RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Effect of Manure Application Timing and Management on the Persistence and Transport of Antibiotics and Antibiotic-Resistant Bacteria in Corn and Soybean Production Systems, **NPB#16-039**

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Industry Summary:

Swine production is a major economic activity in Iowa and the Midwest, and accounts for a significant portion of the gross farm income in the region. The swine industry is also among the most regulated agricultural operations in many states. Environmental stewardship is being increasingly recognized by swine operators as an important criterion for profitable operation. However, contamination of surface water and groundwater from swine manure continues to be a concern for producers. Here we report the results of our study assessing the environmental fate of antibiotics, antibiotic-resistant bacteria, and antibiotic-resistant genes on swine farms.

This work is important to producers because impairment of surface water by pathogens could become a significant roadblock for future expansion of the pork industry. While pathogen contamination of surface waters is the leading cause of water quality impairments in Iowa, the emerging concern about antimicrobial resistance (AMR) is likely to increase scrutiny of the animal production industry, specifically industry-related manure management practices. Our research improves understanding of the occurrence and transport of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) from fields receiving swine manure versus those treated with chemical fertilizer. This information is provided for a range of manure application timing (early fall (EFM), late fall (LFM), spring (SM)) and management practices (chisel plow, no till) to provide practical management information to farmers. Further we considered different manure management strategies (anaerobic digestion, two-phase storage, centrifugation, and ionophore addition) for treatment of manure prior to land application for their potential to reduce ARGs and ARBs.

In considering different manure application timings, we found that swine manure applications in early fall, late fall, and spring resulted in mostly similar patterns of ARB and ARG dissipation in soil. For the ARG, there was evidence for shorter persistence after spring manure

applications; however, the spring period is also the season with greatest precipitation and tile drainage, factors which increase risk of transport to downstream waters. The analysis of resistant genes in soil showed that soils receiving swine manure approximately one year previously (continuous corn) or two years previously (corn soybean) to which had not had antibiotics for many years (controls) were not different in the abundance of ARG in soil. This is evidence suggesting that these ARG are not accumulating in the soil under these farming operations.

For tile drainage samples, previous research has reported significant differences in ARGs in drainage from plots receiving manure and plots not receiving manure in the fall. This study confirms that LFM and SM had a higher percent detection of ARGs when compared to control, however, the same cannot be said for EFM, whose percent detection was comparable to the control. It is possible that this could be attributed to the sampling scheme, as EFM sees the greatest lag time between manure application and when drainage flow sampling commences. Finally, this study saw the largest percent detection of all three ARGs selected (*erm*B, *erm*F, and *tet*M) in drainage from plots receiving spring manure (SM). Greater transport of ARG in tile drainage after SM occurred despite the shorter persistence of ARG in soil after SM application, suggesting that drainage predominates over ARG survival in soils in determination of ARG transport.

The reduction of both resistant *Enterococcus* and resistance genes in two-phase storage with no manure added suggests that this may be a promising management solution for reduction of AMR in manure storage. When compared to anaerobic digestion, our results suggest that two phase storage without manure addition may achieve similar treatment of antibiotic resistance bacteria and genes. This management strategy presents an opportunity for further research and development, and the practicality of installing storage in comparison to anaerobic digestion would need to be further evaluated.

This knowledge will assist farmers, researchers, and extension personnel in the development of better management strategies for manure storage and application for limiting the prevalence of ARGs and ARBs.

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Scientific Abstract:

Antibiotics are commonly used by swine producers at therapeutic levels for disease treatment and at sub-therapeutic levels for disease prevention. These pharmaceuticals reduce animal death rates at swine facilities, thereby enhancing overall production efficiency and increasing profitability. The goal of this study was to improve understanding of the occurrence and transport of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) from fields receiving swine manure versus those treated with chemical fertilizer. The study assessed

ARB and ARG levels in soil and drainage water from plots amended with manure at different times (early fall, late fall, spring) and management practices (chisel plow, no till). The study also investigated different manure management strategies (anaerobic digestion, two-phase storage, centrifugation, and ionophore addition) for treatment of manure prior to land application for their potential to reduce ARGs and ARBs. Swine manure applications in early fall, late fall, and spring resulted in mostly similar patterns of ARB and ARG dissipation in soil. For the ARG, there was evidence for shorter persistence after spring manure applications; however, late fall and spring application had a higher percent detection of ARGs in drainage when compared to the control. The spring period is the season with greatest precipitation and tile drainage, factors which increase risk of transport to downstream waters. Overall our findings suggest that drainage predominates over ARG survival in soils in determination of ARG transport. This knowledge will assist farmers, researchers, and extension personnel in the development of better management strategies for manure storage and application for limiting the prevalence of ARGs and ARBs.

Introduction:

Swine production is a major economic activity in Iowa and the Midwest, and accounts for a significant portion of the gross farm income in the region. The swine industry is also among the most regulated agricultural operations in many states. Environmental stewardship is being increasingly recognized by swine operators as an important criterion for profitable operation. However, contamination of surface water and groundwater from swine manure continues to be a concern for producers. Here we report the results of our study assessing the environmental fate of antibiotics, antibiotic-resistant bacteria, and antibiotic-resistant genes on swine farms.

This work is important to producers because impairment of surface water by pathogens could become a significant roadblock for future expansion of the pork industry. While pathogen contamination of surface waters is the leading cause of water quality impairments in Iowa, the emerging concern about antimicrobial resistance (AMR) is likely to increase scrutiny of the animal production industry, specifically industry-related manure management practices. Our research improves understanding of the occurrence and transport of antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) from fields receiving swine manure versus those treated with chemical fertilizer. This information is provided for a range of manure application timing (early fall, late fall, spring) and management practices (chisel plow, no till) to provide practical management information to farmers. Further we considered different manure management strategies (anaerobic digestion, two-phase storage, centrifugation, and ionophore addition) for treatment of manure prior to land application for their potential to reduce ARGs and ARBs. This knowledge will assist farmers, researchers, and extension personnel in the development of better management strategies for manure storage and application for limiting the prevalence of ARGs and ARBs.

Objectives:

The overall goal of this research project is to further our understanding of the occurrence and transport of antibiotics, antibiotic-resistant bacteria (ARB), and antibiotic resistance genes (ARGs) in tile-drained agricultural fields that have received multi-year applications of liquid swine manure through injection. Key project objectives include 1) determining the effect of manure application timing, tillage, and patterns of rainfall/drainage on the persistence and losses of

antibiotics, ARB, and ARG in soil and drainage water; and 2) determine the effects of alternative manure treatment and storage on the persistence of antibiotics, ARB, and ARG in manure.

Materials & Methods:

Objective 1:

Field experiments were conducted at the Iowa State University Northeast Research Farm (NERF) near Nashua to determine the effect of manure application timing on the persistence of antibiotic resistance. Swine manure from a nearby commercial swine production barn was applied from October 2016 to May 2018 to corn-corn and corn-soybean systems. Each cropping received a single swine manure application either in the spring, early fall, or late fall (Table 1). Each treatment consisted of three replicate plots that were 0.404 ha in size and were equipped with individual tile drains about 1 m below the soil surface (Garder et al, 2014). Manure, soil and water samples were collected throughout the project period.

Table 1. Summary of agronomic systems and swine manure applications for measurements of persistence of AMR in soil.

Crop Rotation	Tillage	Treatment	Manure Application Timing			Application Rate[a] (kg N/ha)
Corn-soy (1)	Chisel Plow	Control	UAN[b] pre-corn plant	All dates[c]	All dates	168
Corn-soy (2)	No-Till	Early-fall	Manure post-soybean harvest	10/6/2016	10/18/17	168
Corn-corn 3.2	Chisel Plow	Spring	Manure pre-corn plant	3/28/2016	4/12/17	224
Corn-corn (4.1)	Chisel Plow	Late fall	Manure post-soybean harvest	11/2/2016	11/8/17	224
Corn-soy (6)	No-Till	Late fall	Manure post-soybean harvest	11/2/2016	11/8/17	168

[a] Volumes of swine manure were adjusted to meet these target N rates.
[b] UAN: urea-ammonium nitrate.
[c] Samples from the control plots were taken at the same times as other treatments were sampled.

