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Narrow Grass Hedge Effects on the Transport of Antimicrobials and Antimicrobial Resistance Genes Following Land Application of Swine Slurry

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NARROW GRASS HEDGE EFFECTS ON THE TRANSPORT OF ANTIMICROBIALS AND ANTIMICROBIAL RESISTANCE GENES FOLLOWING LAND APPLICATION OF SWINE SLURRY

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The objective of this study was to determine the effects of manure amendment and narrow grass hedges on the fate and transport of antimicrobials and 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. The treatment factor manure amendment consisted of two levels: no manure application and manure application to meet the 3 year nitrogen (N) requirements for corn. The treatment factor of grass hedge was established for half of the test plots. Runoff water generated during three 30 min simulated rainfall events was analyzed for antimicrobials and antimicrobial resistance genes (ARGs). The grass hedge proved to be consistently effective in reducing antimicrobial tylosin in runoff ($p=0.016$), while the effect in reducing tylosin resistance gene erm(B) was not significant ($p=0.2465$).

ACKNOWLEGEMENTS

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Chapter 1: Introduction

Antimicrobials in Agricultural Environments

Livestock manure has been widely used as a soil conditioner due to its high organic matter and nutrient content. Land applied manure can also improve the water infiltration properties of the soil, and consequently reduce runoff and erosion (Gilley and Risse 2000). However, livestock manure also contains contaminants such as antimicrobials. Antimicrobials are administered to animals at therapeutic levels for disease treatment and at sub-therapeutic levels for prophylaxis and growth promotion. Commonly used antimicrobials in concentrated animal feeding operations (CAFOs) include tylosin, tetracycline, chlorotetracycline, sulfonamides, and, to a lesser extent, bacitracin (Sarmah *et al.* 2006). A significant portion of these antimicrobials are not adsorbed in the animal gut. For example, up to 75% of tetracycline administered to the animals was not metabolized and was excreted into the environment (Chee-Sanford *et al.* 2009). Antimicrobial residues released with animal wastes often end up in livestock waste management structures (Zhang *et al.* 2013). Furthermore, one study showed that the levels of antimicrobials administered to the livestock had direct impacts on antimicrobial levels in the lagoons treating the wastes from the animals (Peak *et al.* 2007), suggesting that the dosage of antimicrobials in animal feed have direct impacts on antimicrobial levels in livestock waste management structures.

Swine manure slurry is believed to be a major source of antimicrobials in the environment as land application of manure transfers antimicrobial compounds directly into agricultural soils. It has been well documented that manure application was responsible for introduction of sulfamethazine, tetracycline, chlortetracycline, and tylosin in the environment (Heuer *et al.* 2011). Several studies have been conducted to

understand the transport of antimicrobials in the environment following the application of manure from CAFOs (Halling-Sorensen *et al.* 1998; Lee *et al.* 2007; Sanders *et al.* 2008). A study detected veterinary antimicrobial agents such as sulfonamides, sulphamethazine and sulfachloropyridine in various environmental samples (Accinelli *et al.* 2007). Another study reported chlortetracycline concentration of 12ng/L in the animal waste water and 6ng/L sulfamethizole in aquatic environments (Diaz-Cruz and Barcelo 2005). A conceptual model describing the transport of antimicrobials in the environment is presented in Figure 1.1.

Figure 1.1 Anticipated exposure pathways for veterinary antimicrobials in the environment (Sarmah *et al.* 2006).

Since antimicrobials administered to animals makes their way into the manure either as a metabolite or as a parent compound they are present in agricultural soils amended with animal manure (Patten *et al.* 1980). In one study, soil fertilized with liquid manure, which contained 4.0 and 0.1 mg/kg of tetracycline and chlorotetracycline respectively, the resulting antimicrobial concentrations in the top soil (0-10 cm) were found to be 86.2 µg/kg and 4.6 µg/kg, respectively (Hamscher *et al.* 2002). Another study reported plots amended with manure contained on average 27 µg/kg oxytetracycline, 443 µg/kg tetracycline, 93 µg/kg chlorotetracycline, and 4.5 µg/kg sulfamethazine in top soil (0–30 cm) (Hamscher *et al.* 2005). Land application methods can influence the antimicrobial concentrations in soil. In a recent study highest concentration of tylosin in top soil was reported for broadcast manure while incorporation resulted in highest top soil concentrations of chlorotetracycline (Joy *et al.* 2013).

