RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Influence of grass hedges on the transport of antimicrobials, antimicrobial resistance genes, and antimicrobial resistant pathogens after land application of swine manure - **NPB #12-012**

Investigator: Shannon L. Bartelt-Hunt

Institution: University of Nebraska-Lincoln

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Industry Summary:

Pork production is a significant component of the US economy. Swine manure has commonly been applied to land as a soil amendment for crop production, as it provides a valuable source of nutrients including nitrogen, phosphorus and potassium. When antibiotics are used in pork production, some fraction of the antibiotic can be excreted in manure and after land application, can be transported in runoff and enter surface water. Producers typically employ best management practices to minimize transport of these constituents after land application of swine manure. One common type of best management practice is the use of vegetated buffer strips, which have been demonstrated to reduce the transport of nutrients, bacteria and sediment in runoff from manure-amended land. To date, the ability of vegetated buffer strips to remove antimicrobials and antimicrobial resistance genes has not yet been evaluated. In this study, we investigated two objectives. Our first objective was to measure the quantities of bacitracin residues and antimicrobial-resistant pathogens/genes in swine manure and runoff from land-applied swine manure and our second objective was to quantify the removal of antimicrobials and AMR bacteria in runoff using a narrow grass hedge.

To meet these objectives, we conducted a controlled field study at the Rogers Memorial Farm at the University of Nebraska. Manure was collected from animals receiving antibiotics at the US Meat Animal Research Center in Clay Center, Nebraska, and transported to the field site, where it was applied to plots. Manure was applied to plots with and without a narrow grass hedge at a rate to meet the 3 year nitrogen requirement for corn. A series of 3 30-minute rainfall simulation experiments were conducted, and the runoff was collected from the plots. We observed that the grass hedge was consistently effective in removing tylosin from the runoff, and reduced the overall mass loading of tylosin in the runoff by 1 order of magnitude. We also evaluated removal of antimicrobial resistance genes and another gene common to bacteria, 16S rRNA. We observed mixed results for microbial gene removal in runoff, as we did not see a statistically significant removal of the resistance gene, but we did observe statistically significant removal of the other microbial gene. Taken together, our statistical analysis indicates that the grass hedges were effective at removal of microbial genes from runoff.

Our research results provide evidence that commonly used, low-cost best management practices such as narrow grass hedges can remove antimicrobials and microbial genes from runoff before it enters surface water.

For more information, please contact Dr. Shannon Bartelt-Hunt, Department of Civil Engineering, University of Nebraska-Lincoln, e-mail: sbartelt2@unl.edu, phone (402) 554-3868.

Keywords: antimicrobial, antimicrobial resistance genes, grass hedges, runoff

Scientific Abstract: The objective of this study was to determine the effects of narrow grass hedges on the fate and transport of antimicrobials and antimicrobial resistance genes (ARGs) in runoff and in soil following the land application of swine manure slurry. Swine manure slurry was land applied to 0.75m wide by 4.0m long plots established on an Aksarben silty clay loam soil located in southeast Nebraska. Swine manure was applied at a rate to meet the 3-year nitrogen (N) requirements for corn. Swine manure was applied to plots with and without a narrow grass hedge to evaluate the effect of the hedge on antmicrobial and ARG occurrence in runoff following 3 30-min simulated rainfall events. The grass hedge proved to be consistently effective in reducing concentrations of the antimicrobial tylosin in runoff ($p=0.016$), and the total mass of tylosin transport in the runoff was reduced by an order of magnitude in plots with the grass hedge compared to plots without the grass hedge. Because we did not observe significant differences in the total amount of runoff between plots, we can attribute the reduction in mass loading to the decrease in tylosin concentration in plots with the grass hedge. The results of the grass hedge on removal of ARGs in runoff was less clear cut. The effect of the grass hedge on removal of *erm*(B) was not statistically significant (p=0.2465), however, the grass hedge did significantly reduce the amount of 16S rRNA in the runoff. rANOVA results suggest that the narrow grass hedge had a significant effect on removal of microbial genes in runoff (p – 0.0014). To our knowledge, this is the first effort to evaluate the effect of common and low cost best management practices such as narrow grass hedges on the transport of microbial genes in runoff.

Introduction: Pork production is an important component of the U.S. economy with gross earnings of over $97 billion dollars in 2011. Swine manure provides a valuable source of nutrients including nitrogen, phosphorus and potassium, and has been historically used as a soil amendment for crop production. At present, rising fertilizer costs indicate swine manure will continue to represent a valuable component of crop fertility programs. Current swine industry practice is to house animals in confinement facilities with capture and storage of liquid or semi-liquid manure in pits or lagoons. Antimicrobials and other pharmaceuticals are often used at CAFOs at subtherapeutic levels for growth promotion and prophylaxis (Gaskins et al. 2002) and at therapeutic levels for disease treatment. The antimicrobials added in animal feed are often not completely absorbed in the animal gut, resulting in the potential for antimicrobial resistance among commensal and pathogenic bacteria (Salyers et al. 2004).

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

One technique that is currently utilized to limit the occurrence of constituents such as bacteria, nutrients and sediment in runoff from manure-applied fields is vegetative filter strips. Vegetative filter strips are zones of vegetation at the edge of a field through which sediment and pollutant flow are directed prior to discharge to a surface water body. Vegetative filter strips have been previously reported to be very effective for removal of suspended and dissolved constituents in runoff. In a recent study, Lin and co-workers reported as much as a 70% reduction in dissolved and sediment bound herbicides and antibiotics by VBs (Lin et al. 2011). Typically, vegetative filter strips remove constituents from runoff by three principal mechanisms: (1) deposition of suspended material due to reduction in flow velocity; (2) physical trapping of suspended material in litter that has accumulated on the soil surface; and (3) filtration of constituents from runoff that infiltrates into the soil matrix.

One type of vegetative filter strip is a narrow grass hedge, which often consist of stiff stemmed grass strips that are ~1.5 meter wide. Studies have indicated that narrow grass hedges can significantly reduce nutrient losses in runoff (Gilley et al. 2008).

Although narrow grass hedges have been demonstrated to be effective for removal of conventional constituents in runoff, such as sediment and nutrients, their performance for removal of antimicrobials antimicrobial resistance genes and/or antimicrobial resistant pathogens has not been evaluated. It is important to determine the effectiveness of commonly-used conservation practices on the removal of antimicrobials from runoff

Objectives: The occurrence of antimicrobials and AMR bacteria/genes in the environment is of increasing concern for the protection of human and animal health, yet we have little information on the role of common conservation practices for removing these constituents from runoff. In the proposed research, we will build upon a previous study to quantify the concentrations of the parent compound and degradation products of the polypeptide antimicrobial, bacitracin A, and AMR bacteria and genes present in runoff after land application of swine manure. We will evaluate runoff before and after treatment by a narrow grass hedge. Specific objectives of the research include:

1) Measure the quantities of bacitracin residues and AMR pathogens/genes in swine manure and runoff from land-applied swine manure

2) Quantify the removal of bacitracin and AMR bacteria in runoff using a narrow grass hedge.

Materials & Methods:

Manure Collection. Manure was collected from the USDA Meat Animal Research Center (MARC) in Clay Center, NE. Manure slurry from finisher pigs, housed in a mechanically ventilated barn (14 m x 59 m), was collected. Pigs were fed a corn and soybean-based diet and received 39.7 mg of commercial Zinc Bacitracin (BAC) per kg of ration. Underneath the slotted pen floor were pits, which were filled to an approximate depth of 0.5 m with well water. Manure was pushed through slots on the pen floor and was drained once a week from the pits using a pull-plug system. After draining, the plug was replaced and well water was added to refill the pits. In this study, slurry from the pits was pumped, using a submersible pump, into 20-L buckets and transported to the land application site every week. A subsample of the swine slurry was collected in 250 ml amber jars and transported in a cooler to UNL for antimicrobials and ARGs quantification.