Sampling

Manure samples were collected directly from the injector knife at the time of application for each plot before quantifying concentrations of ARB, ARG, and common antibiotics. Separate soil samples were collected from manure injection bands (IB) and the space between them (NB) for 15 plots receiving liquid swine manure to track any lateral movement from injection sites to surrounding soils. Soil samples consisted of three soil cores (15 cm deep and 4 cm in diameter) composited for the IB or NB sampling locations. Soil samples were collected over a six-month span, but the frequency of sampling was weighted heavily near the time of application to accurately quantify the expected rapid decay of analytes. Samples were collected until injection bands could no longer be identified or freezing occurred. Soil probes were cleaned and sterilized with 70% ethanol between plots to reduce contamination. Soil samples were transported to Iowa State University on ice and then sieved through a 22 mm sieve. Samples were stored at 4 °C for less than 48 h prior to enterococci enumeration and at -20 °C for less than 6 months for DNA extraction.

Soil moisture measurements were taken before and after oven drying 5 g soil at 105 °C. Soil temperatures from a nearby weather station were averaged for all sampling dates after manure application and ending with the six-month sampling dates.

Tile drainage samples were collected from the outflow of each designated plot's effluent starting when flow began in the early Spring and concluding with the stoppage of flow in the late Fall. A total volume of 2000 mL in the form of grab samples were taken every week throughout the drainage season, whenever flow was available from the plot. Flow meter readings were recorded, regardless of apparent flow. The number of samples each year varied due to the length of the drainage season and each plot's individual flow. The samples were collected in two 1-liter plastic bottles and transported on ice back to the Water Quality Research Lab (WQRL) at Iowa State University. Samples were then immediately processed upon arrival or stored in a 4ºC refrigerator and processed within 24 hours.

Enterococci Enumeration

Soil, manure, and water *Enterococcus* spp. (enterococci) were grown on three variants of agar: mEnterococcus (Difco, St. Louis, MO), mEnterococcus + 16 mg/L tetracycline (Sigma-Aldrich, St. Louis, MO), and mEnterococcus + 32 mg/L tylosin (Sigma-Aldrich, St. Louis, MO). Antibiotic concentrations in agar were set at resistance breakpoint levels utilized by the National Antibiotic Resistance Monitoring System and outlined by the CLSI antimicrobial susceptibility testing standards (2015). Soil and manure samples with were serially diluted in phosphate buffer solution to obtain counts between 30 and 300 using Luby et al (2014)'s adaptation of the APHA (1998) waste water membrane filtration method. Enterococci were reported as CFU g^{-1} dry soil or mL^{-1} manure.

DNA Extraction and Gene Quantification

qPCR assays quantified copy numbers of *ermB*, *ermF*, *tetO*, *tetM*, and 16S-rRNA genes in soil and manure samples. *ErmB* and *ermF* were chosen because we previously quantified these manure and soils from NERF (Garder et al, 2014, Luby et al, 2016), while *tetO* and *tetM* were chosen based on their abundance in DNA from swine manure and soil sampled at NERF (unpublished data, 2016). Primer sequences for *ermB* and *ermF* are described in Luby et al (2016) and primers for *tetO* and *tetM* are described by Smith et al (2004) and Tamminen et al (2011), respectively.

Both soil and manure DNA samples were extracted using a 96 well MagAttract Power Soil DNA Kit (Qiagen, Valencia, CA) with an EP Motion Liquid Handler. DNA from three 0.25 g soil subsamples was extracted according to the manufacturer protocol and eluted in 100 µL of RNAase-free water. 50 µL from three subsample extracts were composited into a 150 µL sample for IB, NB, or control soils. DNA concentrations were determined using a Biophotometer and ranged from 30 to 55 ng μL^{-1}. Manure DNA was extracted using the same protocol, except 150 µL liquid swine manure and 100 µL qPCR-grade water were loaded into wells instead of 0.25 g soil.

All qPCR reactions were performed in 25 µL volumes using an Opticon CFX96 Touch System (BioRad, Hercules, CA) and consisted of 12.5 µL of SsoAdvanced Universal SYBR Green Supermix (BioRad, Hercules, CA), 2.5 µL of DNA template, and 5 µL of both forward and reverse primers. Primer concentrations and annealing temperatures varied based on their source and were optimized by testing combinations of primer concentrations and temperatures. Reactions were run in triplicate and amplification products were verified by melt curve analysis and gel electrophoresis. All reaction conditions adhered to the following protocol: 30 s at 95 °C

(activation), 40 cycles of 95 °C for 15 s (denaturation), 30 s at the primer specific annealing temperature (table P, appendix), and a temperature gradient from 65 to 95 °C in 0.5 °C/5 s increments for melt-curve analysis of products.

Standards for *ermB* and *ermF* qPCR were generated by growing cells that host plasmids containing the PCR standards (Luby et al, 2016). Plasmid were extracted and DNA concentrations were determined using a biophotometer and serially diluted. *ermB* and *ermF*, standards were prepared as two sets of serial dilutions from plasmid extracts and frozen until use. Standards for *tetO*, *tetM*, and 16S-rRNA were synthesized (GeneArt DNA, Thermo Fisher) with sequences matching the primers as double stranded DNA. Five bases of thymine separated the three target fragments on individual strings. Stand solutions were serially diluted in TE buffer and frozen at -20 ºC until use. Quality control measures for assays were determined separately for each prepared set of *erm* standards.

Limits of quantification (LOQ) for each assay were one-half log unit below the last quantifiable standard between qPCR runs. LOQ's were determined once all plates had been completed; no template controls (NTC) and negative controls as *Pseudomonas stutzeri* DNA (ATCC 14405) were included on each 96-well plate except for 16S-rRNA assays, where NTCs were included as the only negative controls. The melt temperatures of amplified 16S-rRNA gene copies varied slightly, but specific peaks could be observed for GeneArt strings, *Pseudomonas stutzeri* and isolates tested as additional positive and negative controls. Gene target concentrations in soil samples were calculated by adapting the method outlined by Garder et al (2014) - the estimated copies in one µL of template in the reaction was converted to a dry-weight basis using a dry weight-wet weight ratio and the amount of sample analyzed relative to the combined extract.

For water samples, the detection and quantification of ARGs was performed using the Wafergen SmartChip Real-Time PCR system (Michigan State University) and select gene probes. The Wafergen Smartchip Realtime PCR system allows for high throughput qPCR assays within a small reaction volume and allows for many sample and assay combinations. We targeted three genes that have previously been shown to be associated with manure but not highly abundant in the natural environment (Choi et al., 2018). Primers targeting *ermB* and *ermF* genes were selected to represent the erythromycin family, and a probe targeting *tetM* was selected to represent the tetracycline family (Stedtfeld et al., 2018). A probe targeting the 16s ribosomal RNA gene was also used to evaluate total bacteria presence. Additionally, for each gene assay, a set of standards ranging from 0 to 10^8 copies, with a total of 8 dilutions, were included with each set of samples.

Data Analysis and Statistics

Computational analysis and statistics were completed in R 3.4.2. Two primary statistical analyses were performed to further our understanding of the persistence of ARB and ARG in different cropping systems: (1) ARB and ARG decay coefficients (k, Eq 1) by linear regression of natural log-transformed IB concentrations of analytes (resistance genes or enterococci) using days after application as the only predicting variable, and (2) linear mixed effect models producing treatment means including IB and NB samples in a slightly more robust statistical analysis.