Multiple environmental factors may affect the persistence of manure-borne antimicrobials in soil, such as sunlight, temperature, humidity, rainfall, and the nature of soil (Donoho 1984; Sturini *et al.* 2012). The degradation of fecal-borne antimicrobials (e.g., bacitracin, penicillin, streptomycin, tylosin, bambermycins, erythromycin and chlortetracycline) in sandy soil depended on their chemical structure and the incubation temperature (Gavalchin and Katz 1994). Antimicrobials tend to adsorb onto the soil matrix, which can reduce the rate of degradation (Thiele-Bruhn 2003). In a study to determine the persistence of oxytetracycline in soil after the application of 600 µg/mL oxytetracycline of liquid manure, concentrations of >25 µg/g were found for at least 40 days after application and concentrations of < 1 µg/g could be detected in soil even after 1.5 years since manure application (Gonsalves and Tucker 1977). In another study to

determine the persistence of tetracycline and its degradation products in soil, samples were analyzed using enzyme-linked immunosorbent assays (ELISA). Soil analyses revealed that there was no significant decline in the tetracycline concentrations 5 months following manure application (Aga *et al.* 2005).

Antimicrobial Resistance Genes in Agricultural Environments

Antimicrobial residues in the animal system and environment can lead to the emergence of antimicrobial resistance in the native bacteria. Antimicrobial resistance genes (ARGs) are the genetic determinants that confer antimicrobial resistance to bacteria. Under antimicrobial pressure, antimicrobial resistance may emerge in the animal gut or in the environment. ARGs may be transferred to daughter cells through vertical gene transfer or to non-related cells through horizontal gene transfers in the presence of antimicrobials (Peak *et al.* 2007). Vertical gene transfer occurs when the whole complement of intracellular DNA (iDNA) is transferred from parent cells to daughter cells. Horizontal gene transfer takes place through three mechanisms: conjugation, transduction, and transformation (Davison 1999; Ochman *et al.* 2000). Antimicrobial resistance can be proliferated due to the presence of antimicrobials in environment. Manure slurry from pigs fed tylosin, sulfacholorpyridazine, and oxytetracycline was land applied over a 2-year period. Sulfonamide-resistant pathogens, including *Shigella flexneri, Aerococcus spp*., and *Acinetobacter baumannii* were found in the amended soil and soil leachate (Byrne-Bailey *et al.* 2009).

ARGs tend to persist in livestock waste management structures and in agricultural environments. The ARGs in livestock wastes could survive aerobic and anaerobic

digestions under mesophilic and thermophilic conditions in waste treatment facilities (Ghosh *et al.* 2009). Once land applied, ARGs can persist in the environment for a considerable period of time. In a study by Byrne-Bailey and coworkers, the level of sulfonamide resistant bacteria in soil was monitored for 290 days after the land application of manure from pigs on a tylosin-sulfacholorpyridazine-oxytetracycline feed (Byrne-Bailey *et al.* 2009). It was found that the level of the resistant bacteria persisted in soil for the duration of the experiment. Even after antimicrobial resistant bacteria die, intracellular ARGs will be released into the environment and become extracellular ARGs, which can still persist in soil (Zhang *et al.* 2013).

Transport of Antimicrobials and ARGs

Manure-borne antimicrobials and ARGs in soil can be transported to surface water through runoff (Chee-Sanford *et al.* 2009). Although the occurrence of antimicrobials and ARGs in agricultural wastewater have been well documented (McKinney *et al.* 2010), there have been only a few studies to understand the fate and transport of antimicrobials and ARGs in soil and runoff after land application of manure. Antimicrobials may occur in the aqueous and the solid phase within runoff. One study reported that the aqueous concentrations of chlorotetracycline and tylosin were 0.04 and 0.09 µg/L, while the concentrations of chlorotetracycline and tylosin adsorbed on to runoff sediment were 1.5 and 8.0 µg/kg respectively (Davis *et al.* 2006). Similarly, Kim and co-workers reported (Kim *et al.* 2010) the aqueous concentrations of chlorotetracycline and tylosin were 0.01-0.09 µg/L and 0.01-0.24 µg/L in runoff during a

1-hour rainfall simulation, while the concentrations of chlorotetracycline and tylosin on the runoff solids were 6 and 6-12 µg/kg dw runoff solids.

