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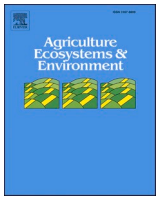

Prairie strips remove swine manure associated antimicrobial resistance genes and bacteria from runoff

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ABSTRACT

Runoff from manured agricultural fields can transport antimicrobial resistance (AMR) contaminants, including genes and bacteria, to downstream ecosystems. Previous work has identified the integration of Conservation Practice 43 (CP 43) prairie strips – a type of vegetative filter strip – within and at the edge of agricultural fields as a potential management solution to reduce the movement of these, and other, manure pollutants, while offering an opportunity for biodiversity conservation. The objectives of this study were to 1) quantify the ability of prairie strips to reduce the presence of antimicrobial resistance genes from manure laden runoff, and 2) characterize the impact of manure on prairie soil microbiomes over time. Simulated rainfall events were used to create artificial runoff on field plots with swine manure amendment and prairie strips as treatment factors. A suite of antibiotic resistance genes and mobile genetic elements were characterized in runoff samples collected during the rainfall simulation, while manure associated bacteria were characterized in soil samples collected over 153 days after the rainfall simulation. Prairie strips placed downslope from manured crop soil significantly reduced the cumulative abundance of resistance genes in both runoff water (p-value < 0.0001) and runoff sediment (p-value < 0.0001). Manure associated bacteria were transported both horizontally, from the manure amended crop soil into the prairie strip soil, and vertically, into the crop and prairie strip soil profiles. The specific manure associated gene *tet(M)* and the specific manure associated bacterial genus *Clostridium* sensu stricto 1 were highly enriched in manured runoff and soil, respectively, and could represent future targets of human health concern. Results from this study provide further support for the use of CP 43 prairie strips as a management practice to reduce the transport of manure associated resistance contaminants off agricultural fields.

1. Introduction

As animal production continues to intensify, an inevitable effect is the accumulation of manure. Manure is often applied to crop fields with the intent of both organic fertilization and animal waste disposal. However, field application of manure directly introduces antibiotic resistant bacteria (ARBs) and antibiotic resistance genes (ARGs) to soil microbial communities and provides an opportunity for the transfer of resistance genes to soil bacteria via horizontal gene transfer facilitated by mobile genetic elements (MGEs) (Chee-Sanford et al., 2009; Xie et al., 2018). Methods of manure application typically include either surface spreading, relying on gravity to incorporate the manure into the soil, or surface incorporation, which utilizes tillage tools to physically integrate or inject the manure into the soil (Laguë et al., 2005). Because these application methods leave manure either on, or relatively near, the soil surface, potential transport methods of manure contaminants downstream can include movement with infiltrating water and surface runoff (Lüneberg et al., 2018). The bacteria and genes mobilized from manure fields can move in suspension unattached, or in association with sediment or manure solids, meaning their removal from transporting waters will primarily occur through straining and filtering during soil infiltration, sedimentation, and adsorption (Jamieson et al., 2002; Reddy et al., 1981).

One established method for reducing the impact of manure derived pollutants to downstream environments are vegetative filter strips (VFSs). VFSs are bands of permanent, dense plant growth that can be

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positioned to intercept runoff from an upland area, such as an agricultural field (Dillaha et al., 1989). Prairie strips, a unique type of VFS, use perennial vegetation composed of diverse, native plant species (i.e., "prairie") as a way to combine both grassland restoration and agroecosystem improvements (Schulte et al., 2017). As well, installation of these prairie strips on farm fields can be supported through cost-share programs; for example, through the USDA Conservation Reserve Program, in which prairie strips are listed as Conservation Practice 43 (CP 43). Presently, prairie strips have been shown to have significant impacts on reducing volumes of surface runoff from agricultural fields while also decreasing the presence of sediments and nutrients within this runoff (Helmers et al., 2012; Zhou et al., 2014). These previously demonstrated reductions of both particulate and dissolved compounds support the hypothesis that prairie strips may be similarly effective in reducing the transport of manure derived genes and bacteria with surface runoff.

While the capture of manure derived contaminants by prairie strips is expected, the impact and persistence of these contaminants following sedimentation and infiltration represents a second stage of relatively unexplored mitigation. The composition of recipient soil microbial communities may be altered through either direct introduction of manure bacteria or alteration of physical or chemical properties of the soil (Lopatto et al., 2019; Rieke et al., 2018b). To fully illustrate the potential mitigative effect of these prairie strips, the types and duration of shifts in prairie strip soil microbial communities following the interception of manure laden runoff must also be characterized.

The movement of antimicrobial resistance (AMR) contaminants from manured agricultural fields to surrounding environments has been well documented, however few strategies have been proposed to combat this transport (Garder et al., 2014; Hsu et al., 2014; Jacobs et al., 2019; Joy et al., 2013; Luby et al., 2016; Neher et al., 2020b, 2020a; Zhang et al., 2017). Prairie strips represent a cost-effective conservation practice already well positioned for adoption based on extensive previous research demonstrating their ability to provide a variety of agroecosystem benefits. Our proposed use of prairie strips would extend these already established benefits to include AMR mitigation and would promote prairie strip installation as a form of manure management. The goal of this study was to evaluate the use of CP 43 prairie strips as a management practice to reduce AMR transport and persistence within agroecosystems at the plot scale. Our objectives were to 1) quantify the ability of prairie strips to reduce the presence of antimicrobial resistance genes from manure laden runoff, and 2) characterize the impact of manure on prairie soil microbiomes over time.

2. Materials and methods

2.1. Study site

Our field study took place at the Iowa State University ARM research farm on an area with no known history of manure application. This farm incorporates CP 43 prairie strips into a corn (*Zea mays* L.) – soybean [*Glycine max* (L.) Merr.] cropping system and is characterized by gently to strongly sloping, well-drained loess soils, with the predominant soil being Marshall silty clay loam and the average slope being 6.6%. The site was cropped to soybeans in the 2018 growing season and plots were constructed immediately following crop harvest.