Soil Sample Collection. The experiment site was located at University of Nebraska Rogers Memorial Farm, 18 km east of Lincoln, Nebraska. The site was cropped using a long term no till management system with controlled wheel traffic. Soil samples were collected from the top 2 cm of plots with and without grass hedges prior to the manure application and were air dried following collection. Soil cores (8-10 cm deep) were also collected from the control and

amended plots without grass hedge using acrylic tubes after the manure application and after the rainfall simulations were completed. Soil cores were transported to the lab at the University of Nebraska-Lincoln and were stored in -20 ºC refrigerator until further analyses.

Experimental Plot Setup. Twenty four 0.75 m by 4 m plots were prepared at the Rogers Memorial Farm: 12 plots without grass hedge and 12 with a narrow grass hedge. Plots were established to provide triplicates of varying manure application rates in plots both with and without a narrow grass hedge. Plots had a mean slope gradient of 3.6 % with overland flow in the direction of the 4 m dimension. The narrow hedges at the end of the test plots were 1.4 m wide switch grass (*Panicum virgatum*), and they were established during 1998 in parallel rows following the contour of the land hedge and spaced at intervals along the hill slope that allowed multiple passes of tillage equipment. The narrow grass hedges were part of a strip-cropping system and row crops were planted between the hedges. Corn was planted during the 2010 season and glyphosate was applied to control the weeds; precautions were taken to protect the grass hedge from herbicide application. A subplot treatment of varying rates of manure application was also included in this study. Based on an annual nitrogen requirement of 151 kg N ha^{-1} yr^{-1} for an expected yield of 9.4 Mg ha^{-1} of corn, swine slurry was applied to meet 0, 1, 2 and 3 times the annual nitrogen requirement, assuming ~70% of the total N in manure slurry is available to crops (Gilbertson et al. 1979). Slurry was weighed in the field and land applied accordingly. Manure rates were applied according in a randomized block design to avoid any bias. Plots were separated by 20 cm-wide sheet metal frames driven approximately 10 cm into the soil.

Rainfall Simulation and Runoff Collection. Rainfall simulations were done to test the effect of narrow grass hedge on the transport of antimicrobials and ARGs in runoff. Water used in the rainfall simulation tests was obtained from an onsite irrigation well. The irrigation water had a mean electrical conductivity (EC) of 0.77 dS m^{-1} and a pH of 7.2. Procedures for rainfall simulation established by the National Phosphorus Research Project (Sharpley and Kleinman 2003) were followed in this study. To ensure saturation and uniform antecedent soil moisture conditions in the plots, water was added to the plots using a garden hose prior to the rainfall simulations. A portable rainfall simulator based on the design by (Humphry et al. 2002) was used to apply rainfall to paired plots. Four rain gauges were placed on the outside edges of the plots and two in between the plots. A 30 minute rainfall event with an intensity of 70 mm $hr^{-1}$was simulated (Humphry et al. 2002). Two additional rainfall simulation tests of the same duration and intensity were conducted at approximately 24-hour intervals.

Runoff from the plot borders were channeled into a sheet metal lip that emptied into a collection trough located across the down gradient border of each plot, runoff was thereof diverted into plastic buckets. Accumulated runoff was continuously agitated to maintain suspension of solids while being pumped into large plastic storage containers using sump pumps. After each simulated rainfall event, storage containers were weighed to determine the total mass of runoff collected. Runoff samples were then transported in a cooler promptly to UNL and were stored at -20 ºC.

Antimicrobial Analysis of Soil, Manure and Runoff Samples. A solvent extraction method was utilized to extract antimicrobials from solid samples (soil and manure). Samples of soil (10g) or manure (0.2 g manure with 5 g clean sand) were well mixed with 14 mL of 5 mM ammonium citrate, buffered to pH 6 using ammonium hydroxide and 6 mL methanol, in 50-mL polypropylene centrifuge tubes. A surrogate (16 ng oleandomycin) was also added to each mixture to monitor the analyte recovery. Mixtures were shaken by hand briefly before putting them on a Burrell Wrist-action shaker for 30 min. Mixtures were centrifuged to separate solids and supernatant, which was decanted into a glass evaporation tube (RapidVap, Labconco Corporation). Extracts from the solids were obtained again using 4 mL of ammonium citrate and 16 mL of methanol and a third time with 20 mL of acetone. Extracts of each sample from the three extractions were pooled and then concentrated, to half the volume, on a RapidVap N2

sample concentrator at 30°C (90% rotation speed). 40 ng of Roxithromycin (internal standard for bacitracin A, bacitracin F, and tylosin) and 40 ng doxycycline (internal standard for chlortetracycline) were added prior to the concentration step. A final volume of 100 mL was obtained by adding purified reagent water to the concentrate. Resulting solutions were cleaned up using preconditioned 200 mg Oasis HLB™ solid phase extraction (SPE) cartridges. SPE cartridges were then eluted into borosilicate test tubes with 130 mM ammonium citrate in methanol. The volume of SPE elute was reduced to approximately 200 μL by a stream of dry nitrogen. The concentrated elute was transferred quantitatively to an autosampler vial with silane-treated insert and mixed with 200 μL reagent water. Recovery of chlortetracycline, bacitracin A, bacitracin F, and tylosin, was determined from extraction and quantification of fortified soils. Fortified blanks and method blanks were analyzed at a frequency of 1 in 20 samples. Method detection limits were determined by extraction and analysis of 8 replicates of clean sand fortified with antimicrobials. Method detection limit of antimicrobials in soil were 0.3 ng/g soil dry weight (dw) and 0.5 ng/g manure solid dw. Recoveries determined using 16 ng/g fortified soil were 57±13% for chlortetracycline, 78±6.5% for tylosin, and 12±46% for bacitracin (i.e., bacitracin A).

Runoff water samples were filtered through a 0.5 μm Gellman A/E binderless glass fiber filters using a vacuum system. To ensure removal of any volatile solids in the filters they were combusted at 550 degree C prior to the filtration step. SPE of the filtrates were performed using 200 mg Oasis HLB cartridges. Cartridges were then stored at -20ºC till the analysis of the extracts. SPE cartridges were processed in a similar manner as those used for the solids, using 3 mL of 0.1% formic acid in methanol, instead of ammonium citrate, for elution. To monitor analyte recovery a surrogate (16 ng oleandomycin) was also added to the methanol solution prior to the elution step. Method detection limits for antimicrobials in runoff extracts were determined by extraction and analysis of 8 replicates of reagent water samples fortified with antimicrobials at 0.01 μg/L. Recoveries determined using 0.004mg/L fortified water were 137±8% for chlortetracycline, 53±7% for tylosin, and 28±2% for bacitracin (i.e., bacitracin A). Electrospray ionization liquid chromatography-tandem mass spectrometry was used to analyze all the samples (Snow et al. 2003; Zhu et al. 2001).

High pressure liquid chromatography was employed to analyze the antimicrobial concentrations. Extracts from all the samples were analyzed with a Waters 2695 high pressure liquid chromatograph (HPLC) and thereafter with Waters Quattro Micro triple quadrupole mass spectrometer. Analytes were separated by placing them through a reverse phase (HyPurity C18, 250 mm x 2.1 mm, 5 μm particle size) column at 50°C. The column had an injection volume of 50-μL. A gradient mobile phase (0.2 mL/min), for separating extracts from runoff, was maintained through the column using A) 1 mM aqueous citric acid and methanol (97:3, v/v) and B) methanol and 1 mM aqueous citric acid (97:3, v/v). Initial gradient conditions (95% A) were held for 2 min and then at 5% A for 16 min and finally returned to 95% A for 5 min to equilibrate the column. Soil and manure extracts were put through the same gradient with an addition of a constant 4% component of 10% aqueous ammonium hydroxide with adjustments to the gradient to replace the aqueous component of mobile phase B.