Transport of antimicrobials in the environment is affected by the physicochemical properties of the compounds as well as the environmental conditions. Different antimicrobials have vastly different solubilities in water (Salvatore and Katz 1993). Hydrophobicity, cation exchange, and cation bridging with the clay particles play a significant role in determining the partitioning coefficient and distribution coefficient (K_d, Table S1) of antimicrobials to soil particles (Tolls 2001) which can vary substantially. With a K_d between 70 - 5000 L/kg, tetracycline is highly immobile in soil, whereas with a K_d between 7 – 300 L/kg, tylosin has intermediate mobility in soil (Tolls 2001). For antimicrobials that are highly sorptive, soil particles are believed to be the major carrier of these compounds in runoff (Davis *et al.* 2006; Dolliver and Gupta 2008; Kim *et al.* 2010).

To limit the transport of sorptive antimicrobials, management practices should address the transport of sediment in runoff. Controlling the flow of surface runoff can impede the transport of sediment and sediment associated contaminants in surface runoff. Vegetative barriers (VB) offer an inexpensive and easy solution to reduce surface runoff and sediment transport. A VB can impede sediment transport by breaking the kinetic energy of the runoff, promote settlement of sediment by ponding of water upstream and improve infiltration properties of the soil (Meyer *et al.* 1995). VBs, also termed as Vegetative filter strips (VFS) or vegetative buffer strips (VBS), are 5 – 15 meter wide strips of densely growing plants seeded next to croplands (Castelle *et al.* 1994). VBs are typically placed at the bottom of hill slopes and along the water bodies. VBs are reported

to be effective in removing dissolved and sediment bound chemical in the runoff. VBs can reduce pesticide losses in runoff with trapping efficiencies of 50% and more (USDA-NRCS 2000). 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). However under high flow conditions, VBs will no longer be effective because runoff may flow over the VB strips (Blanco-Canqui *et al.* 2006).

One type of VBs, narrow grass hedges have been a prevalent conservation practice. VFS consists of native plant materials, while narrow grass hedges often consist of stiff stemmed grass strips that are ~1.5 meter wide. The hedges are often placed at relatively short intervals along the contour of the hill slope. The spacing among grass hedges should be the lesser of either the horizontal distance for 2 m elevation change or the "L" – slope length value in RUSLE 2 (Renard *et al.* 1997) to limit soil loss from the field. Grass hedge width should be greater of 1 m or 0.75 times the change in upslope vertical elevation (USDA-NRCS 2010). The short intervals impede runoff sediments along the hill slope and present within concentrated flow (Meyer *et al.* 1995). In one study, VFS performed poorly in reducing sediment and nutrients in concentrated flow while narrow grass hedges have been effectively used in combination with vegetative filter strips (Blanco-Canqui *et al.* 2004). Narrow grass hedge were placed immediately upstream of the VFS and minimized soil and nutrient losses from interrill and concentrated flow. The stiff stems and upright growth of the grass hedge provides a better filtering of the runoff and managing concentrated flow (Blanco-Canqui *et al.* 2006; Blanco-Canqui *et al.* 2004).

Narrow grass hedges are effective in reducing runoff and runoff sediment. One study reported that narrow grass hedge could reduce runoff by 41% and soil loss by 63% (Gilley *et al.* 2008). As the water pounds the upstream of the grass hedge, nutrients adsorbed to sediment gets deposited and is removed from the runoff. Narrow grass hedges have also been reported to be effective in reducing soluble contaminants in runoff. Gilley and team reported grass hedges reduced the transport of total nitrogen in runoff from 7.62 kg/ha to 4.00 kg/ha and NO^-_3-N from 0.62 kg/ha to 0.20 kg/ha (Gilley *et al.* 2011). Owino and coworkers reported grass hedges significantly reduced the nutrient runoff losses from a clay loam soil by plant uptake and soil infiltration (Owino *et al.* 2006). Improved soil hydraulic properties beneath grass hedges help to enhance infiltration of water into the soil and reduce runoff (Rachman *et al.* 2004; Rachman *et al.* 2004). By ponding runoff on the upper side, grass hedges increase the rate of infiltration thereby reducing the amount of runoff and consequently dissolved nutrients. The effect of narrow grass hedges in combination with other soil conservation practices has also been studied. A single narrow grass hedge in a no till plot reduced runoff concentrations of dissolved P (DP), bioavailable P (BAP), particulate P (PP), total P (TP) and NH_4-N by 47, 48, 38, 40 and 60% respectively when the plots were disked concentrations of DP, BAP, PP, TP, and NH_4-N in runoff decreased by 21, 29, 43, 38, and 52%, respectively (Eghball *et al.* 2000).