Original seeding of the prairie strips occurred after crop harvest in 2014 and utilized the Statewide Mesic 10–30, Iowa Pollinator Mix with additions of milkweed (*Asclepias* spp.), Canada wild rye (*Elymus canadensis* L.), and Indian grass (*Sorghastrum nutans* [L.] Nash) seeds. Resulting plant cover was composed of a mixture of stiff-stemmed, native prairie grasses and forbs, with dominant species including wild bergamot (*Monarda fistulosa* L.), Canada wild rye, and gray-headed coneflower (*Ratibida pinnata* (Vent.) Barnh.).

2.2. Plot construction

Establishment of the plots followed previously described methods with slight modifications (Flater et al., 2022). Briefly, plots were constructed to include or exclude the existing prairie strip plantings that were immediately downslope from the cropped section of the field. Three treatments were evaluated: non-manured crop with a prairie strip installation (Strip + No Manure), manured crop with a prairie strip installation (Strip + Manure), and manured crop without a prairie strip installation (No Strip + Manure) (Fig. 1). The experimental area was subset into three blocks. Three plots were constructed within each block, with each plot representing one of the described treatments. This experimental layout produced three replications of each treatment and a total of nine plots.

All plots were adjacent to each other with the plot longitudinal direction perpendicular to the landscape contour and prairie strip. Plots containing both crop and prairie strip were a total of 1.5 m wide by 3 m long, with the crop section being a 1.5 m by 1.5 m square and the prairie strip section immediately downslope being a 1.5 m by 1.5 m square. Plots containing only crop were a 1.5 m by 1.5 m square. Metal borders were installed to a depth of 15 cm along the plot boundaries to constrain runoff, and a collection trough was established at the downslope edge of the plot.

Swine manure was sourced from a tunnel ventilated deep pit wean to finish facility located in Alden, Iowa that is known to utilize both tetracycline and tiamulin (facility owner, personal communication, 5 December 2018). A preliminary sample from the manure pit was sent to Minnesota Valley Testing Laboratories, Inc. (Nevada, IA), where analysis showed a nutrient concentration of 6 kg N per 1000 L (50.1 lbs N per 1000 gallons). A target application rate of 224 kg total-N ha^{-1} (200 lbs total-N $acre^{-1}$) was chosen for the experiment, a common rate for agronomic corn production in Iowa (Sawyer et al., 2003).

Manure was collected directly from the surface of the pit below the swine confinement the day prior to the first rainfall simulations and stored at 4 °C overnight. Manure samples were taken each day before application to the plots with 12 subsamples subsequently taken from each daily sample and stored at − 20 °C. The crop area of the experimental site was lightly tilled using a bow rake prior to construction of the plots to facilitate uniform manure application. Manure was surface broadcast onto the crop portion of the appropriate plots and then raked into the soil to promote even spreading and simulate light incorporation.

2.3. Rainfall simulation and sample collection

A field-portable rainfall simulator (Miller, 1987) was used to generate runoff events on the various treatment plots following previously described protocols (Kovar et al., 2011; Sauer et al., 2000). Rainfall simulations were conducted over the course of three days in the fall of 2018 (Oct. 24 – 26) with an experimental block, that included a replicate of each treatment plot, being rained on each day. Rainfall occurred within 24 h of manure application to ensure mobilization of contaminants and to simulate scenarios in which rainfall events occur shortly after manure application. Each treatment plot was subjected to a simulated rainfall rate of 76 mm hr^{-1} (5- to 10-yr return period storm). Runoff was characterized by a steady stream of water leaving the collection trough with flow rates that ranged from 9.5×10^{-7} – 1.3×10^{-5} $m^3 s^{-1}$ (Supplemental Fig. S1). Once runoff was achieved, rainfall continued for an additional 30 min during which discrete grab samples were collected.

Six discrete grab samples, representing biological replicates, were taken at 5-min intervals that began 2.5 min after the initiation of runoff to promote steady state conditions. Specifically, samples were taken at times of 2.5, 7.5, 12.5, 17.5, 22.5, and 27.5 min after runoff. Samples were collected in 1 L sterile Nalgene bottles over 1-min durations and their volume was used to determine runoff rates. In total 54 runoff samples were collected. Using a centrifugation technique informed by