The first-order decay equation (Eq 1) has long been used to describe survival of bacterial pathogens and indicator bacteria (Habteselassie et al, 2007) where A_t is the abundance or concentration of genes or bacteria in soil at some time (t) after manure application, A_0 is the abundance or concentration in soil immediately following application of manure and k is the rate coefficient. The linear form of Eq 1 is presented in Eq 2 and decay coefficient (k) was determined by linear

regression. To include zeroes and normalize log transformations, one was added to the concentrations (A) prior to the log-transformation of analytes.

Eq 1 $A_t = [A]0 \bullet e^{-kt}$
Eq 2 $\ln([A+1]_t) = \ln([A+1]_0) - kt$

In addition to first-order models, a linear mixed effect model tested for the effects and significance of days after application, application timing, system, and the random effect of plot for individual analytes in, IB, NB, and control samples. Linear mixed effect models in the lme4 package (Bates et al, 2015) quantify effects of factor, continuous, and random variables for normally, or log-normally distributed data. For this study, each response (enterococci, tetracycline-resistant enterococci, tylosin-resistant enterococci, *ermB*, *ermF*, *tetO*, *tetM*) was log transformed and individually analyzed.

A linear mixed effect (LME) model tested for the significance of days after application, application timing, system, and the random effect of plot for individual analytes in the IB, NB, and control samples. LME models in the lme4 package (Bates et al, 2015) quantify effects of days after application (continuous variable), while treatment, year, and band/no band are categorical (fixed) variables. For this study, each response variable (enterococci, tetracycline resistant enterococci, tylosin resistant enterococci, *ermB*, *ermF*, *tetO*, *tetM*) was log-transformed and individually analyzed (Eq 3).

Eq 3 $\ln(\text{response variable}) \sim \beta(\text{days after application}) + \beta(\text{treatment}) + \beta(\text{year}) + \beta(\text{band}) + (1 \mid \beta\text{Plot})$

Soil moisture content and soil temperature at the time of sampling were initially included as predictive variables, but these were not significant ($p > 0.05$) and were removed from linear mixed effect models.

After main effects were accounted for, variation within treatments (replicate plots) was designated as a random effect. Least-squared means analysis gave estimates of geometric means of response variables for each treatment coefficient (β). In mixed effect models; the least-squared means mimic main-effect means but are adjusted for imbalance (Lenth, 2016). Analysis of these models compared individual treatment β's to one another and determined significant differences based on ratios of least-squared means. Treatment contrasts of mixed effect models for each response variable provide information to discuss overall differences between systems, analytes, and years.

Objective 2:

Manure Collection

Swine manure was collected from a facility in Iowa, directly from the pit below the barns using a transfer pump. Swine at the facility were being administered tiamulin, tylosin, and chlortetracycline (CTC) at the time of collection (personal communication, facility owner, December 13, 2016). Tiamulin was estimated as being administered at a rate of 1.75 g/kg (3.5 lbs/ton) of feed. CTC was estimated as added at a rate similar to 200 g/kg (400 g/ton) of feed, as described by Nitikanchana et al (KSU). The tylosin administration rate at this particular facility is unknown, but we previously reported that a similar commercial finishing facility fed tylosin at subtherapeutic levels of 44.1 g Mg^{-1} feed (~1.85 mg tylosin kg^{-1} body weight) for growth

promotion for 16 out of 20 weeks of each animal rotation or 2.5 turns per year (Garder et al., 2014). Prior to each experiment, manure samples were collected and analyzed for ARB and ARGs as described below.

Manure mitigation experiments

Manure mitigation experiments were designed to evaluate the impact of practices on the persistence of antibiotic resistant *Enterococcus* and selected antibiotic resistance genes. Practices considered included anaerobic digestion, two phase storage, ionophore (Narasin) addition, and centrifugation.

Anaerobic digestion. To evaluate the impact of anaerobic digestion, we added 200 g of liquid swine manure to eighteen 250 ml anaerobic batch reactors sealed with a rubber septum. Six reactors were placed in water baths maintained at moderate (25°C), mesophilic (37°C), and thermophilic (52.5°C) and operated at 150 rpm as previously described by Pandey and Soupir, 2011. Manure samples were collected from three of the six reactors (randomly selected) within each temperature treatment at days 1, 4, 7, 14, 28, 67, 56, 66, 87, 91, 103, 116, and 129 using a 50 ml gas tight glass syringe.

Two phase storage. A simulated two-phase storage system was used to evaluate the impact of longer manure retention times without the addition of fresh manure. To assess the impact of two phase storage, both single and two phase storage systems were simulated. The single stage storage system was represented by adding swine manure (~1L) to three 3.78 L plastic containers, with 10 mL fresh manure being added weekly to the system. To simulate two-phase manure storage, liquid swine manure was added to three 3.78 L containers, with no additional manure additions. This was designed to represent the secondary storage facility where manure is held for six months without additional feces addition prior to land application. Manure samples were collected on days 1, 7, 20, 35, 42, 52, 73, 89, 101, 115.

Ionophore addition. Ionophores are added to swine manure pits for control of pit foaming and methane (CH_4) production. Narasin, an approved feed additive helps to suppress methane production from treatment manures for approximately 60 days. The evaluation of the impact of the addition of ionophores on antibiotics, antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARG) will allow us to measure growth and decay of ARBs and ARGs. A 200 g well mixed manure sample was added to each of the 12- 250 ml anaerobic batch reactors. The bioreactors were then sealed with a rubber septum and maintained at room temperature. Narasin was added to (A) three reactors at a rate of 15 mg/L; (B) three reactors at a rate of 30 mg/L; (C) three reactors served as a control with no Narasin added. Manure samples will be collected at 0,3, 14, 28,42, 56 and 70 days from each reactor.

Centrifugation. Centrifuge was used to separate the manure into solid and liquid fractions. Centrifuge settings were set to encompass a range of centrifugal forces (times gravity, x g) and centrifugation times that could be achieved in the field. After centrifugation, the solids were removed from solution. The supernatant was collected and analyzed.

Analysis

Enterococcus spp. were quantified because of their role as indicators of pathogens, their use in regulatory standards, and their use in past studies on bacteria persistence and transport. *Enterococcus* concentrations, tylosin-resistant enterococci concentrations, and tetracycline-resistant enterococci concentrations in manure as determined by membrane filtration techniques as described in objective 1.

qPCR Analysis Genes associated with microbial resistance to erythromycin and tetracycline in swine manure were quantified in manures using gene primers and qPCR assays described above. DNA was extracted from 250 µL of manure slurry using the MagAttract PowerSoil DNA Kit (Qiagen) and quantified. The qPCR assays were performed using WaferGen Real-Time PCR System (Michigan State University, Research Technology Support Facility) with LightCycler 380 SYBR Green I Master mix (Roche Applied Science). For each 100 nL reaction volume, 25 nL of extracted DNA was used in each reaction and 250 nM of each primer was used. All assays were performed in triplicate. For each targeted gene, *ermB, ermF, tetM*, and 16S rRNA, a synthetic standard was designed and synthesized (Integrated DNA Tecnologies, Iowa City, IA), and various concentrations (10 fold dilutions) of these standards were also included in triplicate on qPCR assays.

Data Analysis

Relative abundance levels, represented as copies of gene per 16S-rRNA gene abundance, were estimated based on the measured qPCR threshold cycle (Ct) values of the appropriate genes and standard curves of known gene copy abundances to Ct values. Standard curves were required to represent high correlation between gene copies and Ct values. Limit of detection (LOD) for each targeted gene was determined using the lowest bound standard for quantified for each gene. Detections less than the LOD were not included in our analysis.