Analytes were analyzed using Multiple Reaction Monitoring (MRM) mode with positive electrospray ionization (ESI). An infusion technique was used to determine the most intense MS/MS transitions (Appendix Table S2). Each analyte was monitored and linear calibration curves, with r^2 values of >0.99, were obtained for analytes and surrogates. Bacitracin A has a tendency to rapidly hydrolyze and degrade in water at near neutral pH. Hence a standard for bacitracin F, degradation product of bacitracin A, was synthesized and used to quantify this compound in the runoff samples (Pavli and Kmetec 2006).

ARGs in Soil, Manure and Runoff Samples. The top 2 cm soil was collected from amended and control plots before manure amendments. Soil cores were collected from the control plots after the manure amendment but before rainfall simulations, and after the 3rd rainfall

simulation. Soil cores (6-10" long) were extruded from acrylic sleeves and separated into top, middle, and bottom sections. The top two inches of soil were homogenized and analyzed for ARGs. For the runoff samples, solids were extracted by centrifuging 500 mL of well-mixed sample for 5 min at 10,000×g at 4°C in sterile 50-mL centrifuge tubes. Supernatants were decanted and pellets were stored at -20°C until DNA extraction. Manure slurry samples were handled in the same fashion, but only 30 mL of manure slurry was utilized.

DNA from runoff solids and soil was extracted using the MoBio UltraClean Soil DNA Isolation Kit (Solana Beach, CA) according to a high yield protocol except that a 40-sec bead beating was used to lyse the cells. Due to high protein contents in manure solids, DNA was extracted from these samples using the MoBio Power Soil DNA isolation kit (Solana Beach, CA) for higher DNA yields and higher A260/A280 ratios. DNA extracts were quantified using a NanoDrop 2000C spectrometer (Wilmington, DE). Regular PCR was run on manure samples for tylosin resistance genes *erm*(A), *erm*(B), *erm*(C) *and* erm(F) (Koike et al. 2007). Because *erm*(B) was the only ARG that was consistently detected in manure slurry and runoff samples, it was quantified using quantitative PCR (Koike et al. 2007) and used as an indicator for all ARGs. The detection limit of the qPCR protocol was determined as the minimum concentration in the linear range of the standard curve. In addition to ARGs, the 16S rRNA gene in each sample was also quantified using qPCR (Suzuki 2000).

Statistical Analysis. Repeated measures analysis of variance (rANOVA) tests were conducted using SAS (Cary, NC) to determine the effects of manure amendment (control vs. amended plots), narrow grass hedge (with vs. without grass hedge), and rainfall event (#1, #2, and #3) on the concentrations of antimicrobial and microbial genes in runoff and soil. If a treatment method was determined as significant ($p \leq 0.05$), least significant difference (LSD) tests were conducted to determine the significance of the differences among the treatment levels. To achieve a normal distribution data was transformed prior to ANOVA analysis. Only soil antimicrobial data was required to be transformed to the base of $\log_{10}$.

Results:

1) Measure the quantities of bacitracin residues and AMR pathogens/genes in swine manure and runoff from land-applied swine manure

Solids were collected from the manure slurry and examined for antimicrobials and ARGs. Among the antimicrobials tested (bacitracin, chlortetracycline, and tylosin), tylosin was the only antimicrobial that was consistently detected in the manure samples. Manure solids had an average moisture content of 76.95%. The average tylosin concentration in the manure slurry was 11.4 □g/kg manure on a wet weight (ww) basis or 49.40 □g/kg on a dry weight (dw) basis (Table 1). Consequently, only tylosin resistance genes were tested in the manure samples. Of the 6 tylosin resistance genes investigated (i.e., *erm*(A), *erm*(B), *erm*(C), *erm*(F) and *erm*(G)), *erm*(B) was the only ARG that was consistently detected in all manure samples. The average absolute abundance of *erm*(B) was 1.83×10^7 copies/mL manure slurry. Hence, *erm*(B) was used as a representative resistance gene to investigate the fate and transport of tylosin resistance genes in this study. In addition, the average absolute abundance of the 16S rRNA gene in manure was 1.44×10^8 copies/mL manure slurry (Table 1).

Table 1. Tylosin, *erm*(B), and the 16S rRNA gene concentrations (average ± standard error) in the swine manure slurries. The averages and standard errors were calculated based on fresh weekly manure samples collected over the 4-week field experiment (n=4).

Antimicrobial		**Microbial Genes**		
(□g/kg ww)	(□g /kg dw)	**(**copy/mL)	**(**copy/g ww)	**(**copy/g dw)
Tylosin		*erm*(B)		
11.40±0.75	49.40±3.18	(1.83 ± 0.66)×10^7	(1.37 ± 0.47)×10^9	(5.81 ± 1.99)×10^9
		16S rRNA gene		
		(1.44 ± 0.52)×10^8	(1.07 ± 0.38)×10^{10}	(4.52 ± 1.60)×10^{10}

2) Quantify the removal of bacitracin and AMR bacteria in runoff using a narrow grass hedge.

Antimicrobial Concentrations in Runoff. Three treatment factors were tested for their effect on runoff water quality: manure amendment (manure application to meet 0 vs. 3 times annual nitrogen demand by corn, or control vs. amended plots), narrow grass hedge (with and without narrow grass hedge), and rainfall events (#1, #2, and #3). Tylosin was detected in the runoff from the amended plots, but not in the runoff from the control plots (Table 2). Among the amended plots, the tylosin concentration in runoff decreased as the rainfall number increased (Table 2 and Figure 1). In addition, concentration of tylosin in the runoff from the amended plots with grass hedges was significantly lower than that from amended plots without grass hedge ($p = 0.0161$, Table 3), demonstrating that grass hedge could effectively reduce tylosin transport in runoff (Figure 1).

Table 2 Tylosin concentrations (average ± standard error) in runoff from control and amended plots with and without grass hedge. The average and standard error were calculated based on triplicate field tests.

Rainfall Event	**Control Plots**		**Amended Plots**	
	w/o Grass Hedge (□g/L)	**w/ Grass Hedge (□g/L)**	**w/o Grass Hedge (□g/L)**	**w/ Grass Hedge (□g/L)**
1	<MDL	<MDL	4.70 ± 1.08	0.35 ± 0.11
2	<MDL	<MDL	2.20 ± 0.81	0.17 ± 0.09
3	<MDL	<MDL	1.91 ± 0.81	0.17 ± 0.03

* MDL – The method detection limit is 0.01 □g/L

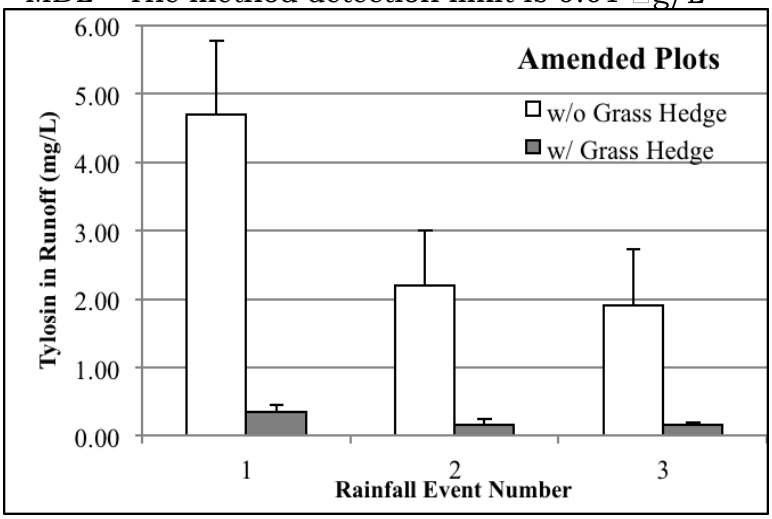

Figure 1 Concentration of tylosin in runoff from amended plots. Error bars represent standard errors from triplicate field experiments.

