tet*(Q) and *tet*(X), behaved differently in this study. The relative abundance of *tet*(Q) decreased by about two orders of magnitude in runoff from both broadcast and injected plots from week 0 to week 3 (Figure 5). That is, the relative abundance started at about 1×10^{-2} copies of *tet*(Q) per copy of the 16S rRNA gene, and then decreased to about 1×10^{-4} copies of *tet*(Q) per copy of the 16S rRNA gene. The decrease mostly occurred during the first two weeks after manure application. In contrast, the relative abundance of *tet*(X) increased by about 3 orders of magnitude in runoff from both broadcast and injected methods after the first week and then maintained at that level for the rest of the 3-week test period.





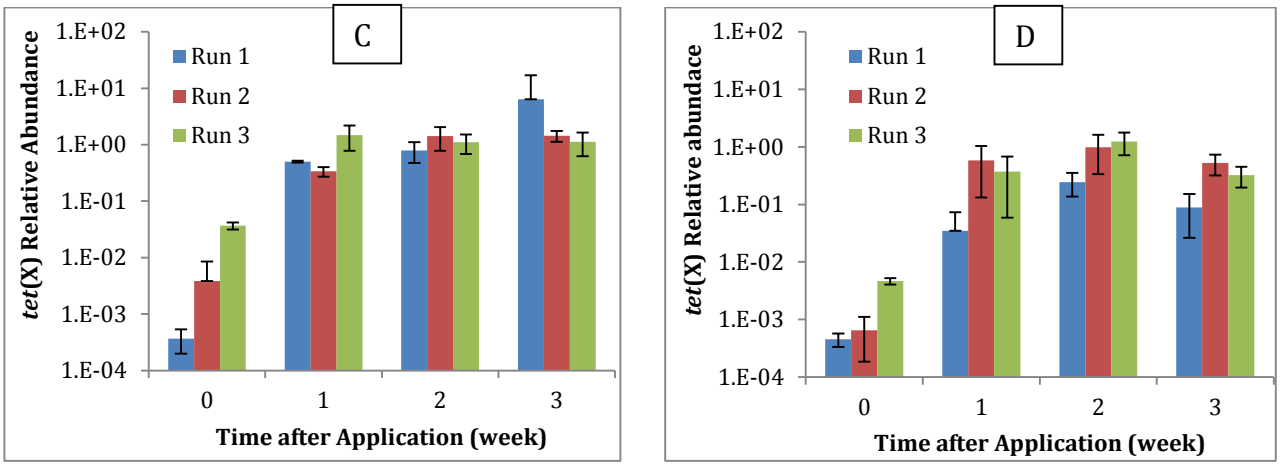
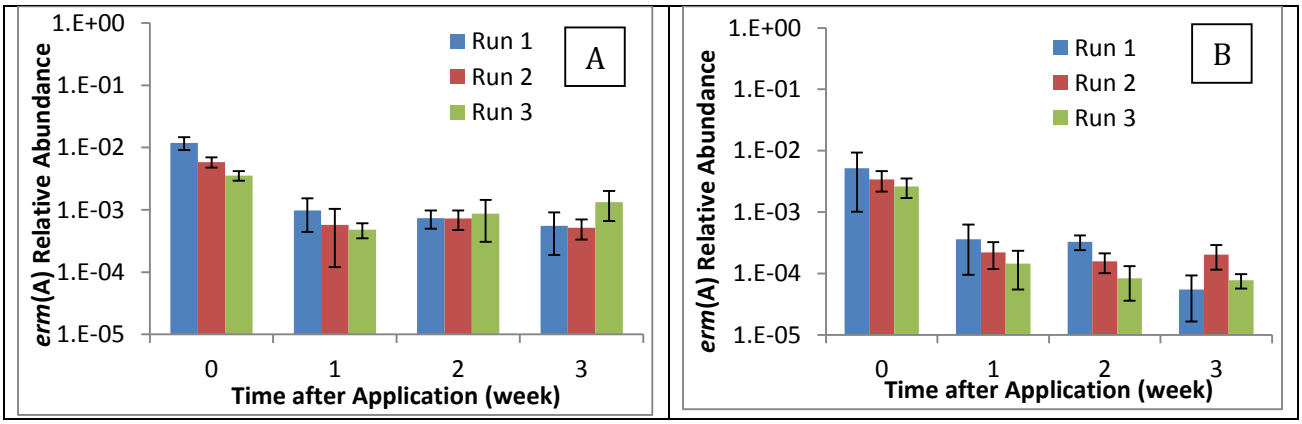


Figure 5. The relative abundances of tetracycline resistance genes, *tet*(Q) and *tet*(X), in runoff from broadcast (A and C) and injected (B and D) plots when three consecutive rainfall events occurred 1 day (0 week), 1 week, 2 weeks, and 3 weeks after manure application. Error bars represent the standard deviations from replicate plots.

