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Bhavneet Soni
University of Nebraska-Lincoln

Shannon L. Bartelt-Hunt
University of Nebraska-Lincoln, sbartelt2@unl.edu

Daniel D. Snow
University of Nebraska-Lincoln, dsnow1@unl.edu

John E. Gilley
USDA & UNL Biological Systems Engineering, john.gilley@ars.usda.gov

Bryan Woodbury
USDA-ARS, bryan.woodbury@ars.usda.gov

See next page for additional authors

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Bhavneet Soni, Shannon L. Bartelt-Hunt, Daniel D. Snow, John E. Gilley, Bryan L. Woodbury, David B. Marx, and Xu Li*

Abstract

Agricultural runoff from areas receiving livestock manure can potentially contaminate surface water with antimicrobials and antimicrobial resistance genes (ARGs). The objective of this study was to investigate the effectiveness of narrow grass hedges (NGHs) on reducing the transport of antimicrobials and ARGs in runoff after land application of swine manure slurry. Plot-scale rainfall simulation tests were conducted on 0.75 m by 4.0 m plots designed to test three treatment factors: manure amendment (control plots receiving no manure vs. amended plots receiving manure based on 3 times N requirement), NGH (plots with a NGH vs. plots without a NGH), and rainfall events (days 1–3). Runoff generated during three 30-min simulated rainfall events was sampled and analyzed for antimicrobials and ARGs. Manure amendment was responsible for the presence of antimicrobial tylosin ($p < 0.0001$) and tylosin resistance gene *erm*(B) ($p < 0.0001$) in runoff. Narrow grass hedges proved to be effective in reducing tylosin ($p < 0.0001$) and *erm*(B) ($p < 0.0347$) in runoff. Manure amendment was responsible for the introduction of tylosin ($p < 0.0482$) and *erm*(B) ($p = 0.0128$) into the soil; however, it had no significant impact on the abundance of the 16S rRNA gene in soil. Results from this study suggest that NGHs could be a best management practice to control the transport of antimicrobials and ARGs in agricultural runoff.

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*Corresponding author (xli4@unl.edu).

Livestock manure is often used as a soil conditioner because of its high organic matter and nutrient content. However, through land application, manure-borne contaminants, such as antimicrobials, pathogens, and antimicrobial resistance genes (ARGs) (Gessel et al., 2004; Accinelli et al., 2007; Byrne-Bailey et al., 2009; Heuer et al., 2011; Milinovich and Klieve, 2011), are introduced to the environment (Joy et al., 2013). Antimicrobials introduced to soil are susceptible to biotic and abiotic degradation, and their fate is affected by environmental factors such as sunlight, temperature, humidity, rainfall, and soil properties (Donoho, 1984; Sturini et al., 2012). Antimicrobial resistance genes introduced to soil can persist in the environment for an extended period of time (Byrne-Bailey et al., 2009; Zhang et al., 2013a; Zhang et al., 2013b). Antimicrobials and ARGs may also be transported from soils to surface water through runoff (Halling-Sorensen et al., 1998; Diaz-Cruz and Barcelo, 2005; Accinelli et al., 2007; Lee et al., 2007; Sanders et al., 2008; Chee-Sanford et al., 2009). Antimicrobial resistance genes are often associated with the solids in runoff (Guber et al., 2005), whereas antimicrobials can occur in both the aqueous and the solid phases in runoff (Davis et al., 2006; Kim et al., 2010).

Vegetative barriers (VBs) are strips of densely growing plants seeded downslope on croplands adjacent to surface water (Castelle et al., 1994). By reducing runoff volume and capturing sediment (Sudhishri et al., 2008), VBs can reduce dissolved and sediment-bound compounds in runoff (USDA–NRCS, 2000; Lin et al., 2007; Lin et al., 2011). Runoff sediment can be impeded by VBs because VBs reduce the kinetic energy of the runoff and sediments are settled by ponding of water upstream from the VBs (Meyer et al., 1995). Dissolved contaminants in runoff are reduced by VBs due to the reduction of runoff volume by enhanced infiltration and improved water holding capacity of the surface soil within VBs (Lin et al., 2011). In addition, agents such as naturally occurring chelators, sorbents, and microbes in

B. Soni, S.L. Bartelt-Hunt, and X. Li, Dep. of Civil Engineering, University of Nebraska–Lincoln, 844 N. 16th St., N117 SEC Link, Lincoln, NE 68588-6105; D.D. Snow, 202 Water Sciences Laboratory, 1840 North 37th Street, Lincoln NE, 68583-0844; J.E. Gilley, 251 LW Chase Hall, Lincoln, NE 68583; B.L. Woodbury, USDA Meat Animal Research Center, Clay Center, NE 68933; D.B. Marx, 342C Hardin Hall North, Lincoln NE 68588-0665. Assigned to Associate Editor Kim Cook.

Abbreviations: ARG, antimicrobial resistance gene; dw, dry weight; NGH, narrow grass hedge; qPCR, quantitative polymerase chain reaction; rANOVA, repeated measures analysis of variance; rRNA, ribosomal ribonucleic acid; SPE, solid phase extraction; VB, vegetative barrier.

VBs can remove and break down the dissolved contaminant in runoff (Pandey et al., 2005).

A narrow grass hedge (NGH) is one type of VB and is constructed using stiff-stemmed grass strips that are ~1.5 m wide and placed at short intervals along the contour of the hill slope. The stiff stems and upright growth of grass hedges provide filtration of the runoff and assist in the management of concentrated flow (Blanco-Canqui et al., 2004; Blanco-Canqui et al., 2006). The relatively short intervals between NGHs can impede runoff sediments within concentrated flows along the hill slope (Meyer et al., 1995). Narrow grass hedges are often effective in removing dissolved (Eghball et al., 2000; Owino et al., 2006; Gilley et al., 2011) and sediment-bound (Gilley et al., 2008) nutrients from runoff.

Although NGHs have been proven effective in reducing nutrients and pesticides in runoff, little is known about their effectiveness in reducing antimicrobials or ARGs in runoff from manure-amended soils. One study reported that a single 9-m-long grass strip could trap more than 99% of the soil, 91% of fecal coliform, and 74% of fecal streptococci in surface runoff (Coyne et al., 1998). Another study reported a 60% reduction in fecal bacteria with various VB formations on the watershed scale (Parajuli et al., 2008). Given that other forms of VBs have been proven effective in reducing dissolved and sediment-bound compounds and bacteria in runoff, it is plausible to expect that NGHs would be effective in limiting the transport of antimicrobials and ARGs in runoff.