Table 3 rANOVA tests on the effects of manure amendment, grass hedge, and rainfall events on the concentrations of antimicrobials and microbial genes in runoff.

	TYL (μg/L)	***erm*(B)** (copy/mL runoff)	**16S rRNA gene** (copy/mL runoff)
Manure Amendment [*, #]			
Control plots	0.003 a	3.43×10^{2}	3.19×10^{6}
Amended plots	1.585 b	2.37×10^{4}	3.09×10^{6}
Grass Hedge			
No Grass Hedge	1.47 a	2.22×10^{4}	5.66×10^{6} a
Grass Hedge	0.12 b	1.89×10^{3}	6.12×10^{5} b
Rainfall Event			
1	1.26 a	1.68×10^{3} a	1.47×10^{6}
2	0.60 ab	2.20×10^{4} b	3.89×10^{6}
3	0.52 b	1.57×10^{4} b	4.06×10^{6}
rANOVA values for [Δ]			
Manure Amendment	0.0075	0.1875	0.9240
Grass Hedge	0.0161	0.2465	0.0014
Rainfall Event	<0.0001	0.0001	0.1132
Manure × Grass	0.0161	0.2598	0.6160
Grass × Rainfall	<0.0001	<0.0001	0.0457
Manure × Rainfall	<0.0001	0.0001	0.8477
Manure × Grass × Rainfa	<0.0001	<0.0001	0.9130

[*] Values reported under "Manure Amendment", "Grass Hedge", and "Rainfall Event" are treatment averages, which were calculated based on all the data for one particular treatment level. For example, 0.003 μg/L was calculated using TYL concentrations of all runoff samples from control plots, regardless whether they were from the plots with or without grass hedge or from which runoff event.
[#] Values followed by different letters are significantly different at the 95% probability level based on LSD tests.
[Δ] rANOVA values are displayed as *p* values.

rANOVA results showed that the effects of the 3-way interaction term, manure amendment × grass hedge × rainfall event, was of high statistical significance ($p < 0.0001$, Table 3). Furthermore, all the 2-way interaction terms and the individual treatment factors also had significant effects on the antimicrobial concentrations in runoff. According to the LSD analysis, the average tylosin concentrations in runoff were significantly different between the control and amended plots, and the plots with and without grass hedge.

ARG and the 16S rRNA gene in Runoff. According to the rANOVA analyses, the 3-way interaction terms and two of the 2-way interaction terms were significant (Table 3). Rainfall event is the only main treatment factor that had a significant impact on the *erm*(B) concentration in runoff ($p = 0.0001$). According to the LSD test, the average abundance of *erm*(B) in the first rainfall event was significantly lower than that in the second and third rainfall event (Table 3).
Effects of manure amendment, grass hedge, and rainfall events on the ARGs in runoff were analyzed by monitoring *erm*(B) in runoff solids. The absolute abundance of *erm*(B) in runoff from all control plots was orders of magnitudes lower than that from the amended plots (Table 4 and Figure 2) ($p = 0.1875$). Among amended plots, the absolute abundance of *erm*(B) in runoff from the plots with the

grass hedge was substantially lower than that from the plots without grass hedge (Table 3 and Figure 2) ($p = 0.2465$). The abundance of resistance gene in the runoff increased after the first rainfall event (Figure 2).

Table 4 The absolute abundance of erm(B) and the 16S rRNA gene (average ± standard error) in runoff from control and amended plots with and without grass hedge.

Rainfall Event	Control Plots		Amended Plots	
	w/o Grass Hedge (copies/mL)	**w/ Grass Hedge (copies/mL)**	**w/o Grass Hedge (copies/mL)**	**w/ Grass Hedge (copies/mL)**
	erm(B)			
1	$(1.25 \pm 0.25) \times 10^3$	$(4.00 \pm 1.40) \times 10^1$	$(1.39 \pm 0.54) \times 10^4$	$(8.65 \pm 2.87) \times 10^3$
2	$(4.47 \pm 1.56) \times 10^2$	$(6.00 \pm 3.00) \times 10^0$	$(8.57 \pm 2.55) \times 10^4$	$(2.18 \pm 1.36) \times 10^3$
3	$(2.46 \pm 1.43) \times 10^2$	$(3.30 \pm 1.60) \times 10^1$	$(6.23 \pm 1.74) \times 10^4$	$(4.13 \pm 1.96) \times 10^2$
	16S rRNA gene			
1	$(1.42 \pm 0.20) \times 10^6$	$(1.12 \pm 0.28) \times 10^6$	$(2.24 \pm 1.09) \times 10^6$	$(4.67 \pm 1.54) \times 10^5$
2	$(6.77 \pm 0.87) \times 10^6$	$(4.36 \pm 2.28) \times 10^5$	$(7.96 \pm 2.27) \times 10^6$	$(3.89 \pm 2.41) \times 10^5$
3	$(8.15 \pm 2.92) \times 10^6$	$(8.17 \pm 2.66) \times 10^5$	$(7.21 \pm 095) \times 10^6$	<MDL

The effect of the narrow grass hedge on the absolute abundance of the 16S rRNA gene in runoff was also investigated. The rANOVA analyses showed that for the 16S rRNA gene, the 3-way interaction term is not significant (Table 3, p=0.9130). The only 2-way interaction term that is significant is Grass × Rainfall ($p = 0.046$). Among individual treatment factors, Grass Hedge is the only significant factor ($p= 0.0014$). This is confirmed by the LSD test results, in which the average abundance of the 16S rRNA gene in runoff samples from plots with and without grass hedge was 6.12×10^5 and 5.66×10^6, respectively.

The absolute abundance of the 16S rRNA gene in runoff from plots with grass hedge was at least one order of magnitude lower than that from plots without grass hedge (Table 4, Figure 3). Similar to *erm*(B), among the amended plots, the 16S rRNA gene increased after the first rainfall event (Figure 3).