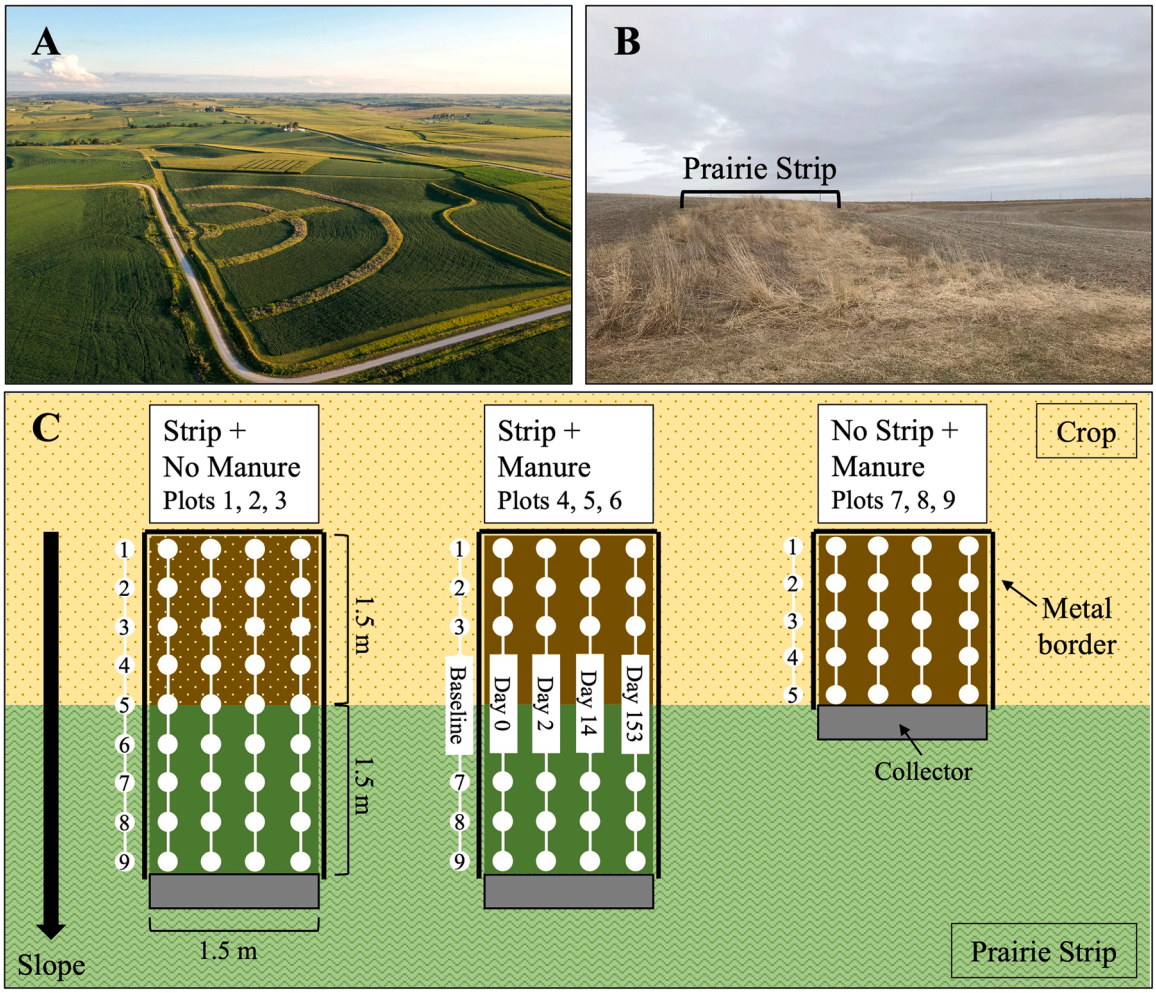

Fig. 1. A: An aerial view of the prairie strips located at ARM research farm during a spring bloom (Photo credit: Omar de Kok-Mercado – Iowa State University). B: Area of in-field prairie strip where experimental plots were constructed. Rainfall simulations were conducted over the course of three days in the fall of 2018 (Oct. 24 – 26). C: Schematic of the three treatment plots within an experimental block. Treatments included non-manured crop with a prairie strip amendment (Strip + No Manure), manured crop with a prairie strip amendment (Strip + Manure), and manured crop without a prairie strip amendment (No Strip + Manure). Soil cores were taken across the length of plot with locations represented by circles. Each soil core (1.9 cm diameter) was taken to a depth of 15 cm with the top 5 cm and the bottom 10 cm being divided into separate samples. Soil cores were taken prior to the simulation (Baseline), immediately after the simulated rainfall (Day 0), and 2, 14, and 153 days after the event (Day 2, Day 14, Day 153), with each sampling time point occurring subsequently along the width of the plots.

Muirhead et al. (2005) and Simmons and Krometis (2005), all runoff samples were centrifuged for 5 min at 4696 x *g* (5000 rpm) to pellet sediment present in runoff. This processing allowed a differentiation between runoff water and runoff sediment.

Soil cores were collected from each plot with the use of a 1.9 cm (0.75 in) core diameter soil probe (JMC Soil Samplers) immediately following the rainfall event (day 0), with subsequent soil cores also collected from each plot 2, 14, and 153 days after the event. Baseline soil cores were collected directly adjacent to each plot prior to any manure application or rainfall event (Fig. 1). Following previously described sampling methods, each soil core was taken to a depth of 15 cm and split into two depths, with the top 5 cm and the bottom 10 cm being divided into separate samples (Fahrenfeld et al., 2014; Joy et al., 2013).

Nine soil cores were taken from the Strip + No Manure treatment plots and the Strip + Manure treatment plots. Of these nine, the first four samples were located within the crop section and occurred at evenly spaced positions across the section, a single sample was collected at the interface between the crop and prairie strip sections, and the last four samples were located within the prairie strip section and occurred at evenly spaced positions until the end of the section was reached. Five cores were taken from the No Strip + Manure treatment plots spanning the crop section at evenly spaced positions. The described sampling method resulted in a total of 690 soil samples. Soil samples were homogenized and then stored at − 20 °C.

2.4. DNA extraction

Following centrifugation, runoff water samples were filtered through 0.22 μm sterile filters within 48 h after collection. Filters were stored at − 80 °C until DNA extractions were performed using a DNeasy PowerWater kit (Qiagen). When possible, 100 mL of each runoff water sample was filtered for DNA extraction. If less than 100 mL of sample was obtained, the total volume of runoff water was filtered for DNA extraction. Previously frozen manure and soil samples were thawed, mixed, and subsampled for DNA extraction. DNA from manure subsamples (250 μL), soil subsamples (250 mg, wet weight), and pelleted runoff sediment (total pellet, ≤250 mg, wet weight) were extracted using MagAttract PowerSoil DNA kits (Qiagen) with robotic handling. All DNA samples were cleaned using OneStep PCR Inhibitor Removal Kits (Zymo) to remove PCR inhibitors and improve downstream analysis. Concentrations of all DNA samples were obtained with the use of the Quant-it dsDNA Assay Kit, high sensitivity (Thermo Fisher Scientific). DNA samples were stored at − 80 °C until further analysis.