The objective of this study was to determine the effectiveness of NGHs in controlling the transport of antimicrobials and ARGs in runoff after land application of swine manure slurry. Swine manure slurry was applied at zero and three times the annual nitrogen (N) requirement of a corn (*Zea mays* L.) crop, and rainfall events were simulated once a day for three consecutive days. Antimicrobial concentrations in manure, runoff, and soil were measured using high-performance liquid chromatography coupled with tandem mass spectrometry. The corresponding ARGs were analyzed using quantitative PCR (qPCR). Outcomes from this study are expected to be useful in determining whether NGHs may be adapted as a best management practice to control the dissemination of antimicrobials and ARGs from land-applied livestock manure to surface water.

Materials and Methods

Manure Collection

Manure was collected from the USDA Meat Animal Research Center in Clay Center, NE. Manure slurry from finisher pigs, housed in a mechanically ventilated barn (14 m by 59 m), was collected weekly from 5 to 28 July 2011. Pigs were fed a corn and soybean [*Glycine max* (L.) Merr.]–based diet at 2.9 to 3.4 kg dry matter intake per animal per day and received 39.7 mg of commercial zinc bacitracin per kg of ration. Pits underneath the slotted pen floor were filled with ~0.5 m deep water. Manure pushed through slots on the pen floor dropped to the pits and was flushed once a week using a pull-plug system. After draining, the plug was replaced, and water was added to refill the pits. In this study, slurry from the pits was pumped into 20-L buckets using a submersible pump and transported to the land application site every week. A subsample of the swine slurry was collected in 250-mL amber jars and transported to the University of Nebraska-Lincoln (UNL) on ice to measure antimicrobial and ARG concentrations. Slurry collected from the pull-plug system used in this study was more dilute than that found in a slotted or perforated floor waste management system with pit storage.

Field Experiments

The field experiment was conducted in the summer of 2011 at the UNL Rogers Memorial Farm (18 km east of Lincoln, NE), which was cropped using a long-term, no-till management system. Field experiments were designed to test three treatment factors: manure amendment (amended plots receiving manure based on 3 times N requirement vs. control plots receiving no manure [0 times N requirement]), NGH (plots with vs. without a NGH), and rainfall events (days 1–3). To ensure a clear distinction between the amended and control plots, an amendment rate of 3 times the annual N requirement for corn was used. Twelve 0.75 m by 4 m plots were established to provide three replicates for each amendment and NGH combination (Supplemental Fig. S1). Plots had a mean slope gradient of 3.6% with overland flow in the direction of the 4-m dimension. Narrow grass hedges, which were strips of switchgrass (*Panicum virgatum* L.), were located immediately adjacent to the bottom of selected test plots (Supplemental Fig. S1). They were established during 1998 in parallel rows following the contour of the land and were spaced along the hill slope at intervals that allowed multiple passes of tillage equipment. The NGHs were part of a strip-cropping system, and row crops were planted between the hedges. Based on an annual N 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 and 3 times the annual N requirement (i.e., control and amended plots), assuming that ~70% of the total N in manure slurry is available to crops (Gilbertson et al., 1979). Slurry was weighed in the field and broadcasted accordingly. Control and amended plots were assigned using a randomized block design. Plots were separated by 20-cm-wide sheet metal frames driven approximately 10 cm into the soil around the perimeter of three sides of the plots (except the bottom side of the plots). The sheet metal frames diverted runoff into a collection trough located at the bottom of the plot.

Rainfall Simulation and Runoff Collection

Rainfall simulations were performed to test the effects of NGH on the transport of antimicrobials and ARGs in runoff. Water used during the rainfall simulations was obtained from an on-site irrigation well, which had a mean electrical conductivity 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 identical antecedent soil moisture conditions in the plots, water was added to the plots using a garden hose before the rainfall simulations. A portable rainfall simulator (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 one was placed in between the plots. A 30-min rainfall event with an intensity of 70 mm h^{-1} was simulated (Humphry et al., 2002). The first rainfall simulation was conducted on control and amended plots a couple of hours after slurry application. Two additional rainfall simulation tests of the same duration and intensity were conducted at approximately 24-h intervals.

Runoff from the plots was channeled into a sheet metal lip that emptied into a collection trough located across the downgradient border of each plot and was collected in plastic buckets. On the plots that contained a NGH, runoff from the plots flowed through a 1.4 m NGH before emptying into the collection trough (Supplemental Fig. S1). Accumulated runoff was pumped into large plastic storage containers using sump pumps. After each simulated rainfall event, the storage containers were weighed to determine the total mass of runoff collected. Runoff samples were then transported in a cooler to the UNL lab and were stored at −20°C.

Soil Collection

Because the fate of manure constituents in soil was not affected by the presence or absence of the downslope NGHs, soil samples were only collected from plots without a NGH. Soil cores were collected before the field testing to assess the background concentrations of antimicrobials and ARGs. Soil cores were also collected from the control and amended plots (without a NGH) after manure application and after the third rainfall simulation. To collect soil core samples, a long soil core sampler (5 cm wide by 30 cm long) was used to obtain relatively undisturbed soil cores (5 cm long) into plastic liners. The plastic liners were then placed in coolers containing ice packs and transferred to UNL. Each soil core (0–5 cm) was homogenized and stored at −20°C until analyzed.

Antimicrobial Analysis of Soil, Manure, and Runoff Samples

Antimicrobials from different environmental matrices were extracted using protocols similar to those reported Joy et al. (2013) and measured on a liquid chromatography coupled to electrospray ionization Quattro Micro triple quadrupole mass spectrometer (Waters Corp.) (Zhu et al., 2001; Snow et al., 2003). Chlortetracycline, bacitracin A, bacitracin F, fenbendazole, and tylosin were included in the analyses. At near neutral pH, bacitracin A rapidly degrades to multiple relatively unstable products through oxidation and hydrolysis on exposure to air and water (Pavli and Kmetec, 2006). One of the more stable degradates in water is bacitracin F (Pavli et al., 2004). A standard for bacitracin F was synthesized from the parent compound, identified by mass spectrometry, and used in the analysis.