To test the effects of manure mitigation treatments, analysis of variance (ANOVA) combined with Tukey's mean separation test were used to compare determine gene abundances in treatments that were significantly different. To determine the correlation of gene relative abundances and time of treatment, a linear regression model was fitted to observed data using the lm package in R.

Results:

Objective 1

Antibiotic resistant enterococci and resistance genes in swine manure

Concentrations of total enterococci and antibiotic-resistant enterococci present in the swine manure at the time of application at the NERF are presented in Table 2. Although all manure was provided by the same commercial facility, there are some variations among the different application dates. Manure collected in the late fall of 2017 and spring of 2018 had lower enterococci concentrations than other manure samples. In most samples the concentrations of tetracycline or tylosin-resistant enterococci were equal or greater than the total enterococci, showing that antibiotic resistant enterococci dominated in the manure. The exceptions were late fall 2017 and spring of 2018 where as few as 21% of the enterococci were antibiotic resistant.

The *ermB* gene (resistance to tylosin and other macrolides) was the most common antibiotic-resistance gene found in the swine manures (Table 3). The *ermF* gene was only detected in two of the six manure loads in this study, which is in contrast to our previous results at this site (Garder et al 2014, Luby et al 2016). *TetO* and *tetM* were detected in all of the manures.

In addition to the ARB and ARG the swine manure contained 4 to 16 µg/g dry manure of tetracyclines and 10.6 to 15.6 µg/g of chlorotetracyclines. In addition tylosin, tiamulin and sulfonamide antibiotics were detected at lower concentrations.

Table 2 Mean concentrations of resistant and total enterococci in manures collected at the time of application. Late fall systems treated as a single manure.

Manure Application		Enterococci	Tetracycline-resistant enterococci	Tylosin-resistant enterococci
		CFU mL^{-1}		
Early Fall	2016	5.05×10^4	5.99×10^4	5.30×10^4
	2017	3.91×10^4	2.50×10^4	2.79×10^4
Late Fall	2016	5.58×10^3	6.50×10^3	5.93×10^3
	2017	5.78×10^4	1.86×10^4	1.23×10^4
Spring	2017	2.07×10^4	1.95×10^4	1.81×10^4
	2018	4.79×10^3	8.71×10^3	8.86×10^3

Table 3. Mean concentrations of ARG in manures collected at the early-fall (EF), late-fall (LF) and spring (S) times of application in corn-soybean (CS), or corn-corn (CC) crop rotations.

System and Year		*ermB*	*ermF*	*tetO*	*tetM*
		copies mL^{-1}			
EF-CS	2016	5.57×10^5	6.81×10^5	1.22×10^5	1.90×10^3
LF-CC + LF CS	2016	2.41×10^5	†ND	4.00×10^4	9.17×10^4
S-CC	2017	7.28×10^5	ND	8.77×10^4	7.17×10^4
EF-CS	2017	2.02×10^6	ND	6.32×10^5	7.09×10^5
LF-CC + LF-CS	2017	2.18×10^6	8.05×10^3	1.31×10^6	7.04×10^4
S-CC	2018	3.44×10^7	ND	9.95×10^6	6.27×10^5

† ND: not detected

Persistence of Antibiotic Resistance After Manure Application – Enterococci

Prior to manure application, soil enterococci and total bacterial populations (estimated by copies of 16S- rRNA gene) were not significantly different between soils with and without histories of manure application ($p > 0.5$), or between soils prior to manure treatment. Generally, the median concentration of antibiotic resistance genes (*tet* and *erm*) were less than 2.7×10^5 copies g^{-1} soil for any single gene. Enterococci were present only at trace concentrations when detected (< 5 cells g^{-1} soil).

Following manure application, concentrations of total and antibiotic-resistant enterococci were elevated above background in the manure injection bands. Concentrations raged from 28 CFU g^{-1} after the early fall manure application in 2016 to 2,181 CFU g^{-1} soil of tylosin-resistant enterococci present after the late fall application in 2017. Regression analysis to estimate the decay coefficient and equivalent half-life (Eq 2) described enterococci survival reasonably well after seven of the eight different manure applications. These analysis produced statistically significant k values ($p < 0.5$) with $R^2 > 0.5$. The resulting half-lives (time for soil concentrations to decrease by half) were generally between 17 and 29 days. Exceptions to this pattern were increased persistence (55 to 67 days) after the early fall manure application in 2016, and decreased

persistence after the late fall application in 2017 (2 to 3 day half-life) and after the spring manure application in 2018 (5 day half-life). Enterococci concentrations in the inter-band soils were generally low and significantly different from the in-band concentrations

Analysis of treatment means estimated from the LME showed that persistence of enterococci (resistant or total) were not different in the spring applications of manure into continuous corn rotations compared to late fall applications into continuous corn. Early fall manure injected after soybeans and before corn had greater ($p < 0.05$) persistence of enterococci (resistant or total) than late- fall applications in the same cropping sequence.

Persistence of Antibiotic Resistance after Manure Application – Resistance Genes

ARGs were consistently detected in control soils (system 1, Table 1) before and after application, but at levels similar to background concentrations of soils with manure history. Both copy numbers and relative abundances of background *ermB* were not significantly different between control and manured soils. In the first year of sampling, annually manured systems (3.2 and 4.1) had more than 1 x 10^5 copies of *ermB* and *tetO* prior to manure application. Early fall corn-soybean and late fall corn-corn systems had significantly more background *tetO* copies than the control ($p < 0.01$); all other background ARGs were not significantly different between soils with manure history and controls. This suggests that ARGs are not accumulating in soils with histories of swine manure for the months prior to manure application, at least for the genes observed in this study.

Like the enterococci, ARG concentrations were elevated on the day following manure injection. At this time concentrations exceeded 1 x 10^6 copies g^{-1} soil and some concentrations exceeded 1 x10^8. The *ermF* gene concentration was lower than concentrations of the other genes, particularly after the early fall (EF) and late fall (LF) applications in 2017 and the spring application in 2018. After these applications, the *ermF* concentrations ranged from 3.85 x 10^3 to 9.60 x 10^3 copies g^{-1} soil which were not distinguishable from the pre-application concentration. The first order decay regression model (Eq 2) estimated half-lives for most post-application scenarios, but in some cases the R^2 fell below 0.2 and the *p* value was not significant. In some cases this appears to be due to low initial concentration (A at day 1) as discussed previously for *ermF*, but in other cases (12 total) the gene concentrations increased or decreased inconsistently. Concentrations of ARG in control soils (no manure application) were generally fairly constant. Concentrations of ARG in soil between the application band (NB) were constant or showed minor decreases, except for *tetO* which had increases in NB soil concentrations in 2016 and 2018 (data not shown). Regardless, ARG concentrations in the IB and NB tended to converge between 75 to 180 days after application.

The half-lives of ARG in soil after the different manure applications ranged from 100 days for *ermB* (EF-CS in 2017) to 5.6 days for *ermB* (S-CC in 2018). Average half-lives of resistance genes are shown in Fig 1. *ErmB* and *tetO* tend to be more persistent than the other genes, but this trend is not statistically significant.