2.5. High-throughput quantitative PCR of runoff samples

High-throughput qPCR was performed using the Biomark HD System and its respective microfluidic 96.96 Dynamic Array Integrated Fluidic Circuits (IFCs) (Fluidigm) according to the manufacturer's Gene Expression protocol without pre-amplification. In order to minimize unnecessary reactions producing nonamplification, a range of diluted DNA from two randomly selected No Strip + Manure runoff water samples was initially screened against a suite of 47 primer sets targeting ARGs, MGEs, and the 16S rRNA gene (Supplemental Table S1). These gene targets were selected to complement the known antibiotics used on the farm where the manure was sourced and based on their previous identification within swine manure (Alt et al., 2021; Smith et al., 2019; Zhao et al., 2018).

Results of the initial screening (unpublished results) supported the dilution of all runoff samples 10-fold to help negate the potential effects of inhibitors and non-specific binding of primers. As well, based on their detection during the initial screening, a final suite of 28 primers sets, targeting 20 ARGs, 7 MGEs, and the 16S rRNA gene, were selected for analysis in all runoff samples. Thermal cycling conditions for all reactions consisted of 95 °C for one minute, 30 cycles at 96 °C for five seconds and 60 °C for 20 s followed by melt curve analysis to confirm that the fluorescence signal originated from specific PCR products. Each assay was prepared in triplicate and a negative control of PCR grade water was included.

Based on amplification of the negative control, a quantification cycle (*Cq*) of 27 was set as the detection limit and only samples with all three replicates amplifying below this limit were regarded as positive. Average *Cq* values were calculated by averaging among amplified technical replicates. The relative abundance of each target gene within an individual sample was then calculated using the Δ*Cq* method (1) with the 16S rRNA gene utilized as an internal control gene (Schmittgen and Livak, 2008).

$$\text{Relative abundance} = 2^{-\Delta Cq}, \Delta Cq = Cq_{ARG/MGE} - Cq_{16S} \quad (1)$$

Where *Cq* is the average of the amplified technical replicates for a sample, ARG/MGE indicates one of the 27 investigated ARG or MGE assays, and 16S indicates the 16S rRNA gene assay.

2.6. Quantitative PCR of manure samples

The final suite of gene targets was analyzed separately in manure samples using a 96-well plate format on a CFX96 Touch Real-Time PCR Detection System (BioRad). Quantitative PCR was performed in triplicate on pooled DNA extracted from all manure subsamples. The pooled manure DNA sample was diluted 100-fold to help negate potential effects of inhibitors and non-specific binding of primers. Total reaction volumes were 20 μL and consisted of 2 μL of DNA (1 – 100 ng), 10 μL of Sso Advanced Universal SYBR Green Supermix (BioRad), 0.5 μL of each primer (forward and reverse at 10 μM), and 7 μL PCR grade water. Thermal cycling conditions for all reactions consisted of 95 °C for three minutes, 40 cycles at 95 °C for 30 s and 60 °C for one minute followed by melt curve analysis to confirm that the fluorescence signal originated from specific PCR products.

No threshold cycle (*Cq*) was set, as no amplification occurred in negative controls. Relative gene abundances were similarly calculated using the Δ*Cq* method (1) with the 16S rRNA gene utilized as an internal control gene (Schmittgen and Livak, 2008).

2.7. 16S rRNA gene amplification and illumina sequencing

Sequencing of the 16S rRNA gene amplicons was performed on a MiSeq instrument (Illumina) using a MiSeq regent Kit v2 (Illumina) at the Genomics Facility at the USDA National Animal Disease Center (Ames, IA). If necessary, DNA samples were diluted to a concentration of 10 ng μL^{-1} to help mitigate the potential influence of inhibitors and the non-specific binding of primers. The V4 region of the 16S rRNA gene was amplified using previously published primers 515 F (FWD: 5'-GTGCCAGCMGCCGCGGTAA-3') and 806 R (REV: 5'-GGACTACHVGGGTWTCTAAT-3') and methods (Caporaso et al., 2011; Kozich et al., 2013).

Sequences were processed using the package simple.dada (version 0.99.0), which runs a simplified wrapper function for the DADA2 (version 1.15.0) pipeline to return merged, denoised, chimera-free, inferred sample sequences (Callahan et al., 2016; Smith, 2020). Sequences were assigned taxonomies at the genus level using the SILVA 16S rRNA sequence database (Release V132) for DADA2 (Quast et al., 2013). Only sequences with a known bacteria classification at the kingdom level were used for further analysis. After processing with DADA2, two soil samples, which had insufficient sequence data, were removed from the dataset (Supplemental Table S2).

2.8. Data Analysis

Data processing, statistical analyses, and figure generation were performed using the RStudio software package with R version 4.1.2 and the packages cowplot (1.1.1), data.table (1.14.2), ggh4x (0.2.1), ggpubr (0.4.0), phyloseq (1.38.0), phylosmith (1.0.6), readxl (1.4.0), rstatix (0.7.0), scales (1.1.1), tidyverse (1.3.1), VennDiagram (1.7.1), viridis (0.6.2), and writexl (1.4.0) (McMurdie and Holmes, 2013; Smith, 2019; Wickham et al., 2019; van den Brand, 2021; Chen, 2021; Dowle and Srinivasan, 2021; Garnier et al., 2021; Kassambara, 2020a; Kassambara, 2020b; Ooms, 2021; R Core Team, 2021; Wickham and Bryan, 2019; Wickham and Seidel, 2020; Wilke, 2020). Comparisons of the total and individual relative abundances of resistance genes between treatments were evaluated using the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test with Bonferroni correction. Quantitative PCR data and code for data processing, statistical analyses, and figure generation are available at https://github.com/LauraAlt/STRIPS_ARM_Rainfall_Manuscript. Sequencing data is available in the NCBI Short Read Archive (SRA) with BioProject PRJNA802329.