A solvent extraction method was used to extract antimicrobials from soil (10 g) and manure (0.2 g manure mixed with 5 g clean sand) samples (Joy et al., 2013). Recovery rates determined using eight replicate 16 ng g^{-1} laboratory-fortified soils were 57 ± 13% for chlortetracycline, 12 ± 46% for bacitracin A, 57 ± 13% for bacitracin F, 47 ± 13% for fenbendazole, and 78 ± 6.5% for tylosin. Laboratory-fortified blanks and method blanks were analyzed at a frequency of 1 in 20 samples. Method detection limits were measured by extraction and analysis of eight replicate laboratory-fortified soils (Keith et al., 1983) and were determined to be 0.3 ng g^{-1} soil dry weight (dw) and 0.5 ng g^{-1} manure solid dw.

Runoff samples were processed using solid phase extraction (SPE) (Joy et al., 2013). Briefly, a 500-mL water sample was spiked with 16 ng of oleandomycin (surrogate). Each sample was then extracted using a 200 mg Oasis HLB (Waters Corp.) SPE cartridge fitted with an in-line glass microfiber filter (Gelman Type A/E [25 mm], Pall Life Sciences) in a Telfon filter holder. After the extraction was complete, the cartridge was washed with 5 mL deionized water and dried under vacuum for 5 min. Each SPE cartridge was eluted with 3 mL ammonium citrate (0.133 mmol L^{-1}) in methanol, evaporated to ~200 μL under a stream of dry nitrogen, and mixed with internal standards (40 ng of both roxithromycin and doxycycline) and 200 μL purified reagent water. Recoveries determined using eight replicates of 4 ng L^{-1} fortified regent water were 137 ± 3% for chlortetracycline, 28 ± 8% for bacitracin A, 77 ± 33% for bacitracin F, 37 ± 20% for fenbendazole, and 53 ± 7% for tylosin. Method detection limits for antimicrobials in runoff were measured by extraction and analysis of eight replicates of fortified reagent water and averaged at ~0.01 μg L^{-1}.

Antimicrobial Resistance Genes in Soil, Manure, and Runoff Samples

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 except that 30 mL slurry was used.

DNA from runoff solids and soil was extracted using the MoBio UltraClean Soil DNA Isolation Kit according to a high-yield protocol except that a 40-s bead beating was used to lyse the cells. Due to high protein contents in manure solids, DNA in these samples was extracted using the MoBio Power Soil DNA isolation kit for higher DNA yields and higher A260/A280 ratios. DNA extracts were quantified using a NanoDrop 2000C spectrometer. Regular PCR was run on manure samples for tylosin resistance genes *erm*(A), *erm*(B), *erm*(C), *erm*(F), and *erm*(G) (Koike et al., 2010). Because *erm*(B) was the only ARG that was consistently detected in manure slurry and runoff samples, it was quantified using SYBR Green–based qPCR (Koike et al., 2010) and used as a representative ARG in this study.

In addition to ARGs, the 16S rRNA gene of total bacteria in each sample was quantified using qPCR (Suzuki et al., 2000). Each DNA extract was measured in duplicate PCR reactions. The detection limit of the qPCR protocol was determined as the minimum concentration in the linear range of the standard curve. Key qPCR parameters and the linear range for each primer set can be found in Supplemental Table S1. DNA extracts with C_t values outside of linear ranges (Supplemental Table S1) were counted as one half the lowest value on the linear range in calculating the average of gene abundance. While estimating the absolute abundance in a specific sample, the amount of original sample (i.e., manure slurry [manure solids], runoff [runoff solids], and soil) from which the DNA extract was obtained was also taken into consideration, leading to various detection limits for each matrix.

Statistical Analyses

Repeated measures ANOVA (rANOVA) tests were conducted using SAS to determine the effects of manure amendment (control vs. amended plots), NGH (with vs. without NGH), and rainfall event (days 1–3) on the concentrations

of antimicrobials and microbial genes in runoff and soil. If a treatment method was determined as significant ($p \leq 0.05$), Fisher's LSD tests were conducted to determine the significance of the differences among the variables.

Results

Antimicrobials and Antimicrobial Resistance Genes in Manure

Among the antimicrobials tested (i.e., bacitracin A, bacitracin F, chlortetracycline, fenbendazole, and tylosin), tylosin was the only antimicrobial that was consistently detected in the manure samples. Consequently, only tylosin resistance genes were tested in the manure samples. The average tylosin concentration associated with the solids in manure slurry was 11.40 μg kg^{-1} wet weight (ww) or 49.40 μg kg^{-1} dw basis (Table 1). Five tylosin resistance genes—*erm*(A), *erm*(B), *erm*(C), *erm*(F), and *erm*(G)—were examined using PCR. The only ARG that was consistently detected in all manure samples was *erm*(B); hence, *erm*(B) was the target ARG for the rest of this study. The absolute abundance of *erm*(B) and the 16S rRNA gene in manure was 1.83×10^7 copies and 1.44×10^8 copies mL^{-1} manure slurry, respectively (Table 1).

Tylosin in Runoff

Three treatment factors were tested for their effects on runoff water quality: (i) manure amendment (manure application to meet 0 vs. 3 times annual N demand by corn or control vs. amended plots), (ii) NGH (plots with and without a NGH), and (iii) rainfall events (days 1–3). For dissolved tylosin in runoff, manure × NGH was the only interaction term that was statistically significant ($p < 0.0001$). Both manure amendment and the presence of a NGH had significant effects on the presence of tylosin in runoff ($p < 0.0001$) (Table 2). Although tylosin concentrations in runoff decreased with successive rainfall events (Fig. 1), the impacts of this treatment factor were not significant ($p = 0.0583$) (Table 2). Consistent with the rANOVA tests, LSD analyses showed that both manure amendment and NGH had significant impacts on the tylosin level in runoff (Table 2).

Table 1. Tylosin, *erm*(B), and the 16S rRNA gene concentrations in the swine manure slurries.

Antimicrobial		Microbial genes		
μg kg^{-1} ww†	μg kg^{-1} dw‡	copy mL^{-1}	copy g^{-1} ww	copy g^{-1} dw
Tylosin			***erm*(B)**	
11.40 ± 0.75	49.40 ± 3.18§	$(1.83 \pm 0.66) \times 10^7$	$(1.37 \pm 0.47) \times 10^9$	$(5.81 \pm 1.99) \times 10^9$
			16S rRNA gene	
		$(1.44 \pm 0.52) \times 10^8$	$(1.07 \pm 0.38) \times 10^{10}$	$(4.52 \pm 1.60) \times 10^{10}$

† Wet weight.