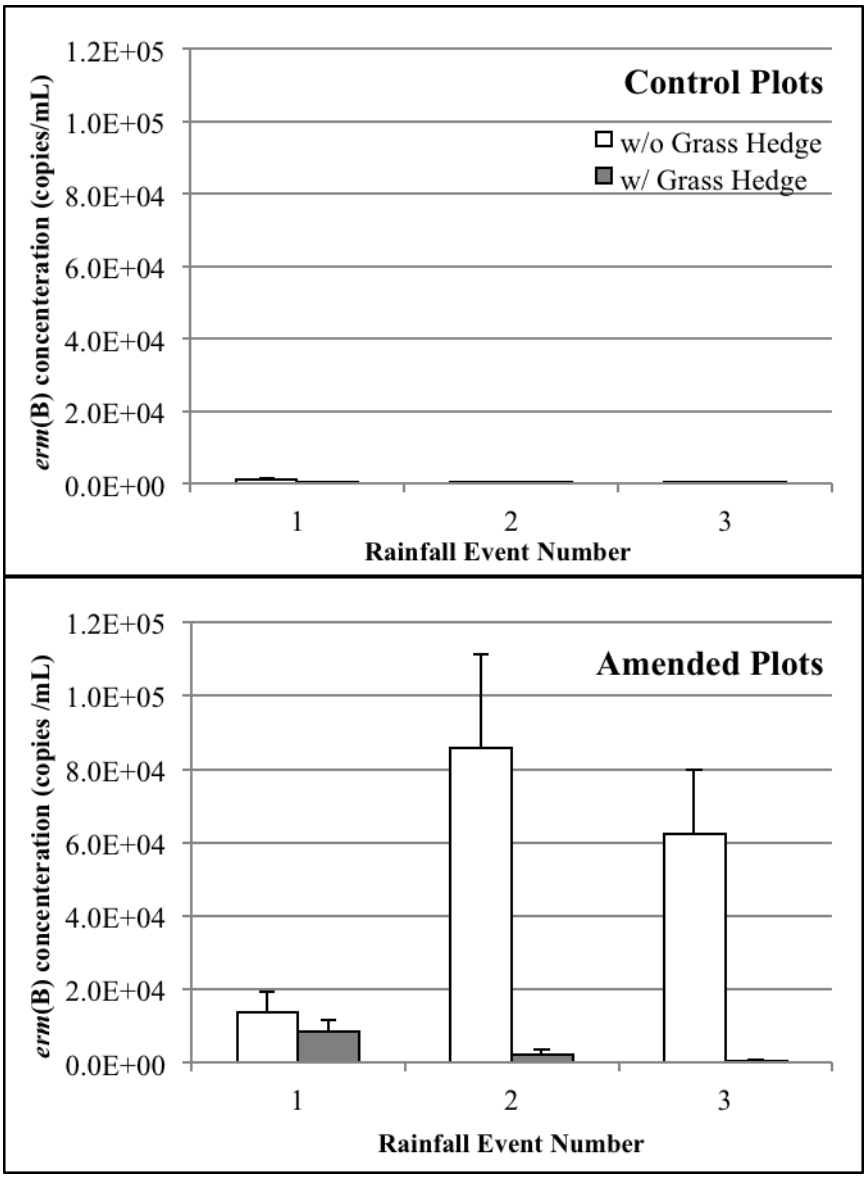

Figure 2 The absolute abundance of *erm*(B) in runoff from control and amended plots with and without narrow grass hedge. Error bars represent standard errors from triplicate field experiments.

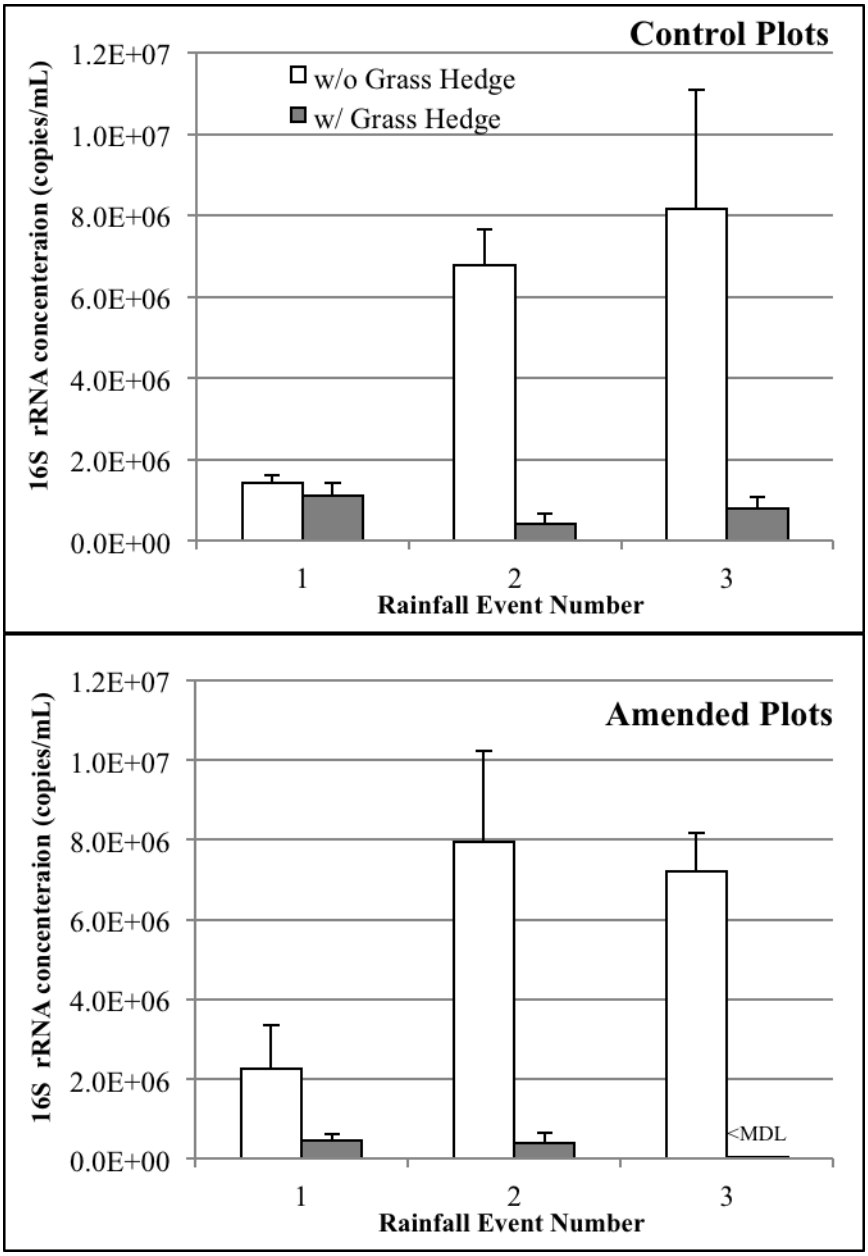

Figure 3 The absolute abundance of the 16S rRNA gene in runoff from control and amended plots with and without narrow grass hedge. Error bars represent standard errors from triplicate field experiments.

In addition to the absolute abundance of *erm*(B) gene, the relative abundance of *erm*(B) was also calculated by normalizing the ARG over the 16S rRNA gene (Figure 4). The relative abundance of *erm*(B) in runoff from amended plots was significantly higher than that from the control plots. Among amended plots, the presence of grass hedge led to a decreasing trend in the relative abundance of *erm*(B) over the rainfall events (Figure 4).