3. Results

3.1. Identification of manure associated resistance genes

Manure, runoff water, and runoff sediment samples were analyzed for the relative abundance of our final suite of resistance genes. In order to distinguish the effect of prairie strips on resistance genes introduced solely via the application of manure and resistance genes that may have been naturally present within the plots, we first identified genes that were considered to be manure associated. Manure associated resistance genes were defined as those present in the manure and absent from all control runoff samples. Control runoff samples included runoff water and runoff sediment samples from the Strip + No Manure treatment plots, as these samples had not been influenced by manure application.

All investigated ARGs and MGEs were detected within the swine manure (Fig. 2). Four of the targeted genes were also detected in the control runoff samples, each of which is associated with encoding MGEs. The detection of these genes within control runoff samples indicated that the plots themselves may have represented a potential source contributing to their presence and made it difficult to resolve the impact that the manure addition might have had on their relative abundance. Therefore, the remaining ARGs and MGEs were considered manure associated, while *intl1(clinical)*, *IS1247*, *IS6100*, and *tnpA-02* were not.

3.2. Total reduction of manure associated resistance genes in runoff

The effect of the three described treatments on the total (sum) relative abundance of the resistance genes in runoff water and runoff sediment was evaluated. Replicate plots within a treatment were

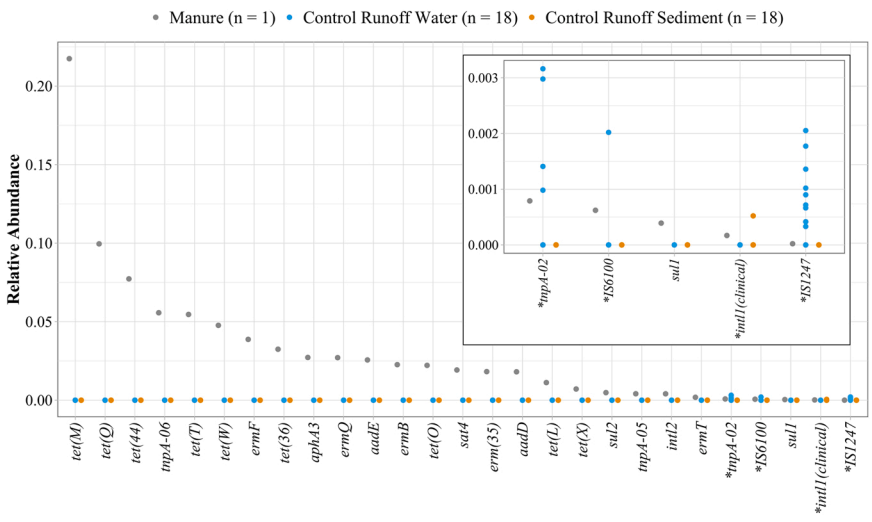

Fig. 2. Relative abundances (relative to the 16S rRNA gene) of all genes analyzed in the pooled swine manure sample (n = 1) and the control, Strip + No Manure runoff water (n = 18) and runoff sediment (n = 18) samples. The inset graph is used to display the bottom five genes with lowest relative abundances in the pooled swine manure sample. Asterisks are used to indicate any gene considered to be not manure associated based on their detection within control runoff samples from the Strip + No Manure treatment plots.

grouped by runoff water or runoff sediment and pairwise comparisons were made between the total relative abundances. Both runoff water and runoff sediment samples followed the same trend between treatments with No Strip + Manure consistently containing the greatest total relative abundance of resistance genes, followed by Strip + Manure, and finally Strip + No Manure (Fig. 3).

Pairwise comparisons revealed that both runoff water and runoff sediment from the No Strip + Manure treatment had significantly higher total relative abundances of resistance genes than the corresponding matrices from the Strip + Manure (p-value < 0.0001) and the Strip + No Manure (p-value < 0.0001) treatments. Conversely, no significant difference was observed between the Strip + Manure and the Strip + No Manure treatments for either runoff water or runoff sediment. Therefore, the total presence of resistance genes was significantly reduced back to control levels from both the manured runoff water and runoff sediment that first passed through prairie strips.

3.3. Individual reduction of resistance genes in runoff

The top five most abundant resistance genes detected within the swine manure were *tet(M)*, *tet(Q)*, *tet(44)*, *tnpA-06*, and *tet(T)*, representing 26%, 12%, 9.2%, 6.6%, and 6.5% of the total relative abundance, respectively (Fig. 4). This high abundance of *tet(M)* was tracked through runoff water, as *tet(M)* was the most abundant resistance gene detected, representing on average 47% of the total relative abundances from the No Strip + Manure treatment samples. Conversely, while *tet(M)* remained the most abundant resistance gene detected in runoff sediment from the No Strip + Manure treatment samples, representing on average 20% of the total relative abundances, *tet(T)*, *aadD*, *tnpA-06*, and *tet(W)* were also highly abundant, each representing 17%, 11%, 11%, and 10% of the average total relative abundance, respectively. Additionally, the *intl1(clinical)* gene was not detected in any runoff water samples, while *tet(44)*, *tet(L)*, and *IS6100* were not detected in any runoff sediment samples.