While narrow grass hedges have been demonstrated to be effective in reducing nutrients and chemical compounds in runoff, studies on their effectiveness in reducing microbiological contaminants are limited. Coyne and co-workers reported that 9 meter long grass strips trapped more than 99% of the soil, 91% of fecal coliforms and 74% of

fecal streptococci in surface water runoff. Whereas 4.5 m long VFS trapped 75% fecal coliform and 68% fecal streptococci (Coyne *et al.* 1998). Another study reported 60% reduction in fecal bacteria with various VBS formation on the watershed scale (Parajuli *et al.* 2008).

There have been few studies performed to investigate the effects of a grass hedge on the transport of antimicrobials and ARGs in runoff. Since other BMPs working on the similar principal have proven to be effective in reducing dissolved and sediment bound compounds and bacterial load in runoff, it is plausible to expect that narrow grass hedges would be effective in limiting the transport of antimicrobials and ARGs in runoff.

The objective of this study was to determine the effects of manure amendment and narrow grass hedges on the fate and transport of antimicrobials and ARGs in runoff and in soil following the land application of swine manure slurry. Swine slurry was applied at 0 and 3 times the annual nitrogen requirement of a corn crop and rainfall events were simulated once a day for three consecutive days. Antimicrobial concentrations in manure, runoff, and soil were measured using high pressure liquid chromatography. Antimicrobials measured in this study included tylosin, chloroteracycline and bacitracin. The corresponding ARGs quantified included *erm*(A), *erm*(B), *erm*(C) and *erm*(F) using qPCR.

Chapter 2: Materials and 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 each week from July 5, 2011 to July 28, 2011. 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.
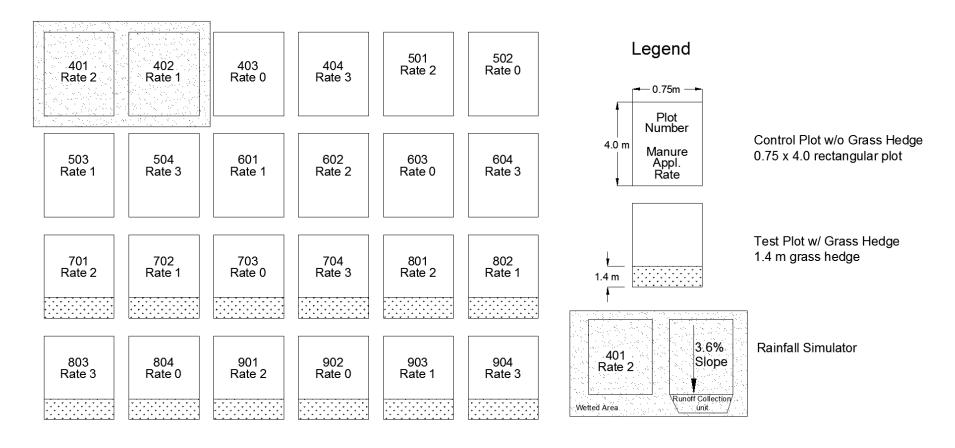
Soil Sample Collection

The field experiment was conducted by Dr. John Gilley of the USDA ARS in the summer of 2011. 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 Roger's Memorial Farm: 12 plots

without grass hedge and 12 with a narrow grass hedge (Figure 2.1).   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

Figure 2.1 Schematic showing plot layout, hedge and no-hedge and manure application rates based on 0, 1, 2 or 3 year corn N requirements. Each row of plots was used each week of the experiment.

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

Properties of the antimicrobials analyzed in this study are shown in Table S.1. Solvent extraction method was utilized to extract antimicrobials from solid samples (soil and manure). Samples of soil (10g) or manure (0.2g manure with 5g 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 finaly 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 analysed 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 4ºC in sterile 50-mL centrifuge tubes. Supernatants were decanted and pellets were stored at -20ºC 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) (S. Koike 2007). Because *erm*(B) was the only ARG that was consistently detected in manure slurry and runoff samples, it was quantified using quantitative PCR (S. Koike 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). Key qPCR parameters and the linear range for each primer set can be found in Table 2.1.

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 $\log_{10}$.