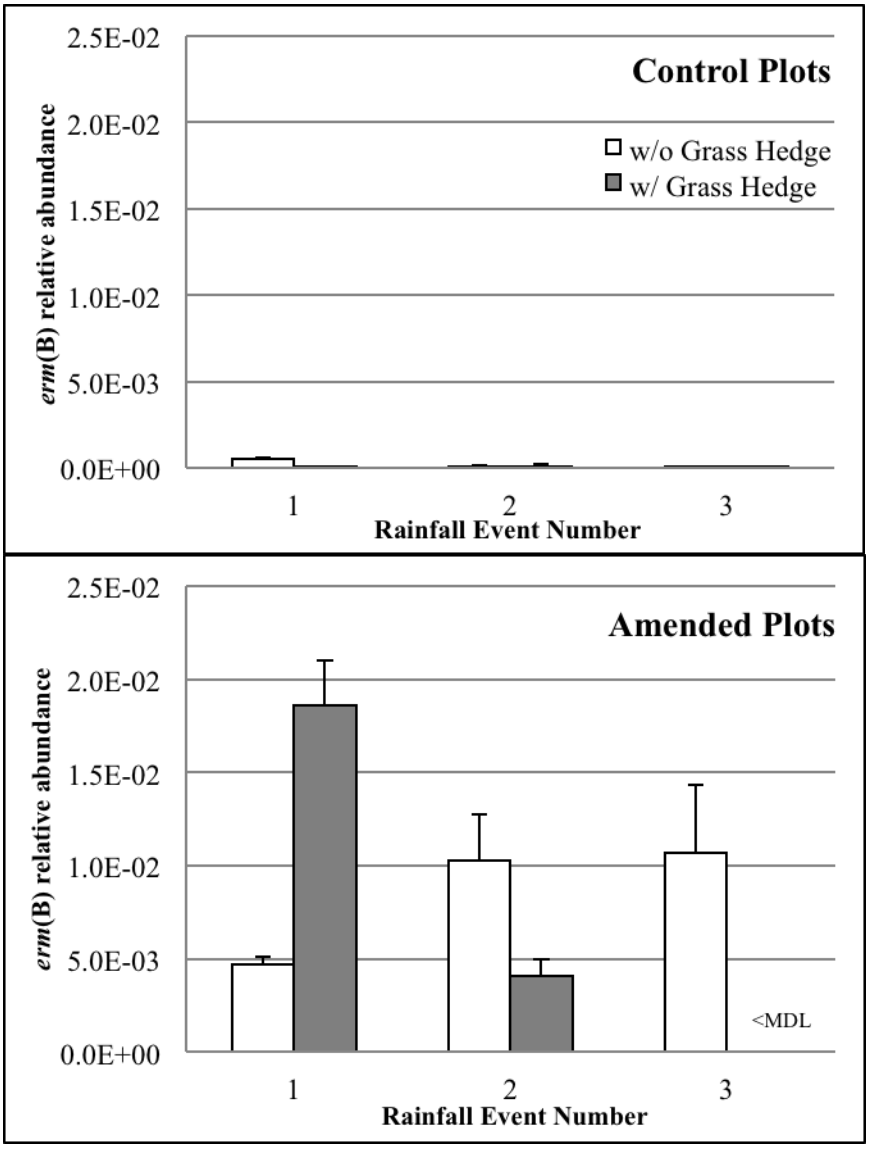

Figure 4 The relative abundance of *erm*(B) in runoff from three rainfall events. Error bars represent standard errors from triplicate field experiments.

Antimicrobial in Soil. Soil from the control and amended plots were tested for antimicrobials. No tylosin was detected in any soil sample collected prior to the land application of manure. In contrast, after land application of manure, the average tylosin concentration in the top soil of the amended plots was 8.70 ± 5.81 □g/kg of soil ww or 11.46 ±□□□□□□□g/kg soil dw. After the three rainfall events, the average tylosin concentration in the top soil was 7.27 ±□□□□□□□g/kg soil dw or 5.09 ± 1.57 □g/kg of soil ww (Figure 5). No tylosin was detected in the soils from the control plots at the two sampling times (Figure 5).

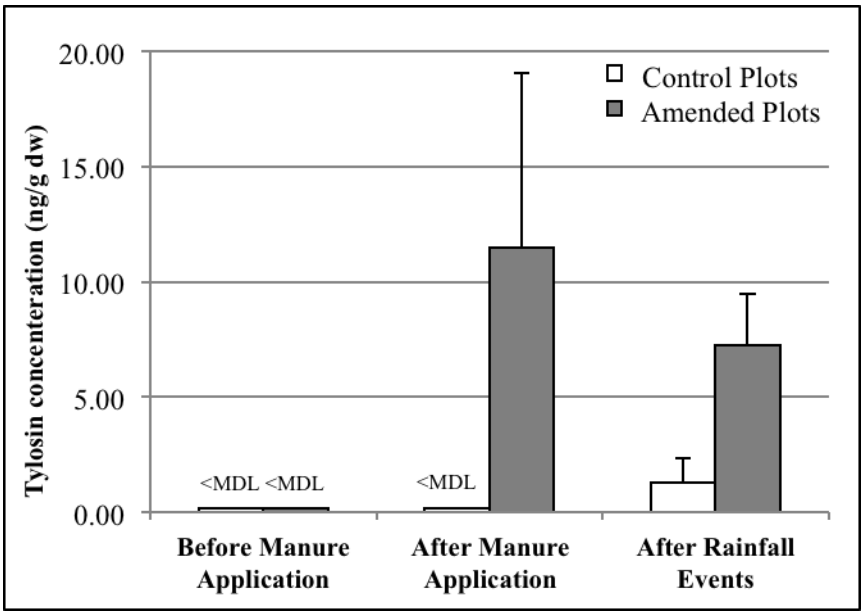

Figure 5 Concentration of tylosin in soils from control and amended plots. Error bars represent standard errors from triplicate field experiments. Method detection limit (MDL) was 0.3 ng/g soil dw.

rANOVA tests were conducted to investigate the effects of two main treatment factors, manure amendment (control vs. amended plots) and event (before manure application, after manure application, and after the three rainfall events), on the level of tylosin in top soil (Table 5). The tests showed that the 2-way interaction term of Manure × Event had a significant effect on the tylosin concentration in the soil (*p=0.016*, Table 5). The two individual treatment factors also had significant impacts on the tylosin concentrations in soil.

Table 5 rANOVA tests on the effects of manure amendment and events on the concentrations of antimicrobial and microbial genes in soil.

	Tylosin (μg/g)	***erm*(B)** (copy/g soil dw)	**16S rRNA gene** (copy/g soil dw)
Manure Amendment [*, #]			
Control plots	0.03 a	1.09×10^{4}	2.15×10^{9} a
Amended plots	4.10 b	1.24×10^{7}	3.00×10^{9} b
Event			
Before Manure Application	0.01 a	4.13×10^{3}	2.87×10^{9}
After Manure Application	0.98 ab	1.04×10^{7}	2.81×10^{9}
After Rainfalls	3.17 b	8.30×10^{6}	2.04×10^{9}
rANOVA values for [Δ]			
Manure Amendment	0.0141	0.2026	0.4494
Event	0.0038	0.5831	0.6914
Maure × Event	0.0163	0.5842	0.4681

[*] Values reported under "Manure Amendment", and "Event" are treatment averages, which were calculated based on all the data for one particular treatment level. For example, 0.03 μg/g was calculated using TYL concentrations of all soil samples from control plots, regardless whether they were before manure application, after manure application or after the rainfall events.
[#] Values followed by different letters are significantly different at the 0.05 probability level based on LSD tests.
[Δ] rANOVA values are displayed as *p* values.

ARG and the 16S rRNA gene in Soil. rANOVA tests showed that neither manure amendment nor rainfall events had significant effects on the abundance of *erm*(B) and the 16S rRNA gene (Table 5). The abundance of *erm*(B) increased in the top soil after manure application (*p=0.2026*) and decreased after rainfall events (*p=0.5831*) (Table 5). Plots receiving no manure had an average abundance of *erm*(B) at 2.63×10^4 copies/g soil dw after manure application and at 8.16×10^3 copies/g soil dw after rainfall events. Similarly, no significant change in the 16S rRNA gene copy number were observed after manure application (*p= 0.4494*) or after rainfall events (*p = 0.6914*, Table 5).
The absolute abundance of *erm*(B) in most of the triplicate field plots prior to the manure application were outside or at the lower end of linear range (Figure 6). The absolute abundance of *erm*(B) was back calculated from the Ct values of the qPCR results. Absolute abundance of *erm*(B) in the control and amended plots prior to manure application at 3.57×10^3 and 9.34×10^3 copies/g soil dw. Among amended plots, the absolute abundance of *erm*(B) in top soil increased to 2.07×10^7 copies/soil dw after manure application, and then dropped to 1.09×10^7 copies/soil dw (Table 6, Figure 6).
The 16S rRNA gene, prior to manure application, was detected at 2.67×10^9 copies/g soil dw, in the amended plots. There was no change in the 16S rRNA gene level in soil after manure application and after rainfall events (Figure 6, Table 6).