Once again, replicate plots within a treatment were grouped by runoff water or runoff sediment and pairwise comparisons were made

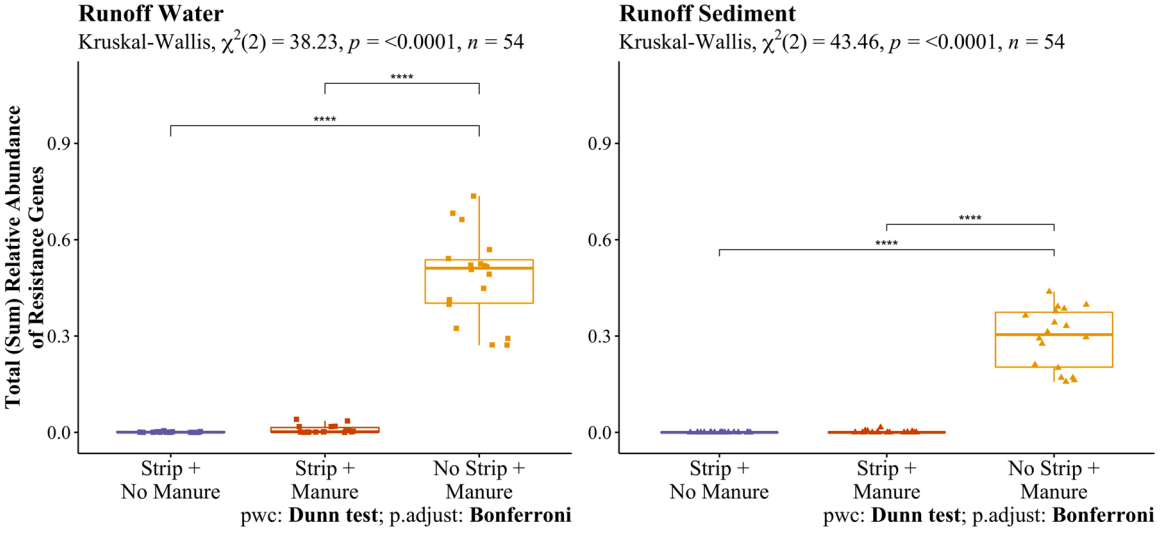

Fig. 3. Total (sum) relative abundances (relative to the 16S rRNA gene) of resistance genes present in runoff water and runoff sediment samples within each treatment. The middle line on the boxplots represents the median, while the upper quartile of the box represents the 75th percentile and the lower quartile the 25th percentile. The whiskers denote the range of the quartiles. Significant differences between treatments and within each matrix based on the non-parametric Kruskal-Wallis test followed by Dunn's multiple comparisons test with Bonferroni correction are denoted above the boxplots (* p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001).

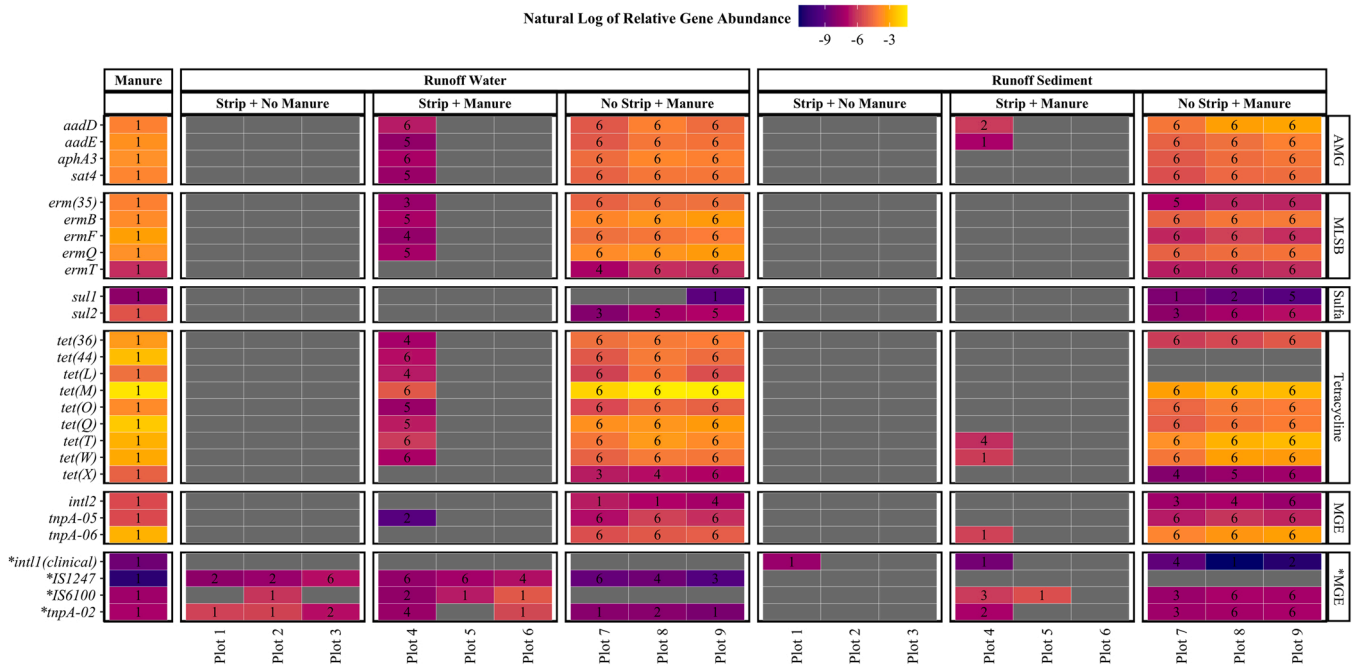

Fig. 4. Heatmap representing the detection frequency and median relative abundance (relative to the 16S rRNA gene) of individual manure associated resistance genes in swine manure (n = 1), runoff water (n = 6), and runoff sediment (n = 6) samples from each plot within each treatment. Medians for each sample were calculated from samples with detectable levels of the indicated genes, while numbers listed within each cell represent the total samples with detectable levels of the indicated gene. Asterisks are used to indicate any gene considered to be not manure associated based on their detection within control runoff samples from the Strip + No Manure treatment plots.

between gene relative abundances using Dunn's multiple comparisons test with Bonferroni correction (Supplemental Table S3). When compared individually between treatment and runoff matrix, nearly all resistance genes followed the same trend observed when compared in total. Significant gene reductions occurred in the manured runoff water and runoff sediment that had passed through a prairie strip, with many