Table 2. 1 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	GGTTGCTCTTGCACACTCAAG	65	10^1-10^9	0.996	94.4	(Koike *et al.* 2007)
	ermB-R	CAGTTGACGATATTCTCGATTG					
16S rRNA	1369 F	CGGTGAATACGTTCYCGG	56	10^3-10^9	0.979	82.4	(Suzuki *et al.* 2000)
	1492 R	GGWTACCTTGTTACGACTT					

Chapter 3: Results

Antimicrobials and ARGs in Manure

Solids were collected from the manure slurry and examined for antimicrobials and ARGs. Among the antimicrobials tested (i.e., 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 % wet weight (ww) basis. The average tylosin concentration in the manure slurry was 11.4 µg/kg ww or 49.40 µg/kg dw basis (Table 3.1). 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 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 3.1).

Table 3.1 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		
(µg/kg ww)	(µg /kg dw)	(copy/mL)	(copy/g ww)	(copy/g dw)
Tylosin			erm(B)	
11.40±0.75	49.40±3.18	$(1.83 \pm 0.66)\times10^7$	$(1.37 \pm 0.47)\times10^9$	$(5.81 \pm 1.99)\times10^9$
			16S rRNA gene	
		$(1.44 \pm 0.52)\times10^8$	$(1.07 \pm 0.38)\times10^{10}$	$(4.52 \pm 1.60)\times10^{10}$
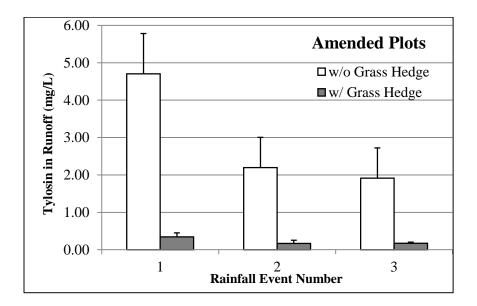
Antimicrobials in Runoff

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 3.2). Among the amended plots, tylosin concentration in runoff decreased as the rainfall number increased (Table 3.2 and Figure 3.1). 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 3.3), demonstrating that grass hedge could effectively reduce tylosin transport in runoff (Figure 3.1).

Table 3.2 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 (μg/L)	w/ Grass Hedge (μg/L)	w/o Grass Hedge (μg/L)	w/ Grass Hedge (μg/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 μg/L

Figure 3.1 Concentration of tylosin in runoff from amended plots. Error bars represent standard errors from triplicate field experiments.

rANOVA results showed that the effects of the 3-way interaction term, manure amendment $\times$ grass hedge $\times$ rainfall event, was of high statistical significance ($p < 0.0001$, Table 3.3). 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.

Table 3.3 rANOVA tests on the effects of manure amendment, grass hedge, and rainfall events on the concentrations of antimicrobials and microbial genes in runoff.

	TYL	*erm*(B)	16S rRNA gene
	(µg/L)	(copy/mL runoff)	(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 × Rainfall	<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 0.05 probability level based on LSD tests.

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

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 3.3). Rainfall event is the only main treatment factor that had a significant impact on the *erm*(B) concentration in runoff (*p* =
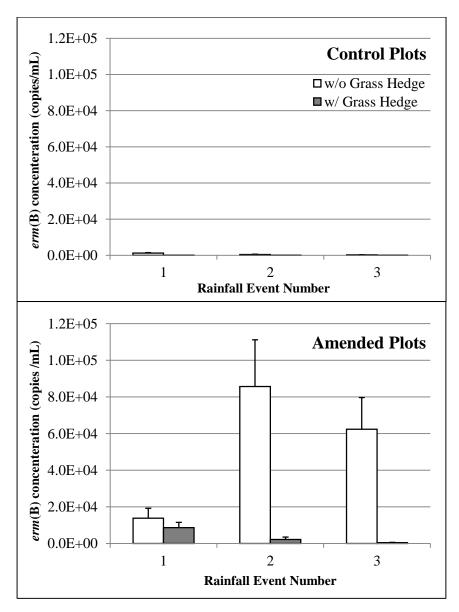
.0001). According to the LSD test, the average abundance of *erm*(B) in the first rainfall

event was significantly lower than that in the second and third rainfall event (Table 3.3).

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 3.4 and Figure 3.2) (*p* = *.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 3.3 and Figure

3.2) (*p* = *.2465*). The abundance of resistance gene in the runoff increased after the first

rainfall event (Figure 3.2).