The most common mechanism of resistance to lincosamides involves the dimethylation of a specific adenine of the 23S rRNA. This alteration of the antibiotic target site is invariably catalyzed by an rRNA methyltransferase encoded by the *erm* genes. Two *erm* genes, *erm*(A) and *erm*(B), were included in this study. They behaved similarly (Figure 6). For both the broadcast and injection methods, the relative abundance of the two *erm* genes in runoff followed a decreasing trend from week 0 to week 3 (Figure 6). The decrease was less pronounced in the runoff from the broadcast plots than in the runoff from the injected plots. For example, the relative abundance of *erm*(A) decreased about 1 order of magnitude in runoff from the broadcast plot, while decreased about 2 orders of magnitude in runoff from the injected plots. Among the three consecutive rainfalls in each week, the relative abundance of *erm* genes in the first runoff was typically higher than that in the second and third runoff.



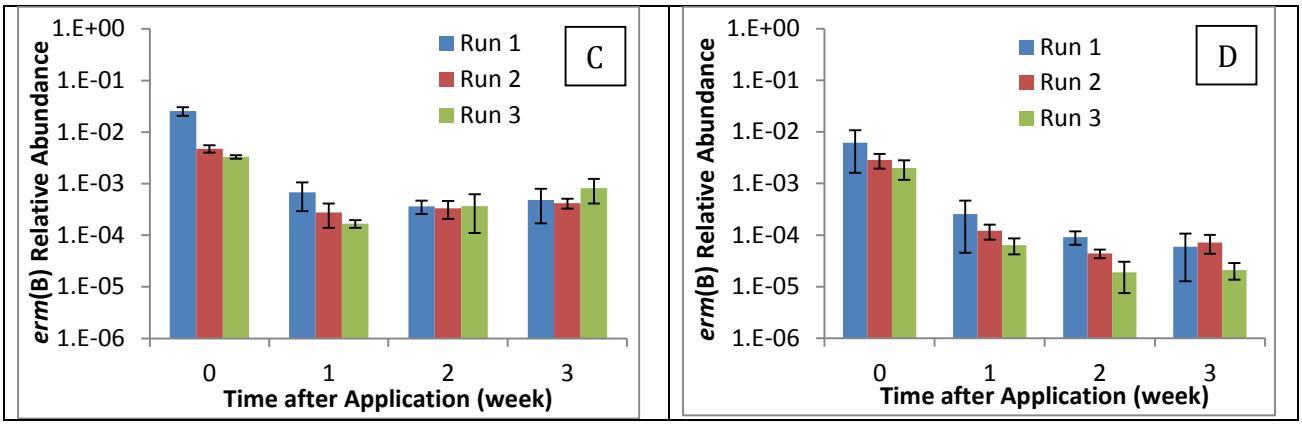


Figure 6. The relative abundances of lincosamide resistance genes, *erm*(A) and *erm*(B), in runoff from broadcast (A and C) and injected (B and D) plots when three consecutive rainfall events occurred 1 day (0 week), 1 week, 2 weeks, and 3 weeks after manure application. Error bars represent the standard deviations from replicate plots.

I. Discussion

Nutrient

The mean runoff loads from plots where slurry was applied had significantly greater DP, PP, TP, and EC values than check plots where no manure slurry was applied. However, slurry injection resulted in greater rates of soil loss than broadcast application, likely due to the tillage required in the injection application process.

Analysis of runoff water quality parameters revealed that time since slurry application had a significant impact on NH_4-N levels in runoff. As time following manure application increases, the nutrients present in organic forms in the manure begin to mineralize to plant-accessible forms. Therefore, the concentration of nutrients, once applied to soil, is subject to change over time.