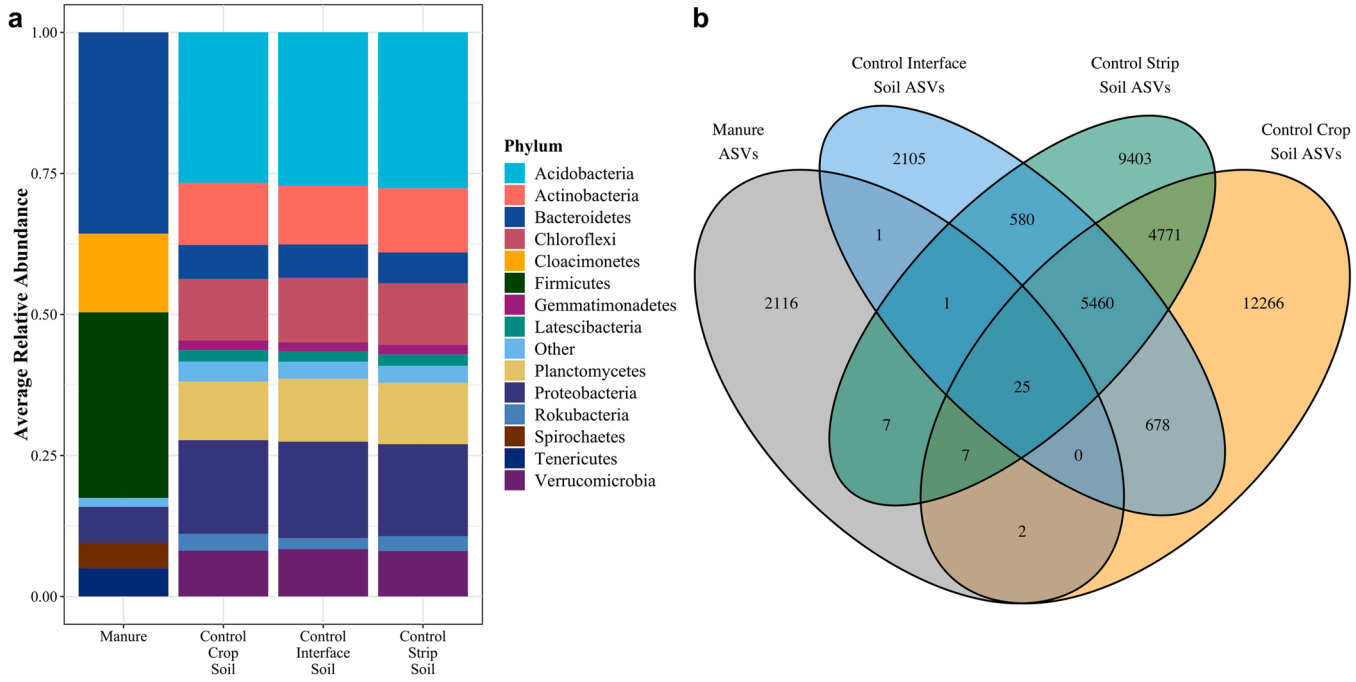

Fig. 5. a: Average relative abundances (relative to the 16S rRNA gene) of phyla among swine manure (n = 36), control crop soil (n = 173), control interface soil (n = 36), and control strip soil (n = 144). Control soil samples are those that did not receive manure and included baseline soil samples from all treatment plots and soil samples from the Strip + No Manure treatment plots at all collection timepoints. The Other grouping represents phyla that individually make up less than 1% of the total abundance. b: A Venn diagram displaying the number of ASVs unique to the various control soil microbial communities (represented by all non-manured soil samples) as well as ASVs unique to the swine manure microbial community. Manure associated bacteria were defined as ASVs observed only within the manure microbial community and include 2,116 total ASVs.

gene targets becoming undetectable in samples from two of the Strip + Manure Treatment plots. In the runoff water and runoff sediment from the third Strip + Manure treatment plot, plot 4, a total of 22 of the 27 resistance genes were still detected. However, the inclusion of this plot did not impact the statistical differences observed between treatments for any of the individual genes. The only exceptions to this overall trend occurred in the runoff water and included the genes *IS1247*, *IS6100*, and *tnpA-02*, which were not considered to be manure associated, and *sul1* which, although considered to be manure associated, was only detected in one No Strip + Manure runoff water sample.

3.4. Identification of manure associated bacteria

Similar to the manure associated resistance genes, manure associated bacteria were defined as ASVs present in the swine manure and absent from all control soil samples. These bacteria offered a second effective proxy of manure influence, as the previously described manure associated resistance genes were often not detectable within the manured soil samples. Control soil samples were again comprised of those that did not receive manure and therefore included baseline soil samples from all treatment plots and soil samples from the Strip + No Manure treatment plots at all collection timepoints.

The manure microbial community consisted primarily of members from the Bacteroidetes, Firmicutes, and Cloacimonetes phyla, which contributed 36%, 33%, and 14% of the total relative abundance, respectively (Fig. 5a). In total, there were 23,774 ASVs observed to be unique to the control soil samples, 25 ASVs observed to be shared between the control soil samples and manure samples, and 2,116 ASVs observed to be unique to the manure samples (Fig. 5b). These 2,116 ASVs unique to the manure microbial community represented the manure associated bacteria.

3.5. Persistence of manure associated bacteria in soil

As the manure associated resistance genes were often not detectable within the manured soil samples, manure associated bacteria were used as an alternative indicator of manure influence. The movement of manure was tracked horizontally, with surface runoff into the prairie strips, and vertically, with infiltration into the soil profile, by characterizing the presence of manure associated bacteria in all soil samples from the Strip + Manure treatment plots at depths 0–5 cm and 5–15 cm. Out of the 2,116 total ASVs identified as manure associated bacteria, 499 were detected in the manured soil, with Firmicutes, Bacteroidetes, and Cloacimonetes being the dominant represented phyla.