Table 3.4 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
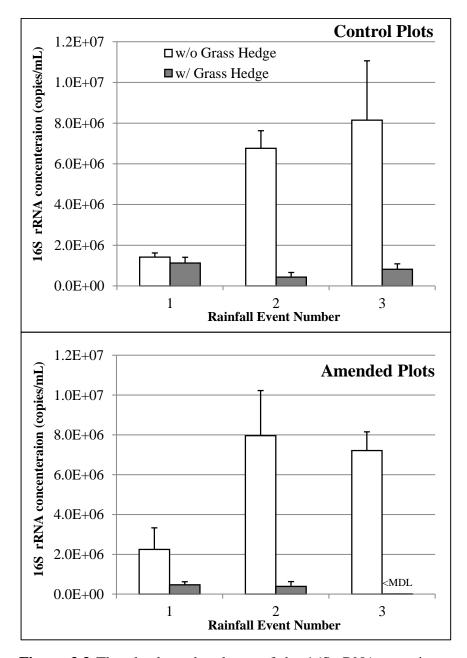
Figure 3.2 The absolute abundance of *erm*(B) in runoff from control and amended plots with and without narrow grass hedge. Error bars represent standard errors from triplicate field experiments.

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 3.3, 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.
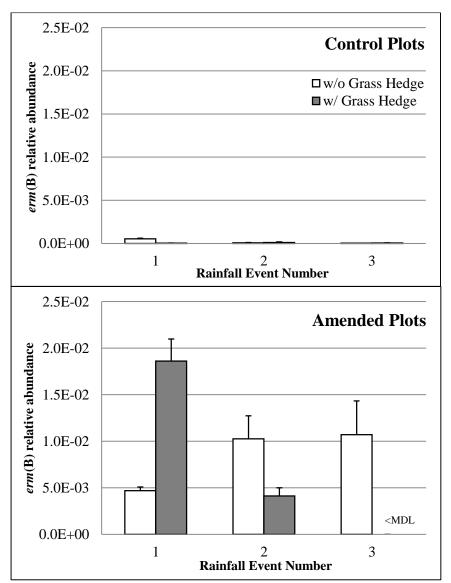
The absolute abundance of the 16S rRNA gene in runoff from plots with grass hedge was at least one order of magnitude lower than that from plots without grass hedge (Table 3.4, Figure 3.3). Similar to *erm*(B), among the amended plots, the 16S rRNA gene increased after the first rainfall event (Figure 3.3).

Figure 3.3 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 3.4). The relative abundance of *erm*(B) in runoff from amended plots was significantly
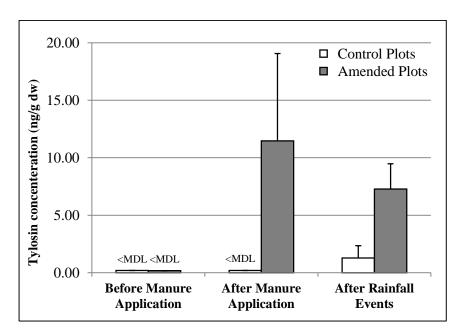
higher than that from the control plots. Among amended plots, the presence of grass hedge led to a decreasing trend in the relative abundance of *erm*(B) over the rainfall events (Figure 3.4).

Figure 3.4 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 µg/kg of soil ww or 11.46 ± 7.60 µg/kg soil dw. After the three rainfall events, the average tylosin concentration in the top soil was 7.27 ± 2.21 µg/kg soil dw or 5.09 ± 1.57 µg/kg of soil ww (Figure 3.5). No tylosin was detected in the soils from the control plots at the two sampling times (Figure 3.5).

Figure 3.5 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 3. 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 3.5). The two individual treatment factors also had significant impacts on the tylosin concentrations in soil.

Table 3.5 rANOVA tests on the effects of manure amendment and events on the concentrations of antimicrobial and microbial genes in soil.