Table 6 Absolute abundance of *erm*(B) and the 16S rRNA gene (average ± standard error) in top soils of the amended plots, before manure application, after manure application and after three rainfall events. Standard errors were calculated based on triplicate field experiments.

Gene	**Before Manure Application (copies/g soil dw)**	**After Manure Application (copies/g soil dw)**	**After 3 Rainfall events (copies/g soil dw)**
erm(B)	$(9.34 \pm 2.18) \times 10^3$	$(2.07 \pm 0.84) \times 10^7$	$(1.09 \pm 0.86) \times 10^7$
16S rRNA	$(2.67 \pm 0.52) \times 10^9$	$(3.96 \pm 0.89) \times 10^9$	$(2.38 \pm 0.97) \times 10^9$

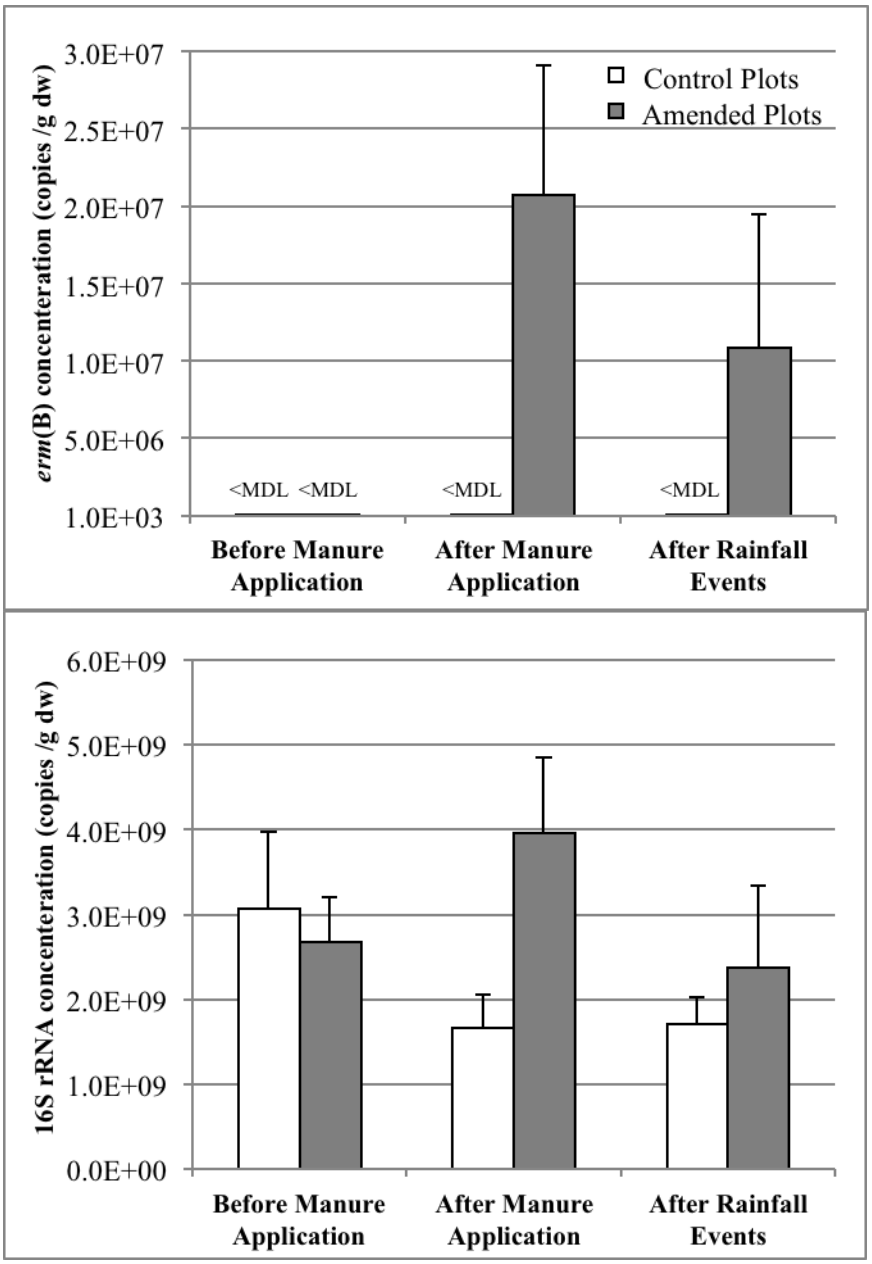

Figure 6 The absolute abundance of *erm*(B) and the 16S rRNA gene in soil (copy/g dw) before manure application, after manure application and after three rainfall events in control and amended plots. Error bars represent standard errors from triplicate field experiments.

The relative abundance of *erm*(B) in soil was calculated by normalizing ARG over the 16S rRNA gene (Figure 7). Among amended plots, as in the case of absolute abundance, the relative abundance increased substantially after the manure application and remained at a high level after the rainfall events.

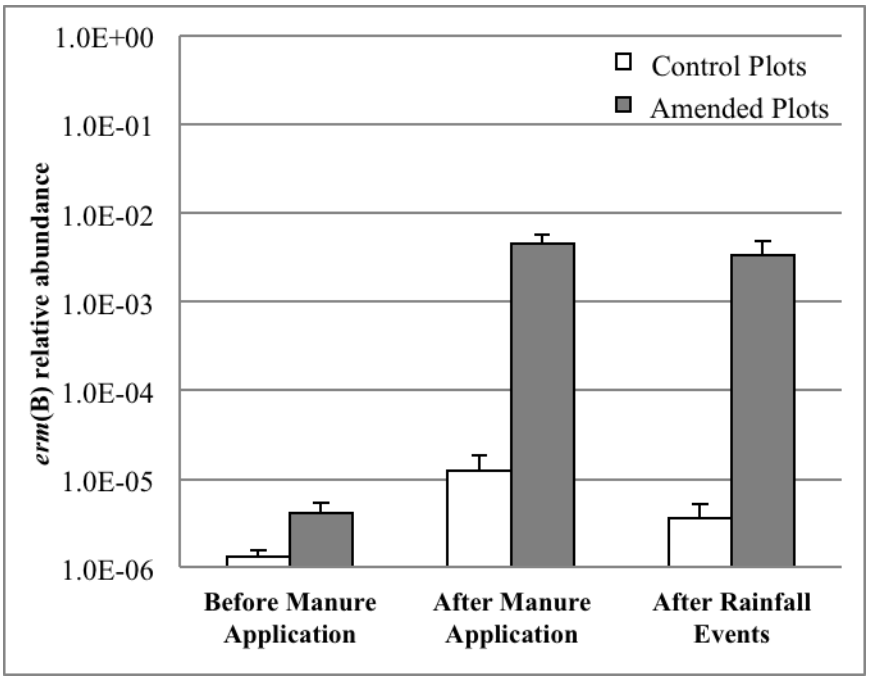

Figure 7 Relative abundance of *erm*(B) genes in soil before manure application, after manure application, and after three rainfall events in control and amended plots. Error bars represent standard errors from triplicate field experiments.

IX. Discussion: Manure slurry was analyzed for bacitracin, tylosin, and chlorotetracycline. Although bacitracin was administered to animals, it was not detected in any manure samples collected over the 4-week period. Bacitracin is known to have a short half-life and loses its antimicrobial activities at room temperature (Sarmah et al. 2006). Various microbiologically active components of bacitracin (bacitracin A) and their degradation products such as bacitracin F (Pavli et al. 2004) were also tested in the chemical analysis but none of them were detected in the manure samples. As the only antimicrobial compound that was detected consistently in all manure samples, tylosin had an average concentration of 11.4 □g/kg manure wet weight (ww). In another study conducted with manure from the same source, the tylosin concentration was reported at 290 □g/kg ww (Joy et al. 2013). Antimicrobial concentration in animal wastes is dependent on the dosage and frequency of antimicrobial being administered to the animals. It is also influenced by how and when the manure was collected.