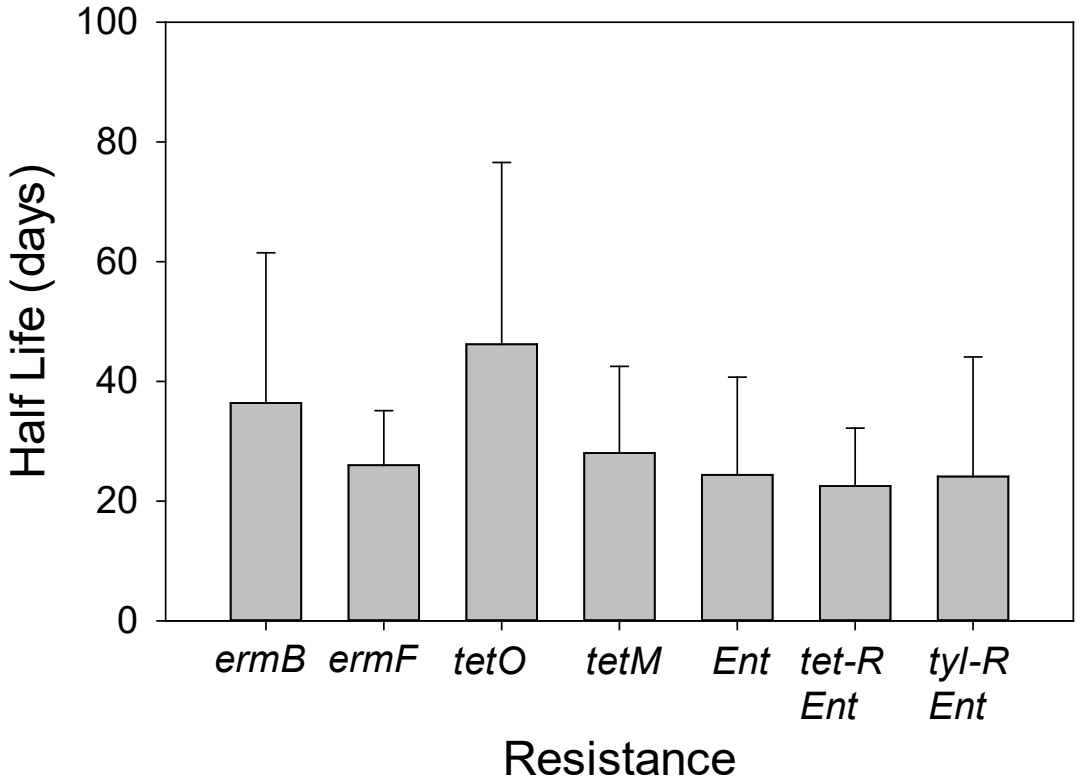

Figure 1. Half-lives of resistance genes (*ermB*, *ermF*, *tetO* and *tetM*) averaged over different manure application timings and compared to similar averages of tylosin-resistant (tyl-R Ent) and tetracycline-resistant (tet-R Ent) *Enterococcus*. Vertical bars indicate the standard deviation.

The average half-lives for all ARG over all years indicates that there is a modest benefit (shorter ARG half-life) after the spring manure application into continuous corn. Moreover, the LME models indicated significant effects of the systems (combinations of application timing and cropping rotation) for some genes, but these effects were not consistent with all or most genes. The LME model confirmed that the average ARG half-life after the spring manure application into continuous corn (13 days) was significantly different ($p < 0.05$) from the ARG half-life of 57 days after early fall application into the corn-soybean system (Figure 2).

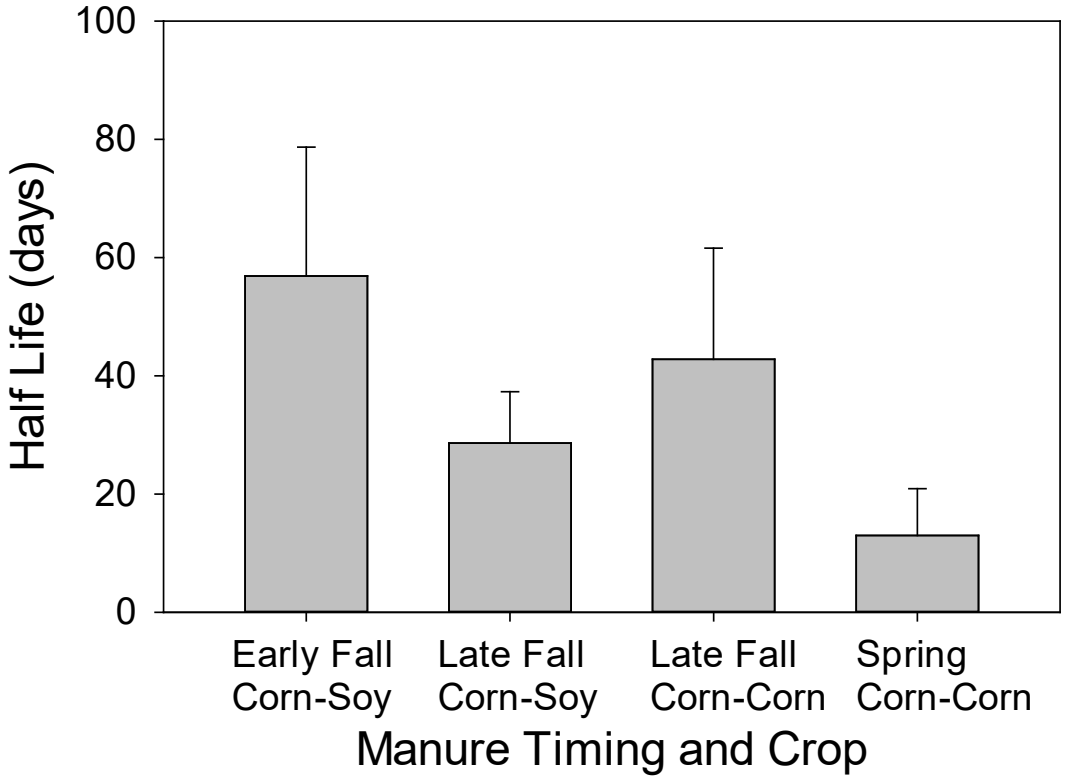

Figure 2. Half-lives of resistance genes (*ermB, ermF, tetO and tetM*) in soil averaged over different manure application timings and crop rotations. Vertical bars indicate the standard deviation.

Tile drainage analysis

In water samples, concentrations of ARB were consistently low. Total enterococci concentrations varied between each sampling year and were generally low. Drainage samples from 2016 had the highest median concentration with 7 CFU/100mL, followed by 2018 and 2017 at 6 CFU/100mL and 1 CFU/100mL, respectively (Table 4). Samples from 2017 were significantly different (Wilcoxon Ranked Sums, $\alpha = 0.05$) than 2016 and 2018, however, 2016 and 2018 were not significantly different. This indicated that years may not be combined for analysis.

Tetracycline resistant *Enterococcus* were significantly different ($\alpha = 0.05$) in 2016 than other sampling years. Tetracycline resistant *Enterococcus* were detected in 63.5% of all samples, whereas only 37.3% of samples in 2018 and 16.8% of samples in 2016 had detection (Table 7). Although the 2016 median concentration was <1.0 CFU/100mL, the maximum value was 72 CFU/100mL (Table 5). Similar to total enterococci, 2018 was the next highest year with far lower detected growth of tetracycline resistant *Enterococcus*. The maximum concentration was nearly 3.5 times lower at 22CFU/100mL, and the median concentration was also >1CFU/100mL. 2017 manure samples had extremely low detection with a median concentration of < 1 and a maximum of only 4 CFU/100mL.

Tylosin-resistant enterococci concentrations were extremely low over all three years of this study (data not shown). Median concentrations for all years were <1 CFU/100mL, and the maximum value observed over all three years was 8 CFU/100mL. The highest detection

percentage followed previous trends, with 2016 being the highest (25.4%) followed by 2017 (16.8%) and 2018 (13.4%), Table 8. Due to the low overall concentrations and detection, further analysis was not performed.

Table 4. Median total *Enterococcus* concentrations by year in tile drainage water.

	Enterococcus					
	2016		**2017**		**2018**	
Manure Application	**Median**	**Std Deviation**	**Median**	**Std Deviation**	**Median**	**Std Deviation**
Early Fall	6	223	12	16	5	13
Late Fall	11	25	>1	9	5	16
Spring	9	19	3	16	7	13
Control	5	20	>1	7	8	15
Total	7	22	1	13	6	14

Table 5. Median tetracycline-resistant *Enterococcus* concentrations by year in tile drainage water.