	TYL	*erm*(B)	16S rRNA gene
	(µg/g)	(copy/g soil dw)	(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 △			
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.
△ 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 16S rRNA gene (Table 3.5). The
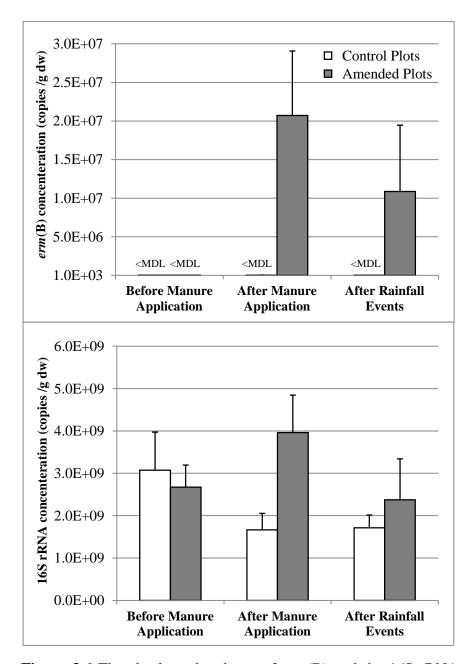
abundance of *erm*(B) increased in the top soil after manure application (*p=0.2026*) and decreased after rainfall events (*p=0.5831*) (Table 3.5). Plots receiving no manure had an average abundance of *erm*(B) at 2.63×10^4 copies/g soil dw after manure application and at 8.16×10^3 copies/g soil dw after 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 3.5).

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 3.6). 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 3.6, Figure 3.6).

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 3.6, Table 3.6).

Table 3.6 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.
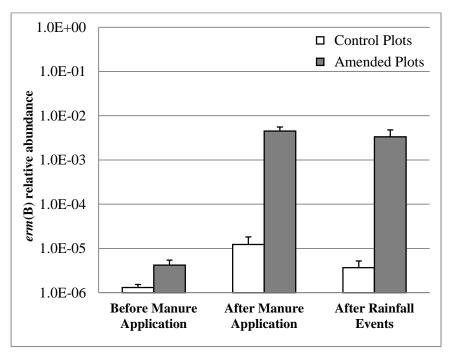
Gene	Before Manure Application (copies/g soil dw)	After Manure Application (copies/g soil dw)	After 3 Rainfall events (copies/g soil dw)
erm(B)	$(9.34 \pm 2.18) \times 10^3$	$(2.07 \pm 0.84) \times 10^7$	$(1.09 \pm 0.86) \times 10^7$
16S rRNA	$(2.67 \pm 0.52) \times 10^9$	$(3.96 \pm 0.89) \times 10^9$	$(2.38 \pm 0.97) \times 10^9$

Figure 3.6 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 3.7). 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 3.7 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.

Chapter 4: 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 μg/kg manure wet weight (ww). In another study conducted with manure from the same source, the tylosin concentration was reported at 290 μg/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 effected 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 1.6×10^4 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).

Land applied manure is often considered as the main source of antimicrobials and ARGs in agricultural runoff. In this study, tylosin concentrations in the runoff from the amended plots were considerably 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 0.081 and 6.111 µg/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 4.1).

Table 4.1 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 ($\mu g/m^2$)	w/ Grass Hedge ($\mu g/m^2$)
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 #1	0.47	0.31

The narrow grass hedge was very effective in reducing the dissolved antimicrobial load from the runoff. Narrow grass hedge lowered total antimicrobial

loading in runoff by an order of magnitude (Table 4.1). 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 dissolved phosphorous load were 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; 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 3.2, Figure 3.1). Tylosin has an affinity towards soil particles and directly adsorb to the surface and clay content of the soil (Sassman *et al.* 2007) and as the runoff pass thru the grass hedge the aqueous phase antimicrobial had a higher surface contact with soil and vegetative surfaces in the grass hedge and got adsorbed to them and is removed from runoff.

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 3.2). While Joy et al. reported that the absolute abundance of ARGs (*tet*(Q), *tet*(X) *erm*(B), *erm*(F)) in runoff from plots applied by broadcast method decreased with rainfall events (Joy *et al.* 2013). Runoff appears to provide a liquid medium for an increased horizontal and vertical transfer of resistance genes following the first rain fall event.

The grass hedge significantly reduced the amount of 16S rRNA gene in the runoff (Table 3.4 and Figure 3.3). rANOVA results suggests that the narrow grass hedge had a significant statistical effect (*p = 0.0014*) on microbial genes in runoff. Narrow grass hedge reduces the amount of suspended and dissolved solids in the runoff. Microbial population and DNA is 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 hedge were able to remove more than 90% microbial DNA from runoff. We are not aware of another studies on the effects of narrow grass hedges on the microbial genes in runoff. However some studies were conducted to investigate the effect of vegetative filter strips on pathogen content in runoff. One study has shown that grass filter strips (15 to 30 feet in length) remove 75 to 91% of fecal coliforms and 68 to 74% of fecal streptococci in runoff from manure amended plots (Coyne *et al.* 1998). Another study showed that there was no decline in the total and fecal coliform numbers in the water as it moved downslope through the vegetative filter (Entry *et al.* 2000). While it has been suggested that animal confinement areas should have a 66 to 99 foot vegetative filter strip between animals and surface water in order to minimize the contaminant load in runoff (Entry *et al.* 2000), our results show that a series of narrow grass hedges will be as effective with less loss of cultivable land.