It is difficult to compare the ARG levels in manure with the data reported in the literature, because ARG concentrations in manure are affected by various factors such as antimicrobial conditions, moisture content, and the age of manure. Presence of ARGs in swine manure have been reported in the literature as copies per gram of wet manure or fresh manure, which makes it even more difficult to compare the absolute abundance of ARGs as water content may vary widely. Using the same qPCR protocol, a recent study reported *erm*(B) at 1.6 x 10^4 copies/mL of manure slurry (Joy et al. 2013). The *erm*(B) level measured in this study was within the tylosin resistance genes range, 10^4 and 10^9 copies/mL fresh swine manure, reported in other studies (Chen et al. 2010; Chen et al. 2007).

In this study, tylosin concentrations in the runoff from the plots amended with manure were higher than those in the runoff from the control plots, which were largely below the MDL. Among amended plots, tylosin concentration in the runoff ranged between 0.08 and 6.1 □g/L, which are similar to previously reported values of 0.01 and 6 μg/L (Davis et al. 2006; Dolliver and Gupta 2008; Kim et al. 2010). For runoff from the amended plots, the tylosin concentration in the runoff decreased in subsequent runoff events. As much as 47 % of the

total antimicrobial load from the plots without a grass hedge were carried off in the initial rainfall event (Table 4).

Table 4 Mass loadings of tylosin exported in runoff from the amended plots with and without grass hedge during three rainfall events (average ± standard error). Averages and standard errors were calculated based on triplicate field experiments.

Rainfall event	Tylosin	
	w/o Grass Hedge (μg/m²)	**w/ Grass Hedge (μg/m²)**
1	48.47 ± 23.25	2.74 ± 1.77
2	33.69 ± 13.41	3.61 ± 3.29
3	20.50 ± 12.63	2.48 ± 0.59
Sum	102.65	8.87
Fraction from #1	0.47	0.31

The narrow grass hedge was very effective in reducing the dissolved antimicrobial load from the runoff. The narrow grass hedge lowered total antimicrobial loading in runoff by an order of magnitude (Table 4). Our results are comparable to the results from a study investigating the effects of narrow grass hedge on the runoff nutrient load which found that the dissolved phosphorous load was reduced by an order of magnitude from 0.69 to 0.08 kg/ha (Gilley et al. 2008). The dissolved antimicrobial load could have likely been reduced because of the enhanced infiltration and water holding capacity of the soils resulting from grass roots and plant evapotranspiration (Rachman et al. 2004). Since the total runoff from both the plots with and without the narrow grass hedge were approximately the same reduction in mass loading was due to the lower concentration of tylosin in runoff from the plots with a grass hedge (Table 3, Figure 3).

Although this study did not quantify tylosin bound to runoff solids, the grass hedges were thought to be effective in lowering solid bound tylosin in runoff because of their effectiveness in retaining runoff solids. Gilley et al. found that grass hedge reduced the runoff significantly; consequently soil erosion and nutrient transport (DP, TP NO_3-N, NH_4-N and TN) were also reduced by the use of the grass hedge (Gilley et al. 2008). A study by Hussen et al. found that the stiff grass hedge reduced the sediment loading in the outflow to 3.2 to 6.0% of the inflow concentrations (Hussein et al. 2007).

In contrast to the trend observed for antimicrobial, the abundance of ARG did not decrease as rainfall events proceeded: the absolute abundance of *erm*(B) increased in the second rainfall event and leveled off in the third rainfall event (Figure 2). Joy et al. (2013) reported that the absolute abundance of ARGs (*tet*(Q), *tet*(X) *erm*(B), *erm*(F)) in runoff from plots amended with swine manure applied by broadcast method decreased with rainfall events (Joy et al. 2013).

The grass hedge significantly reduced the amount of 16S rRNA gene in the runoff (Table 4 and Figure 3). rANOVA results suggests that the narrow grass hedge had a significant statistical effect ($p = 0.0014$) on microbial genes in runoff. The narrow grass hedge reduced the amount of suspended and dissolved solids in the runoff. Microbial population and DNA can be adsorbed to the surface of solids and reduction of solids in runoff leads to lower absolute abundances of the microbial genes in the runoff. The grass hedges were able to remove more than 90% of microbial DNA from runoff. We are not aware of another studies on the effects of narrow grass hedges on the transport of microbial genes in runoff.
To date, the following publications have resulted from this study:

Gilley, J.E.; Bartelt-Hunt, S.L.; Li, X.; Marx, D.B.; Snow, D.D.; Parker, D.B.; Woodbury, B.L. (2013). Narrow Grass Hedge Effects on Nutrient Transport Following Swine Slurry Application, Transactions of ASABE, 56: 1441-1450.

Soni, B.; Bartelt-Hunt, S.L.; Snow, D.D.; Woodbury, B.L.; Li, X. (2014). Influence of Narrow Grass Hedge on Antibiotic and Antibiotic Resistance Gene Transport Following Land Application of Swine Manure, in preparation.

X. References:

Chen, J., et al. (2010). *Microb Ecol*, 60(3), 479-486; Chen, J., et al. (2007). *Appl Environ Microb*, 73(14), 4407-4416; Davis, J. G., et al. (2006). *J Environ Qual*, 35(6), 2250-2260; Dolliver, H.A.S. and Gupta, S.C. (2008). *J. Environ. Qual.* 37: 1238-1244; Gaskins H. et al. (2002). *Animal Biotechnology* 13, 29–42; Gilbertson, C. B., et al. (1979). "Animal waste utilization on cropland and pastureland: A manual for evaluating agronomic and environmental effects." *Utilization Research Report No. 6.*, USDA, Washington D.C.; Gilley, J.E. et al. (2008). *Transactions of the ASAE* 51(3): 997-1005; Humphry, J.B. et al. (2002). *Appl. Eng. Agricul.* 18(2):199-204; Hussein, J., et al. (2007). *Soil Sci Soc Am J*, 71(5), 1516-1523; Lin, C. H., et al. (2011). *J Environ Qual*, 40(3), 791-799; Joy, S. R., et al. (2013). *Environ. Sci. Technol.* 47(21): 12081-12088; Kim, S. C., et al. (2010). *J Hazard Mater*, 175(1-3), 836-843; Koike, S. et al. (2007). *Appl. Environ. Microbiol.* 73, (15), 4813-4823; Lin, C. H., et al. (2011). *J Environ Qual*, 40(3), 791-799; Pavli, V. and V. Kmetec (2006). *Biol. Pharmacol. Bull.* 29: 2160-2167; Pavli, V., et al. (2004). *J. Liq. Chromatog. R T*, 27(15), 2381-2396; Rachman, A., et al. (2004). *Soil Sci Soc Am J*, 68(4), 1386-1393; Salyers, A. A. et al. (2004). *Tr. Microbiol.* 12, (9), 412-416; Sarmah, A. K., et al. (2006). *Chemosphere*, 65(5), 725-759; Sharpley, A. and Kleinman, P. (2003). *J Environ. Qual.* 32(6), 2172-2179; Snow, D. D., et al. (2003). *Liquid Chromatography/Mass Spectrometry, MS/MS and Time-of-Flight MS*, 850, 161-174; Suzuki, M. T., et al. (2000). *Appl Environ Microb*, 66(11), 4605-4614; Zhu, J., et al. (2001). *J Chromatogr A*, 928(2), 177-186