‡ Dry weight.

§ Values are averages ± SE calculated based on fresh weekly manure samples collected over the 4-wk field experiment ($n = 4$).

Table 2. Statistical tests on the effects of manure amendment, narrow grass hedge, and rainfall events on the concentrations of tylosin and microbial genes in runoff.

	Tylosin	*erm*(B)	16S rRNA gene
	μg L^{-1}	copy mL^{-1} runoff	
Manure amendment†			
Control plots	0.003a‡	3.21×10^2a	4.19×10^6
Amended plots	1.583b	2.53×10^5b	1.61×10^7
Narrow grass hedge (NGH)			
Plots without a NGH	1.47a	2.43×10^5a	1.66×10^7a
Plots with a NGH	0.12b	1.09×10^4b	3.65×10^6b
Rainfall event			
1	1.26	3.43×10^5a	1.92×10^7
2	0.59	2.31×10^4b	5.61×10^6
3	0.52	1.44×10^4c	5.61×10^6
rANOVA values for§			
Manure amendment	<0.0001	<0.0001	0.1125
NGH	<0.0001	0.0347	0.0390
Rainfall event	0.0583	0.0007	0.2093
Manure × NGH	<0.0001	0.1987	0.1305
NGH × rainfall	0.1081	0.1310	0.8931
Manure × rainfall	0.0580	0.4329	0.0146
Manure × NGH × rainfall	0.1036	0.8718	0.0191

† Values reported under "Manure amendment," "NGH," 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^{-1} was calculated using tylosin concentrations of all runoff samples from control plots regardless whether or not they were from the plots with or without NGH or from which runoff event.

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

§ Repeated measures ANOVA (rANOVA) values are displayed as *p* values.

erm(B) and the 16S rRNA Genes in Runoff

The effect of the three treatment factors on the ARGs in runoff were analyzed by monitoring *erm*(B) in runoff. According to the rANOVA analyses, none of the interaction terms was significant, whereas all three treatment factors had statistically significant impacts on the level of this ARG in runoff (Table 2). This observation was supported by LSD test results (Table 2). The absolute abundance of *erm*(B) in the runoff from the control plots was several orders of magnitude less than that from the amended plots (Fig. 2; Supplemental Table S2), indicating that manure application caused the occurrence of *erm*(B) in runoff. The absolute abundance of *erm*(B) in runoff from the plots with a NGH was an order of magnitude less than that from plots without a NGH (Fig. 2; Supplemental Table S2), suggesting that NGH effectively reduced ARG levels in runoff. Finally, the absolute abundance of *erm*(B) in runoff from amended plots decreased with multiple rainfall events (Fig. 2; Supplemental Table S2).

The rANOVA analyses showed that the three-way interaction term had significant effects on the absolute abundance of the 16S rRNA gene in runoff ($p = 0.0191$) (Table 2). The only treatment factor that showed a significant impact was NGH ($p = 0.0390$) (Table 2). Least significant difference test results confirmed the levels of the 16S rRNA gene in runoff from plots with a NGH were significantly lower than those from plots without a NGH (Table 2; Fig. 2).

The relative abundance of *erm*(B) was calculated by normalizing the ARG over the 16S rRNA gene (Supplemental Fig. S2). The relative abundance of *erm*(B) in runoff from amended plots was significantly higher than that from the control plots. Among amended plots, the relative abundance of *erm*(B) from plots without a NGH decreased with rainfall events, whereas that from plots with a NGH did not exhibit a pronounced decreasing trend (Supplemental Fig. S2).

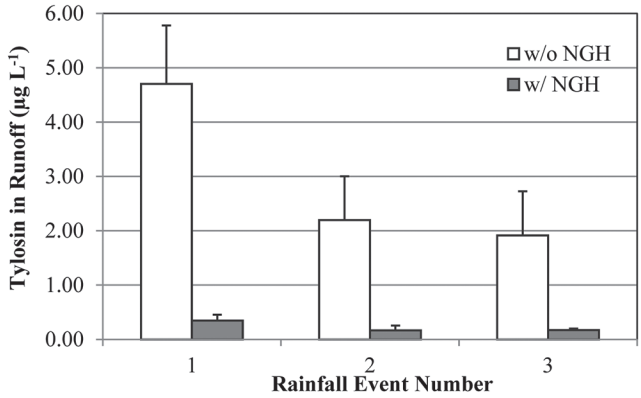

Fig. 1. Concentrations of dissolved tylosin in runoff from manure-amended plots with and without a narrow grass hedge (NGH). Error bars represent SE from triplicate field experiments.

Tylosin in Soil

Soil samples from all plots were tested for antimicrobials. Tylosin was not detected in any soil sample collected before the land application of manure. After land application, the average tylosin concentration in the topsoil of the amended plots was 11.46 ± 7.60 μg kg^{-1} soil dw or 8.70 ± 5.81 μg kg^{-1} of soil ww. After the three rainfall events, the average tylosin concentration in the topsoil was 7.27 ± 2.21 μg kg^{-1} soil dw or 5.09 ± 1.57 μg kg^{-1} of soil ww (Fig. 3). No tylosin was detected in the soils from the control plots at the two sampling times (Fig. 3).