The effect of overland flow rate on nutrient transport in runoff was also investigated. It was determined that increasing runoff rates had a significant impact on each of the measured water quality parameters, including DP, PP, TP, NO_3-N, NH_4-N, and TN. Values for each of the measured nutrient concentrations increased significantly with increasing overland flow rate. Manure application method, time since slurry application, and runoff rate should each be considered when estimating nutrient transport loads from areas on which swine slurry is applied.

Antimicrobials

The concentrations of antimicrobials were consistently lower in runoff from the injected plots than from the broadcast plots. When broadcast, the manure was spread on the soil surface, which made it easy for soluble antimicrobials to be flushed away with surface runoff. This finding agreed with one of our previous studies (Joy *et al.* 2013), where both injection and incorporation led to lower antimicrobial loadings in runoff than did broadcast. The lower concentrations of antimicrobials observed with increasing time between manure application and rainfall events was likely due to environmental degradation of the antimicrobials in soil.

Antimicrobial Resistant Bacteria and Genes

The observed pattern of decreasing fecal indicator numbers was not seen with total coliforms, which remained the same regardless of manure application method, and showed only a three-fold reduction over the six week sample collection period. The “total coliform” group includes a number of bacteria

that are associated with plants, and it is likely that the plant-associated bacteria contributed to the measurements we made from this set of agronomic fields. The *E. coli* numbers likely provide a more accurate representation of the fecal bacteria associated with swine manures applied to fields for this experiment.

The higher percentage of tetracycline resistant bacteria at week 6 compared to week 0 can be attributed to either an increased survival rate of the tetracycline resistant bacteria compared to the non-tetracycline resistant bacteria, or the selective growth of tetracycline resistant bacteria compared to the non-tetracycline-resistant bacteria. Since the overall bacteria numbers were declining, and since the *E. coli* fecal indicator numbers suggest that the fecal bacteria were not multiplying in the soil, the data from this experiment supports the idea that the tetracycline resistant fecal bacteria survive longer in the manure-amended soil compared to the non-resistant bacteria.

This idea is partially supported by the results from the tetracycline resistance gene analyses. Between the two *tet* genes that were consistently detected in the manure and runoff samples, the relative abundance of *tet*(Q) initially decreased and then stabilized through week 3, while the relative abundance of *tet*(X) initially increased before stabilizing at a relatively high level. The *tet* gene analyses suggest that tetracycline resistant bacteria carrying certain resistance genes (e.g., the ones carrying *tet*(X), which codes an oxidoreductase that can modify tetracycline molecules) may survive longer compared to non-resistant bacteria while resistant bacteria with other resistance genes (e.g., the ones carrying *tet*(Q), which codes a ribosomal protection protein that protects ribosome from the translation inhibition of tetracycline) may die off faster than non-resistant bacteria.

The behavior of *tet*(X) in this study was different from the ones reported in our previous study. In the previous study, swine manure was stored under anaerobic conditions to simulate the storage condition in deep pits (Joy *et al.* 2014). Over the course of 40 days, the relative abundance of *tet*(Q) and *tet*(X) both decreased. Different from the previous study, the manure was land applied and the microbes were exposed to the atmosphere in this study. Hence, the presence of oxygen appeared to affect the survival of tetracycline resistant bacteria.

The time between land application and rainfall events had significant impacts on the transport of AMR genes in runoff. Our results demonstrated that the survival of AMR bacteria in soil and the relative abundance of AMR genes in soil changed with time. Depending on the resistance genes, different time intervals would lead to different transport phenomena of AMR bacteria/genes in runoff. This study is a one step further from our previous study where rainfall simulation was conducted immediately after manure application (i.e., only one time interval) (Joy *et al.* 2013). In that study, land application methods appeared to have significant impacts on the transport of *erm* genes in runoff but not on *tet* genes in runoff.

On average, *E. coli* isolates from both broadcast and injection runoff displayed resistance to an average of two drugs (range 0-5 for broadcast, 0-10 for injected). In the runoff from the broadcast plots, 18% of the isolates were sensitive to all 12 antibiotics tested (pan-sensitive), compared to 28% pan-sensitive isolates in runoff from injected plots. So, although the *E. coli* isolate with the highest number of resistances (10/12) was found in an injected plot, overall the *E. coli* isolates from the injected plot were more likely to be sensitive to one of the 12 drugs measured in this study.

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