Reporting genes with low abundance in the samples was challenging, because the MDL for each qPCR protocol depended on the sample preparation procedure. DNA extracts with C_t values outside of linear ranges (Table 2.1) were counted as the half of the lowest value on the linear range. While calculating the absolute abundance in a specific sample, the amounts of original samples (i.e., manure slurry [manure solids], runoff [runoff solids], and soil) from which the DNA extract were obtained were also taken into consideration, leading to varied detection limits. For example, the absolute abundance of *erm*(B) in the runoff from the control plots did not fall in the linear range, whereas the absolute abundance of ARGs in the first runoff from amended plots were on the order of 10^4 copies per mL of runoff.

Other than the loss through runoff, the degradation may also contribute to the decrease of tylosin concentration in soil after the rainfall events. Tylosin has a short half-life of 7 – 8 days in soil (Hu and Coats 2007), and 4.5 days in manure amended soils (Carlson and Mabury 2006) suggesting it may be degraded over the 4-day field tests. Tylosin A may hydrolyze into various compounds, such as tylosin A adol, tylosin D, and isotylosin A, under alkaline and acidic conditions between pH 2.0 and 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 while the microbial degradation is very rapid during the first 3 days (Carlson and Mabury 2006) Furthermore, the variation among soil tylosin concentrations following manure application (7.60 µg/kg) was larger than the variation among soil tylosin concentrations after the rainfall events, suggesting that the rainfall events led to more homogeneous distribution of tylosin in soil.

Similarly, *erm*(B) increased from below MDL before manure application to 10^7 copies/g of soil dw after the manure application and remained at the same level after the rainfall events. One study reported an increase in the level of ARGs in soil amended with cattle manure: *tet*(B), *tet*(C), *tet*(L), and *tet*(M) increased over the first 50 days after land application and then returned to initial levels, while *tet*(W) decreased an order of magnitude over the course of the 175-day experiment (Alexander *et al.* 2011). Joy et al. also reported that absolute abundance of *tet*(Q), *tet*(X), *erm*(B) and *erm*(F) genes increased in the top soil following rainfall simulations over a period of 3 days (Joy *et al.* 2013).

In contrast, there was no change in the soil 16S rRNA gene abundance with the application of manure or the rainfall events (i.e., at the order of 10^9 copies/g soil dw throughout the experiment). This is understandable, as all bacteria contain the 16S rRNA gene and the indigenous soil bacteria outnumbered the manure-borne bacteria introduced with land application.

The chemical compounds and organic matter trapped in the grass hedges and adsorbed onto the vegetative surfaces may act as a biofilm reactor. Grass hedges have been reported to adsorb chemicals in runoff and improve the pH and EC of the runoff water (Gilley *et al.* 2011), hence it is plausible that grass hedge itself may provide for a good breeding ground for the microbes. Also with high organic matter trapped in the grass hedge zone and high liquid gas interface provided by the vegetative surfaces it provides for a perfect breeding ground for microbes to multiply. In retrospection a chemical and microbiological analysis of the soil samples and vegetative surfaces from the narrow grass hedge region, where most of the solids were trapped and adsorbed,

would have complemented the results of this study and should be included in the future

studies.

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Appendix

Table S.1 Properties of the antimicrobials used in this study.

Antimicrobial	Chemical Structure	Properties
Chlortetracycline		K_d = 501-3715 L/kg (Teixido *et al.* 2012) Solubility = 500 mg/L $t_{1/2}$ = 21 days (Carlson and Mabury 2006)
Tylosin		K_d = 1,300 L/kg (Clay *et al.* 2005) Solubility = 6,000 mg/L $t_{1/2}$ = 6-8 days (Carlson and Mabury 2006; Hu and Coats 2007)
Bacitracin (Bacitracin A)		Environmental fate data for Bacitracin A are not available in the literature

Table S.2 Molecular weight, retention times, and MRM transition of antimicrobials, internal standards (IS), and surrogate (S) compound.

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