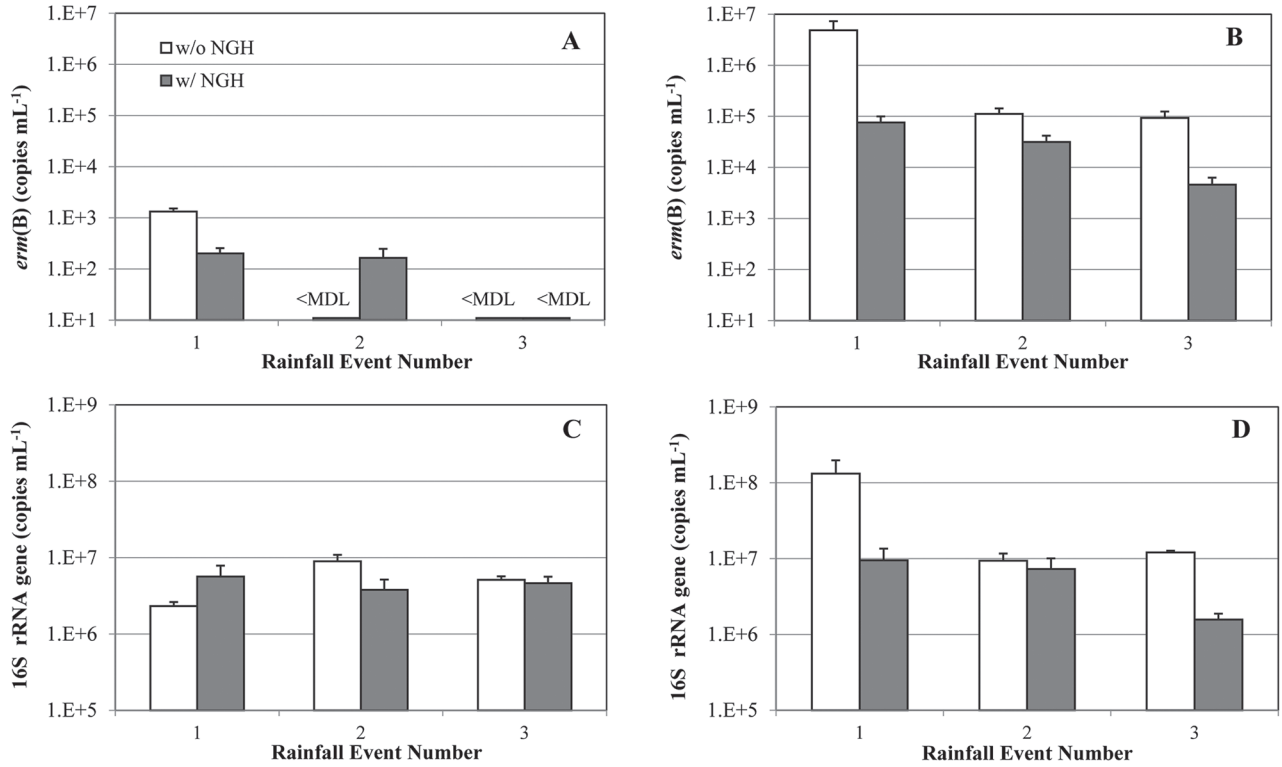

Fig. 2. The absolute abundance of *erm*(B) and the 16S rRNA gene in runoff from the control (A and C) and the amended (B and D) plots with and without a narrow grass hedge (NGH). Error bars represent SE from triplicate field experiments.

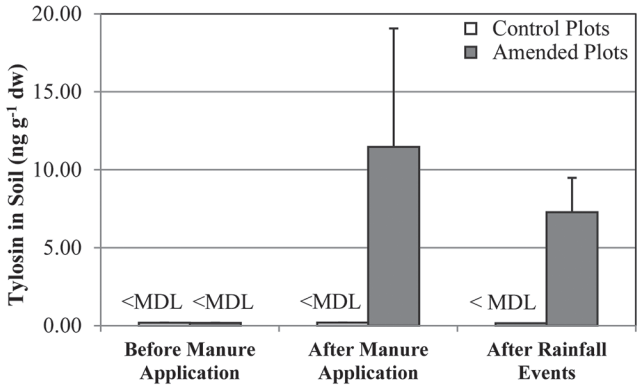

Fig. 3. Concentrations of tylosin in topsoils from control and amended plots. Error bars represent SE from triplicate field experiments. The method detection limit (MDL) was 0.3 ng g^{-1} soil dry weight (dw).

Statistical 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 3). The rANOVA tests showed that manure application was the only treatment that significantly affected tylosin levels in soil ($p = 0.0482$) (Table 3). Least significant difference tests confirmed that the differences in tylosin levels between the control and the amended plots were statistically significant.

erm(B) and the 16S rRNA Genes in Soil

The absolute abundance of *erm*(B) in the control plots was below the detection limit throughout the experiment, whereas that in the amended plots was substantial (Fig. 4), indicating that manure application had a significant effect on the ARG levels in soil ($p = 0.0128$) (Table 3). The abundance of *erm*(B) increased in topsoil after manure application and decreased after rainfall events (Fig. 4; Supplemental Table S3). According to LSD tests, the changes in *erm*(B) level between events were significant (Table 3). There was no significant change in the 16S rRNA abundance after manure application ($p = 0.6437$) or after rainfall events ($p = 0.6984$) (Table 3). The absolute abundance of the 16S rRNA gene in soil remained at around 10^9 copies g^{-1} soil dw throughout the course of experiment (Supplemental Table S3).

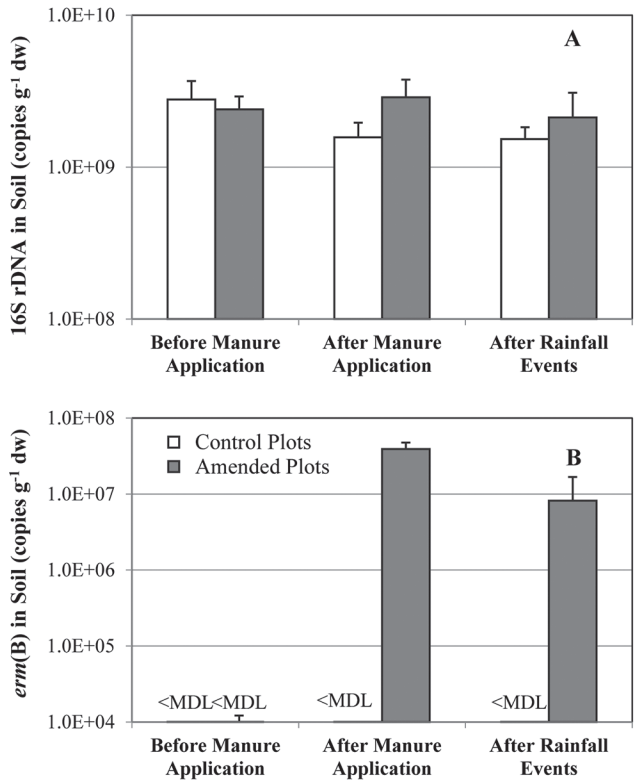

Fig. 4. The absolute abundance of *erm*(B) (A) and the 16S rRNA gene (B) in topsoil at different time points. Error bars represent SEs from triplicate field experiments. dw, dry weight.