	***Enterococcus* + Tetracycline**					
	2016		**2017**		**2018**	
Manure Application	**Median**	**Std Deviation**	**Median**	**Std Deviation**	**Median**	**Std Deviation**
Early Fall	1	8	>1	>1	>1	2
Late Fall	2	17	>1	1	>1	>1
Spring	1	15	>1	>1	>1	1
Control	>1	5	>1	>1	>1	>1
Total	1	12	>1	>1	>1	3

Table 6. Detection of *Enterococcus* by year in tile drainage water.

	Enterococcus					
	2016		**2017**		**2018**	
Manure Application	**Samples with Detection**	**Percent Detection**	**Samples with Detection**	**Percent Detection**	**Samples with Detection**	**Percent Detection**
Early Fall	80	82.5%	57	75.0%	99	87.6%
Late Fall	56	93.3%	34	73.9%	46	86.8%
Spring	36	81.8%	24	80.0%	51	92.7%
Control	30	85.7%	23	60.5%	45	95.7%
Overall	202	85.6%	138	72.6%	241	89.9%

Table 7. Detection of tetracycline-resistant *Enterococcus* by year in tile drainage water.

	***Enterococcus* + Tetracycline**					
	2016		**2017**		**2018**	
Manure Application	**Samples with Detection**	**Percent Detection**	**Samples with Detection**	**Percent Detection**	**Samples with Detection**	**Percent Detection**
Early Fall	59	60.8%	13	17.1%	41	36.3%
Late Fall	45	75.0%	10	21.7%	21	39.6%
Spring	28	63.6%	8	26.6%	23	41.8%
Control	18	51.4%	1	2.6%	15	31.9%
Overall	150	63.5%	32	16.8%	100	37.3%

Table 8. Detection of tylosin-resistant *Enterococcus* by year in tile drainage water.

	***Enterococcus* + Tylosin**					
	2016		**2017**		**2018**	
Manure Application	**Samples with Detection**	**Percent Detection**	**Samples with Detection**	**Percent Detection**	**Samples with Detection**	**Percent Detection**
Early Fall	24	24.7%	11	14.5%	15	13.3%
Late Fall	25	41.7%	9	19.6%	7	13.2%
Spring	8	18.2%	11	36.7%	8	14.6%
Control	3	8.6%	1	2.6%	6	12.8%
Overall	60	25.4%	32	16.8%	36	13.4%

Drainage samples from plots receiving manure application in the late fall had the highest median concentration of tetracycline-resistant *Enterococcus* (2 CFU/mL) and the highest percent detection (75.0%) and were the only treatment that was significantly different from the no-manure control (Wilcoxon Ranked Sums, $p = 0.1$). Spring manure had the next highest percent detection at 63.6%, followed closely by Early Fall application at 60.8%. Tylosin ARBs mirrored tetracycline ARBs and also had the highest percent detection in 2016 in late fall application (41.6%).

In 2017 and 2018, samples associated with spring manure application had the highest percent detection of tetracycline-resistant *Enterococcus* (26.7%, 41.8%) and tylosin-resistant *Enterococcus* (36.7%, 14.6%), although these datasets were not nearly as large as 2016 (Tables 7 and 8).

Antibiotic Resistant Genes in Drainage

Overall, detection of all resistance genes (*erm*B, *erm*F, *tet*M) was very low across all years, and thus all years were grouped together for analysis. *Erm*F had the highest percent detection across all samples at 4.7%, followed by *erm*B at 4.1% detection, and *tet*M with the least at 2.3% detection.

Plots receiving manure in the spring consistently had higher detection frequency across all three resistance genes when compared to plots receiving manure in the fall or to control plots receiving no manure. *Erm*B and *erm*F both were detected in 14.7% of SM samples, and *tet*M was detected in 9.8% of SM samples. Drainage from plots receiving manure in the late fall had the highest percent detection across all ARGs tested, with *erm*F detected most often (7.1%), followed by *erm*B (4.5%), and *tet*M (2.5%). Both early fall application and control plots had < 2% detection for all ARGs. Overall, *tet*M had the highest median copy number with 3×10^4 copies/100mL followed by *erm*B and *erm*F both at 7×10^3 copies/100mL.

Objective 2:

Anaerobic digestion. With anaerobic digestion, a reduction of at least two orders of magnitude in total *Enterococcus* was observed, regardless of digestion temperature. Reduced counts of tylosin and tetracycline resistant enterococci were associated with higher digestion temperatures, with the greatest reduction in both tylosin and tetracycline resistance observed at the highest digestion temperature, 57ºC.

Concentrations of ARGs significantly increased at digestion temperatures of 37 and 57ºC between day 1 and day 116 (p-value = 0.0001). Figure 3 shows *ermB* relative abundances at each of the three temperatures as a function of time. Notably, the relative abundance of these resistance genes comprise a small fraction of total genes in the microbial population. Based on the abundance of 16S-rRNA genes, which are housekeeping genes often used to estimate the total number of cells, these *ermB, ermF* and *tetM* genes represent mean 2.7%, 12.6%, and 10% of 16S-rRNA gene population, respectively. In contrast to enterococci counts, the total abundance of 16S-rRNA genes in anaerobically digested manure generally increased over time. An exception to this increase in gene count was on day 90, where 16S-rRNA gene counts decreased, but were followed by an increase on day 129.

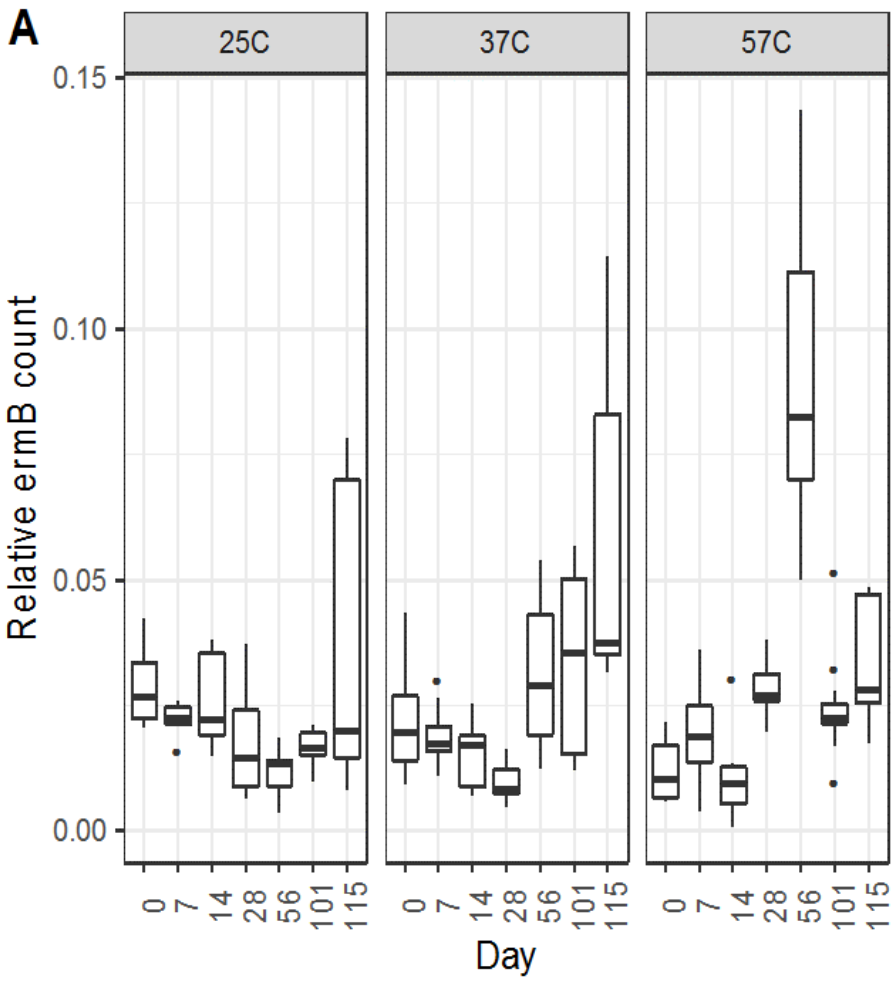

Figure 3. Relative abundance of *ermB* genes in manure samples treated by anaerobic digestion at three temperatures.