Discussion

Although bacitracin was administered to the pigs before sampling, bacitracin A, the parent compound, was not detected in any manure samples collected over the 4-wk period. Bacitracin F, a degradation product of bacitracin A (Pavli et al., 2004), was not detected in the manure samples either. Instead, tylosin was the only antimicrobial that was consistently detected in all manure samples, with an average concentration of 49.4 μg kg^{-1} dw. The presence of tylosin is likely attributed to the manure residues in the pits before the experiment. In our previous studies, manure from the same facility where sows and gilts were fed 75 mg tylosin per kg ration contained 32.5 mg tylosin per kg dw manure solids (Joy et al., 2013, 2014).

Little was known about the effectiveness of NGHs on reducing dissolved antimicrobial loadings and concentrations in runoff. In this study, NGHs lowered tylosin loadings in runoff by more than an order of magnitude (Table 4). A recent study also demonstrated that vegetative buffer strips made of tall fescue could reduce tylosin in runoff and reported that the reduction of tylosin in runoff followed a first-order exponential decay relationship with buffer strip length (Lin et al., 2011). Rachman and coworkers (2004a, 2004b) found that the soil within the a switchgrass hedge had lower bulk density and clay content and higher silt content and porosity than surrounding soils, which led to increased infiltration as measured using saturated hydraulic conductivity. Enhanced infiltration likely accounted for increased removal of dissolved tylosin loadings in runoff.

Adsorption of tylosin within the NGH system may account for the reduction in dissolved tylosin concentrations in runoff. Tylosin has an affinity toward soil particles by directly adsorbing to clay surfaces within the soil (Sassman et al., 2007). As the runoff passes through NGHs, soil and vegetative surfaces in the grass hedge provide a relatively large surface area for dissolved tylosin to adsorb. Narrow grass hedges have been reported to lower dissolved chemicals in runoff and to improve the pH and electrical conductivity of the runoff water (Gilley et al., 2011).

Finally, the dissolved tylosin concentrations in runoff decreased significantly with successive rainfall events for plots without a NGH, whereas no such trend was observed for plots with a NGH (Table 4). Although this study did not quantify tylosin adsorbed onto runoff solids, NGHs were thought to be effective in lowering solid bound tylosin in runoff because of their effectiveness in retaining runoff solids and reducing soil erosion (Gilley et al., 2008). One study reported that a stiff grass hedge can reduce the sediment loading in the outflow to 3.2 to 6.0% of the inflow concentrations (Hussein et al., 2007).

Table 3. Statistical test results on the effects of manure amendment and rainfall events on the concentrations of tylosin and microbial genes in soil.

	Tylosin	*erm*(B)	16S rRNA gene
	μg g^{-1} soil dw†	copy g^{-1} soil dw	copy g^{-1} soil dw
Manure amendment‡			
Control plots	0.38a§	6.38×10^4a	1.65×10^9
Amended plots	6.25b	1.15×10^7b	2.07×10^9
Event			
Before manure application	0.00	2.60×10^4a	2.25×10^9
After manure application	5.73	1.42×10^7b	1.93×10^9
After rainfalls	4.20	3.11×10^6c	1.39×10^9
rANOVA values for¶			
Manure amendment	0.0482	0.0128	0.6437
Event	0.2323	0.0002	0.4587
Manure × event	0.2533	0.0030	0.6984

† Dry weight.

‡ 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, 0.38 μg g^{-1} was calculated using tylosin concentrations of all soil samples from control plots regardless of sampling times.

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

¶ Repeated measures ANOVA (rANOVA) values are displayed as *p* values.

Because NGHs can effectively retain runoff solids, it is reasonable to expect that suspended and sediment-bound microbes would also be impeded by NGHs. Results showed that *erm*(B) and the 16S rRNA gene were removed from runoff by NGHs (Table 2). One study showed that grass filter strips (4.6–9.1 m [15–30 ft] in length) could remove 75 to 91% of fecal coliforms and 68 to 74% of fecal streptococci in runoff from manure-amended plots (Coyne et al., 1998).

In addition to the loss to runoff, degradation may contribute to the decline of tylosin in manured soil. Tylosin has a half-life of 7 to 8 d in soil (Hu and Coats, 2007) or 4.5 d in manure-amended soils (Carlson and Mabury, 2006), suggesting it may be degraded substantially over the 4-d field tests in this study. Tylosin A may hydrolyze into tylosin A adol, tylosin D, and isotylosin A under a broad pH range of 2.0 to 12.8 (Paesen et al., 1995; Sassman et al., 2007). Both abiotic and microbial processes contribute to the degradation and transformation of tylosin. Abiotic processes are much slower, whereas the microbial degradation is very rapid during the first 3 d (Carlson and Mabury, 2006). Interestingly, the variation among soil tylosin concentrations after rainfall events was smaller than that before the rainfall events (Fig. 3), suggesting that rainfall resulted in more homogeneous distribution of tylosin in soil.

In this study, ARG *erm*(B) in soil decreased after the three rainfall events. One study reported that four of the five ARGs tested increased in soil amended with cattle manure over the first 50 d after land application (Alexander et al., 2011). Another study reported that the absolute abundance of ARGs increased in the topsoil after rainfall simulations over a period of 3 d (Joy et al., 2013). Different from ARGs, there was no significant change in the 16S rRNA gene abundance in soil either after land application or after the rainfall events. This is expected because all bacteria contain the 16S rRNA gene and the indigenous soil bacteria outnumbered the manure-borne bacteria introduced by land application.

Table 4. Mass loadings of tylosin in runoff from the amended plots with and without a narrow grass hedge during three rainfall events.

Rainfall event	Tylosin	
	Without narrow grass hedge	With narrow grass hedge
	μg m^{-2}	
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 event 1	0.47	0.31

† Values are average ± SE calculated based on triplicate field experiments.

In conclusion, this study demonstrated the effectiveness of NGHs in reducing antimicrobial and ARG levels in agricultural runoff. Livestock wastes are often land applied as soil conditioners due to their high organic matter and nutrient contents. Introduction of veterinary antimicrobials and corresponding ARGs into the environment through land application is inevitable. As a cost-effective best management practice, NGHs have been demonstrated to be effective in reducing nutrient loads in agricultural runoff. Results from this study show that NGH can also reduce dissolved antimicrobials and ARGs in agricultural runoff after land application of swine manure.

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Supplemental Information

Narrow Grass Hedges Reduce Tylosin and Associated Antimicrobial Resistance Genes in Agricultural Runoff

Bhavneet Soni[a], *Shannon L. Bartelt-Hunt*[a], *Daniel D. Snow*[b], *John E. Gilley*[c], *Bryan L. Woodbury*[d], *David B. Marx*[e], *and Xu Li*[a,*]*

[a]Department of Civil Engineering, [b]School of Natural Resources, [e]Department of Statistics University of Nebraska-Lincoln; [c]USDA-ARS; [d]USDA Meat Animal Research Center

[*]Corresponding author:
844 N. 16th Street, N117 SLNK
Lincoln, NE 68588-6105
Phone: (402) 472-6042
Fax: (402) 472-8934
E-mail: xuli@unl.edu

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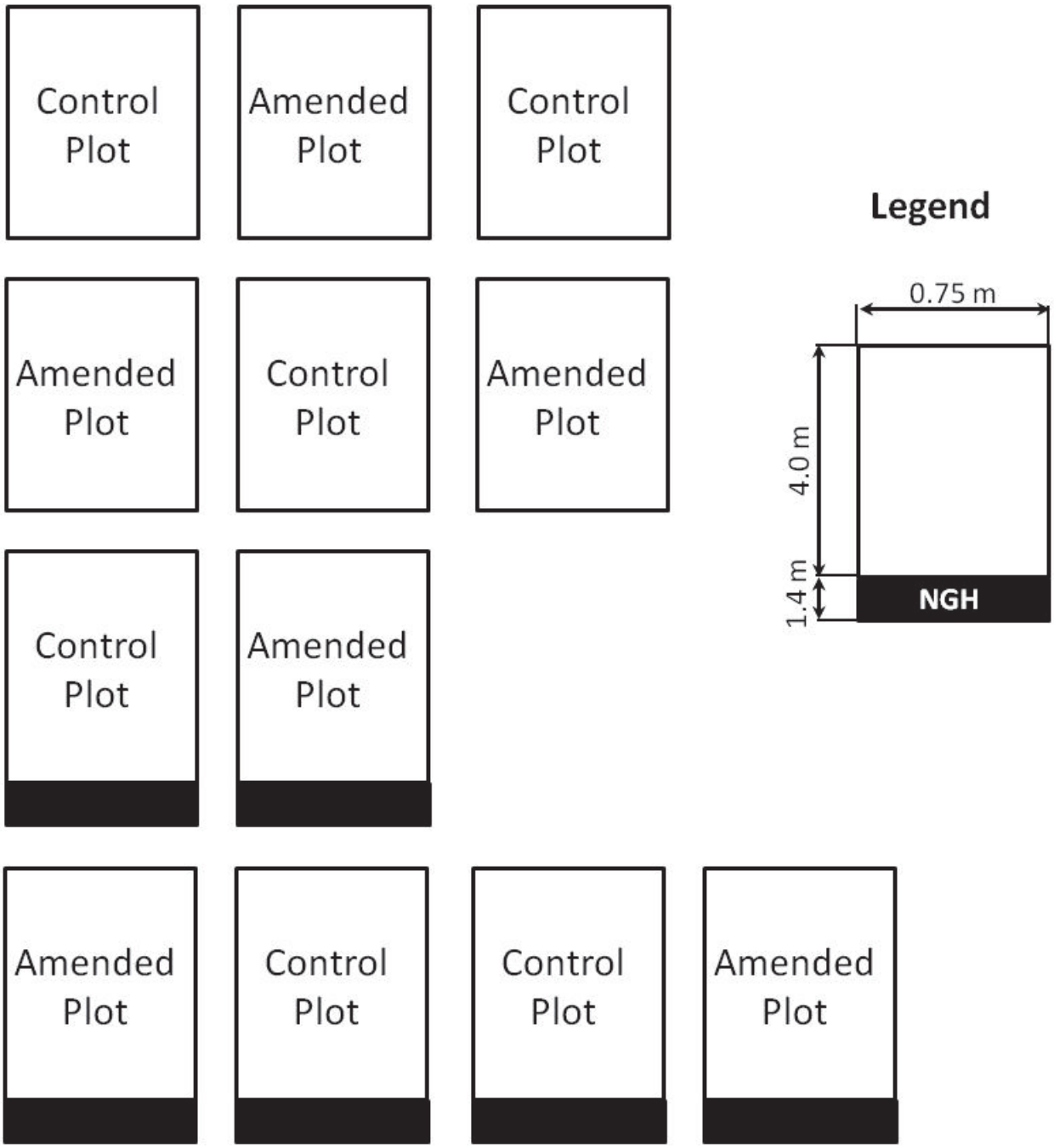
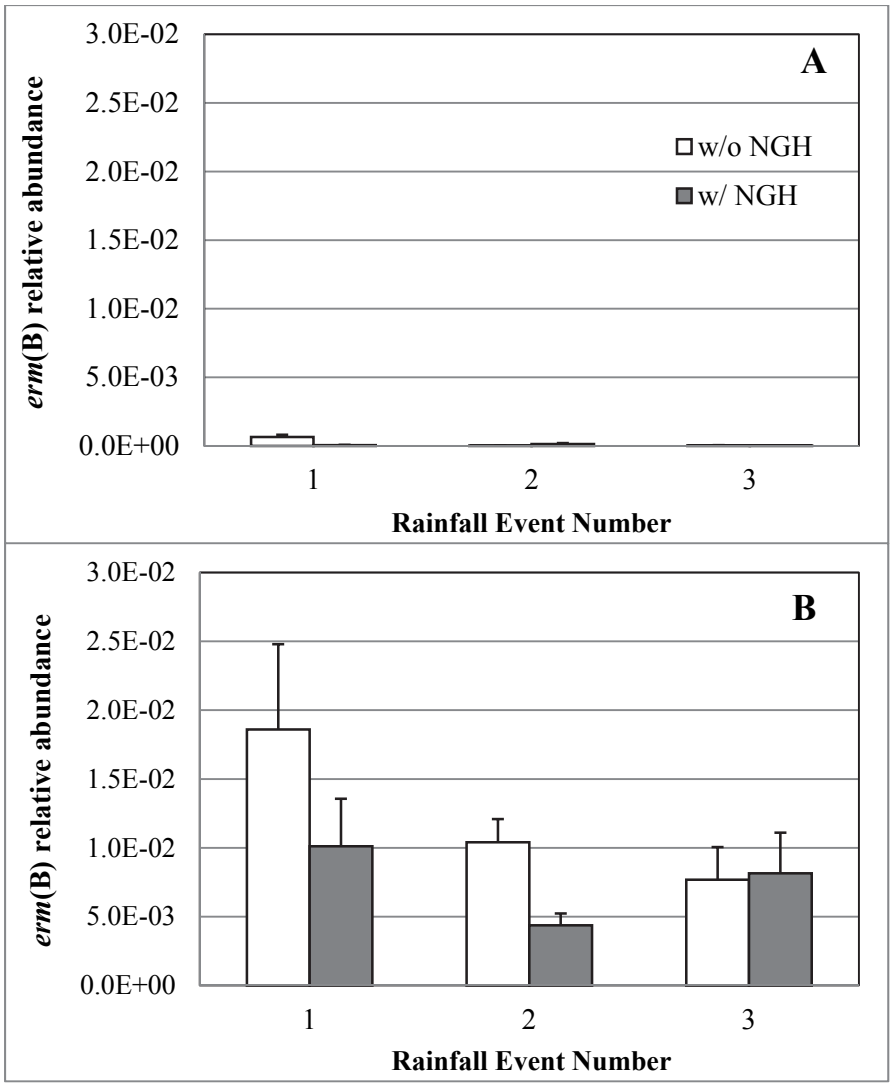
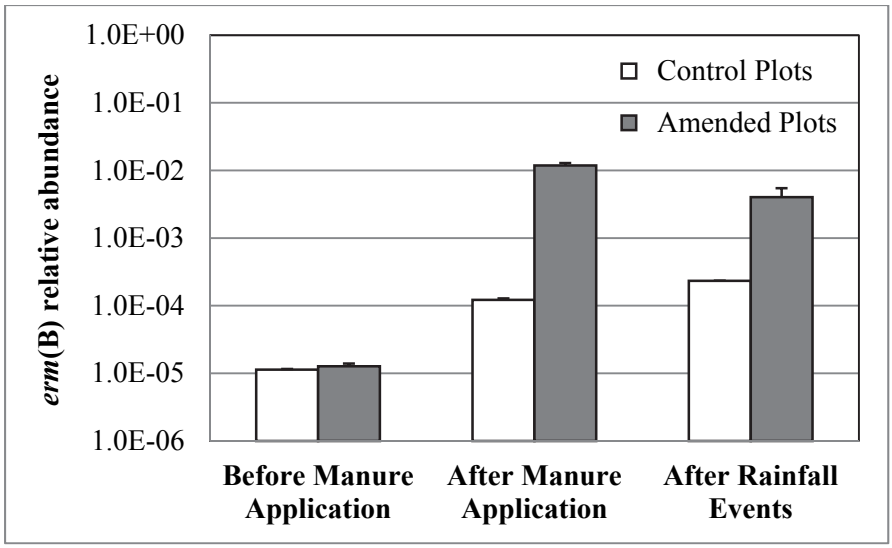