Two phase storage. The one-phase storage with the addition of fresh manure resulted an increase in enterococci levels, relative to the two-phase storage system. Additionally, with the fresh manure addition, we observed 9-fold and 6-fold increase in *ermB* and *ermF* (data not shown) gene concentrations between days 7 and 115 (Figure 4). In comparison, when no additional manure was added, we observed reduction of both tylosin and tetracycline resistant enterococci. The presence of ARGs increased when fresh manure was added and decreased when no manure was added. An exception was *tetM* genes, which were observed to be similar over time in manure added treatments (one phase reactor), and slightly reduced in no manure added treatments (two phase reactor), data not shown.

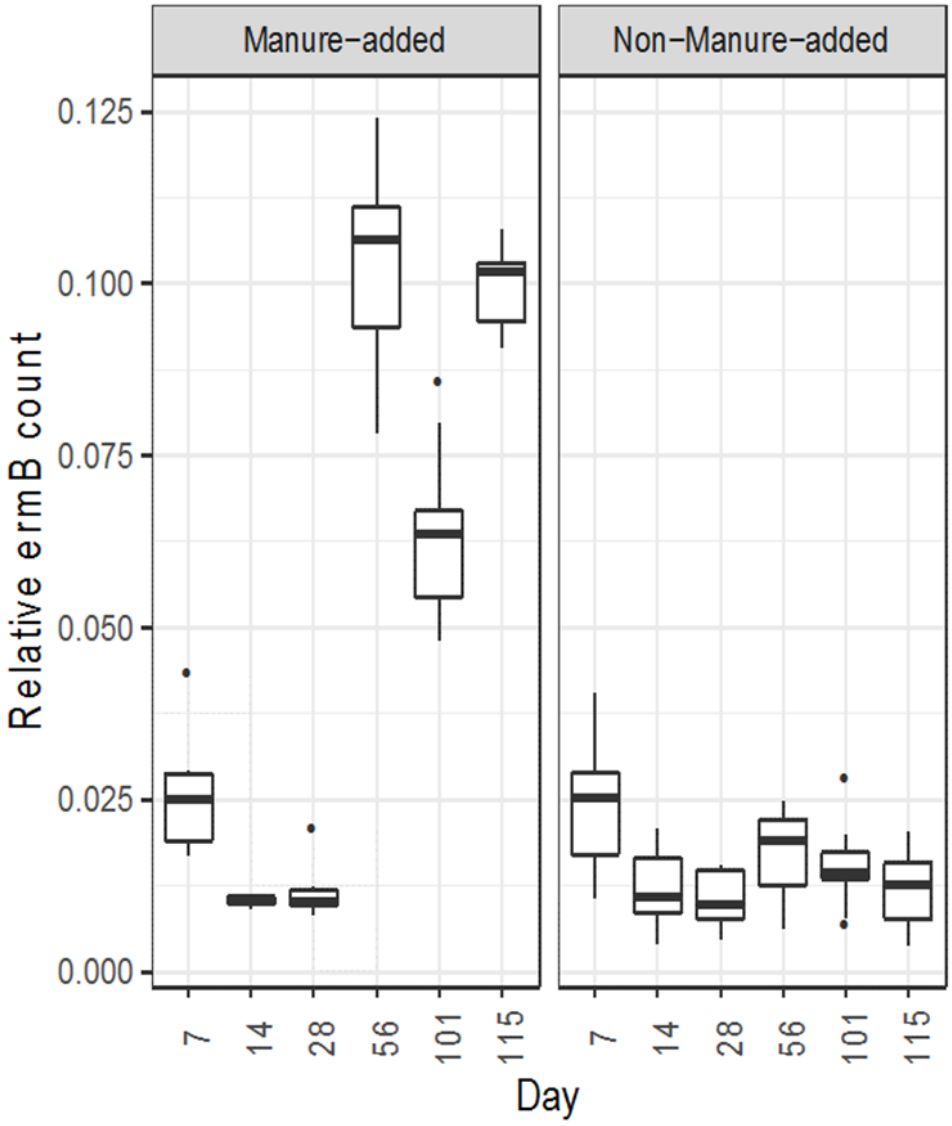

Figure 4. Relative abundance of *ermB* genes in manure samples with manure added and compared to samples where no additional manure was added to the system.

Centrifugation. Separation of solid and liquids in manure allows land application of liquids and composting of solids. In our study, we evaluated the presence of AMR in the liquid phase resulting from four centrifugation speeds and compared to untreated manure. We observed that total and resistant enterococci decreased with increasing centrifugation speeds, with very few or undetectable concentrations in the liquid separated at 7100 x g. Reduction of enterococci below 25 CFUs per mL were generally observed above 6000 x g centrifugation speeds.

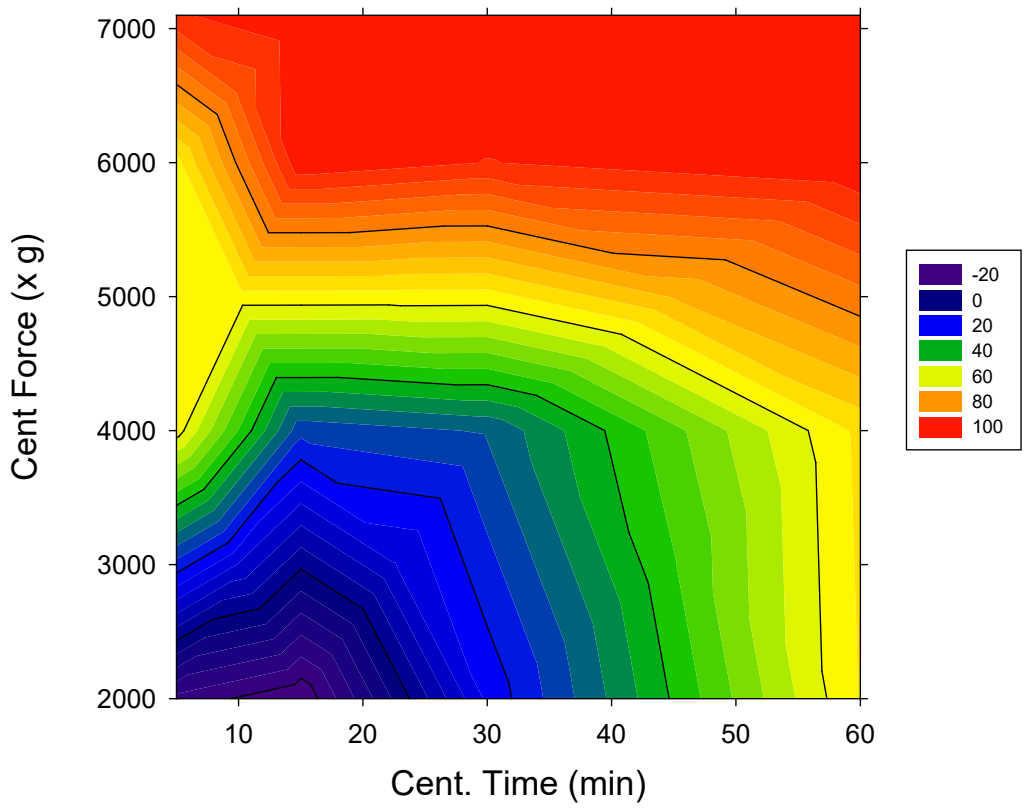

Figure 5. Percent reduction of tetracycline resistant enterococci in centrifuged supernatant as a function of centrifugation force and time.