Supplemental Fig. S1. Schematic showing plot layout. The plots are not drawn to scale. Each row of plots were tested in the same week.



Supplemental Fig. S2. The relative abundance of *erm*(B) in runoff during the three rainfall events from control (A) and amended (B) plots. Error bars represent standard errors from triplicate field experiments.



Supplemental Fig. S3. The relative abundance of *erm*(B) 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.

Supplemental Table S1. Relevant information of the qPCR reactions used in this study.

Target Gene	Primer	Sequence (5'-3')	Annealing Temp (^{o}C)	Linear Range (copies/20µL)	R^2	Efficiency (%)	Reference
erm(B)	ermB-F ermB-R	GGTTGCTCTTGCACACTCAAG CAGTTGACGATATTCTCGATTG	65	10^1-10^9	0.996	94.4	(Koike et al., 2010)
16S rRNA	1369 F 1492 R	CGGTGAATACGTTCYCGG GGWTACCTTGTTACGACTT	56	10^3-10^9	0.979	82.4	(Suzuki et al., 2000)

Supplemental Table S2. The absolute abundance of *erm*(B) and the 16S rRNA gene (average ± standard error) in runoff from control and amended plots with and without NGHs.

Rainfall Event	Control Plots		Amended Plots	
	w/o NGH (copies/mL)	**w/ NGH (copies/mL)**	**w/o NGH (copies/mL)**	**w/ NGH (copies/mL)**
		erm(B)		
1	$(1.33 \pm 0.19) \times 10^3$	$(2.02 \pm 0.53) \times 10^2$	$(4.85 \pm 2.44) \times 10^6$	$(7.61 \pm 2.35) \times 10^4$
2	< MDL	$(1.65 \pm 0.84) \times 10^2$	$(1.12 \pm 0.32) \times 10^5$	$(3.14 \pm 1.92) \times 10^4$
3	< MDL	< MDL	$(9.30 \pm 3.14) \times 10^4$	$(4.59 \pm 1.73) \times 10^3$
		16S rRNA gene		
1	$(2.31 \pm 0.31) \times 10^6$	$(5.63 \pm 2.22) \times 10^6$	$(1.32 \pm 0.66) \times 10^8$	$(9.48 \pm 4.07) \times 10^6$
2	$(8.97 \pm 1.89) \times 10^6$	$(3.78 \pm 1.36) \times 10^6$	$(9.37 \pm 2.29) \times 10^6$	$(7.28 \pm 2.79) \times 10^6$
3	$(5.12 \pm 0.55) \times 10^6$	$(4.63 \pm 0.99) \times 10^5$	$(1.21 \pm 0.06) \times 10^7$	$(1.57 \pm 0.30) \times 10^6$

Supplemental Table S3. The 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)	< MDL	$(3.91 \pm 1.43) \times 10^7$	$(8.18 \pm 2.20) \times 10^6$
16S rRNA	$(2.40 \pm 0.34) \times 10^9$	$(2.88 \pm 0.71) \times 10^9$	$(2.12 \pm 0.77) \times 10^9$

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