Compared to 16S-rRNA data, we did not observe that centrifugation reduced ARG in the liquid phase. Similar to our observations with manure treated with anaerobic digestion, centrifugation decreased concentrations of both total and resistant enterococci within the separated liquid, but this result was inconsistent with resistant ARG which were enriched in the liquid phase regardless of centrifugation speed ($p < 0.001$).

Ionophore addition. The addition of Narasin did not impact enterococci or ARB concentrations through the course of the experiment, with concentrations remaining relatively stable over the 77 days.

Discussion:

Soil ARB and ARG persistence. The persistence of ARB and ARG are important to the understanding of risks posed by current antibiotic use in swine production. This study shows that application of manure results in elevated levels of ARB and ARG in soil after application. These elevated levels of ARB and ARG increase the potential for off-site transport of resistance in surface runoff waters or in subsurface (tile) drainage. In addition, elevated ARB and ARG concentrations increase the potential for genetic exchange between the manure-derived community and the soil community (Heuer et al, 2011).

Our results for ARG are similar to the results from Marti et al (2014) which reported *ermB* gene half-lives of 29 to 50 days in soil after swine manure application in Ontario. Their results also showed a trend towards shorter half-lives in spring manure applications, which is similar to our results. Previous work by our research group observed the initial increase in ARG after manure

application and the decline to background within 12 months (Garder et al, 2014; Luby et al 2016). These results narrow the period that ARB and ARG concentrations are elevated to 200 days or less, which for a fall swine manure application would include most of the subsequent growing season.

The utilization of half-lives to describe ARB and ARG persistence is a useful tool, but some consideration of underlying assumptions is needed. First, the choice of *Enterococcus* and the four resistance genes as representative of antibiotic resistance is considered. *Enterococcus* has long been used as an indicator of fecal pollution at beaches and in drinking water, but there are no regulatory standards for *Enterococcus* presence in soil. Recent research in our laboratories with the same soils and manures used in these studies show that the swine manure community is dominated by Firmicutes, which include the bacterial genera *Bacillus*, *Enterococcus*, *Staphylococcus* and *Streptococcus* (Riecke et al. 2018). Firmicutes are also present in soil. *Enterococcus* is capable of genetic exchange with each of these bacteria, and very likely with other members of the Firmicutes (Werner et al, 2013). While *Enterococcus* is only a component of the manure bacterial community it may interact with the soil community and other Firmicutes especially, and is therefore relevant to the antibiotic resistance discussion. Previous work shows that *ermB* and *ermF* are abundant in swine manure and in soil associated with animals (Choi et al 2018). That same study showed that primers for qPCR were effective in quantifying all the variant forms of *ermB* or *ermF*. The diversity of tet genes is also recognized, with 46 different tetracycline resistance genes described (Roberts and Schwarz, 2016). Nevertheless, the primers used in this study target representative members of the ARG present in swine manure.

In this research, *ermB*, *tetM*, and *tetO* behaved in a mostly similar pattern (Figure 2), with *tetO* marginally more persistent and *ermF* less persistent. The half-lives of these genes were also similar to the antibiotic-resistant *Enterococcus*. These similarities among the persistence of these four genes is unlikely to be fully representative of all ARG persistence. Unpublished research in our group indicates that some manure-borne ARG definitively persist longer than others. Observable differences in persistence of these genes is due to the survival of the bacteria that host them in manure or soil.

Swine manure applications in early fall, late fall, and spring resulted in mostly similar patterns of dissipation of either enterococci and antibiotic-resistance genes. For the ARG, there was evidence for shorter persistence after spring manure applications. However the effect of manure application into soil following corn (CC, spring application) versus application into soil after soybean (CS, fall application) is not known. The persistence of antibiotic resistance (above background) after swine manure application should be 200 days or less. While the shorter persistence of ARG after spring application suggests some benefit in risk management, the spring period is also the season with greatest precipitation and tile drainage, factors which increase risk of ARG transport.

The analysis of these for resistance genes in soil showed that soils receiving swine manure approximately one year previously (continuous corn) or two years previously (corn soybean) to which had not had antibiotics for many years (controls) were not different in the abundance of ARG in soil. This is evidence suggesting that these ARG are not accumulating in the soil under these farming operations.

Water ARB and ARG persistence. Throughout the experiment, we observed that antibiotic resistance in drainage coming from plots receiving manure was generally low. The highest yearly median concentration of the total fecal indicator bacteria, *Enterococcus*, was less than one third of

the US EPA recreational water quality limit of 33 cfu/100mL. Both tetracycline and tylosin resistant enterococcus median concentrations were less than 1 CFU/100mL. Total enterococci and ARB concentrations found in this study are systematically lower, yet comparable, to other studies performed at the same location in previous years. Luby et al. (2016) reported higher total enterococcus concentrations ranging from >1 to 110 CFU/100mL and similar low tylosin-resistant enterococci concentrations of <1 CFU/100mL. Similarly, a study also done at the same location by Garder et al. (2014) reported average enterococci concentrations of about 100 CFU/100mL or below. Neither Garder et al. or Luby et al. measured tetracycline ARBs.

Similar to ARBs, antibiotic resistant gene detection in this study were observed to be low, and lower than other comparable studies at the same site. Luby et al. (2016) was able to report detection of *erm*B and *erm*F (*tet*M not studied). *Erm*B showed up to 82% detection and *erm*F showed up to 44% detection in drainage from manured, as compared to this study which reports the highest detection (14.8%) in a manured system for both *erm*B and *erm*F. Garder et al. (2014) reported even higher percent detection for *erm*B and *erm*F. This study found copy numbers comparable to Garder et al. (2014), but higher than Luby et al. (2016). However, it should be noted that because this study reported far less data over the limit of quantification, the dataset is not robust enough to draw any definitive conclusions between studies.

Previous research has only compared differences between plots receiving manure and plots not receiving manure amendments. This study considers differences between the timing of the application of the manure in either early fall (EF), late fall (LF), or spring (SM). Previously, there have been no significant differences detected in tylosin-resistant enterococci in tile water between plots receiving manure and plots not receiving manure (Garder et al. 2014; Luby et al. 2016). This study found similar results with low tylosin ARB detection. Tetracycline ARB were more often detected, however, only one manure application time in one year (2016 LFM) was found to be significantly different than the control.

Although research has not shown differences between manure application in ARBs, there has been significant differences shown in ARGs in drainage from plots receiving manure and plots not receiving manure in the fall (Luby et al., 2016). This study confirms that LFM and SM had a higher percent detection of ARGs when compared to control, however, the same cannot be said for EFM, whose percent detection was comparable to the control. It is possible that this could be attributed to the sampling scheme, as EFM sees the greatest lag time between manure application and when drainage flow sampling commences. Finally, this study saw the largest percent detection of all three ARGs selected (*erm*B, *erm*F, and *tet*M) in drainage from plots receiving spring manure (SM). Greater transport of ARG in tile drainage after SM occurred despite the shorter persistence of ARG in soil after SM application, suggesting that drainage predominates over ARG survival in soils in determination of ARG transport. In this study, due to overall low detection, it was challenging to quantify this impact.

Manure Mitigation Strategies. The reduction of both resistant *Enterococcus* and resistance genes in two-phase storage with no manure added suggests that this may be a promising management solution for reduction of AMR in manure storage. When compared to anaerobic digestion, our results suggest that two phase storage without manure addition may achieve similar treatment of antibiotic resistance bacteria and genes. This management strategy presents an opportunity for further research and development, and the practicality of installing storage in comparison to anaerobic digestion would need to be further evaluated.

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