The relative abundance of manure associated bacteria was highest in the top 0–5 cm of crop soil on day 0 with these bacteria contributing an average of 10% of the total bacterial community (Fig. 6). Manure associated bacteria were less abundant in the bottom 5–15 cm on day 0 in crop soils, comprising an average of only 1.0% of the total bacterial community. On average, the relative abundance of manure associated bacteria remained relatively unchanged from day 0 to day 2 at both depths, however by day 14 the relative abundance of these bacteria had dropped by 83% and 80% for the 0–5 cm depth and 5–15 cm depth, respectively. By day 153, at both depths, the average relative abundance of manure associated bacteria had drastically decreased, showing over 99% reduction from day 0. While substantial reduction had occurred by the final sampling day, multiple phyla of manure associated bacteria were still detected in crop soils at both depths. Of these persisting bacteria detected in the 0–5 cm depth of the crop soils, 61% belonged to the genus *Clostridium* sensu stricto 1 of the phylum Firmicutes. Conversely in the 5–15 cm depth, the dominant persisting bacteria on day 153 included a relatively even split between the genus *Clostridium* sensu stricto 1, representing 31%, and the genus *Bacteroides* of the phylum Bacteroidetes, which represented 35%.

Unlike in the crop soils, manure associated bacteria were present at much lower abundances in the prairie strip soils, with average relative abundances of 0.004% in the 0–5 cm depth and 0.05% in the 5–15 cm depth on day 0. As well, contrary to what was observed in crop soils, manure associated bacteria were often present at an equal or higher relative abundance in the 5–15 cm depth when compared to the 0–5 cm depth. By day 153 the relative abundance of manure associated bacteria in the prairie strip soils had dissipated by 45% in the 0–5 cm depth and

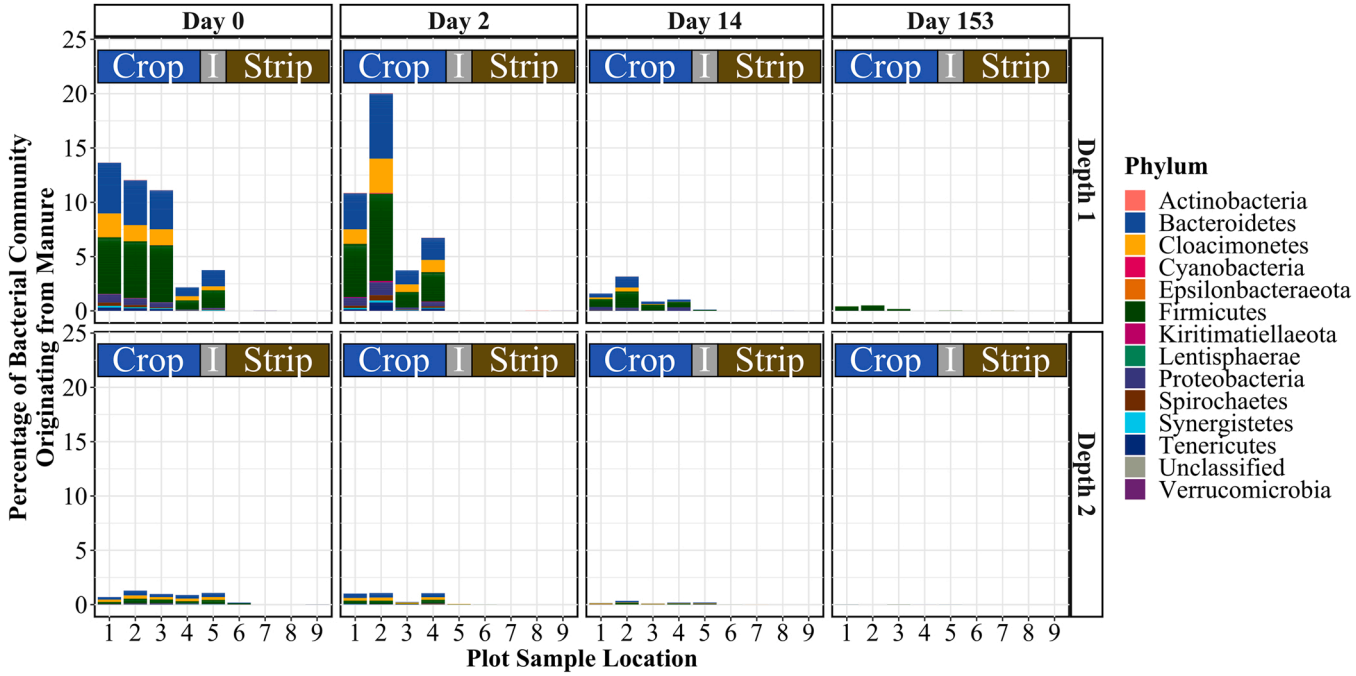

Fig. 6. Tracking of manure associated bacteria through Strip + Manure soil samples at depth 1 (0–5 cm) and depth 2 (5–15 cm) immediately (Day 0), 2, 14, and 153 days after simulated rainfall events. Abundance of manure associated bacteria is depicted as a percentage of the total bacterial community and is averaged across plot replicates (n = 3). Plot sample locations correspond to soil cores taken across the length of the plots. Labels are provided to indicate which sample locations fall within crop sections, interface sections (I), and prairie strip sections. The Unclassified grouping represent bacterial ASVs which did not match known phyla.

had dissipated completely in the 5–15 cm depth. Of the remaining manure associated bacteria present on day 153 in the 0–5 cm depth strip soils, all were unclassified members of the order Clostridiales (Firmicutes).

4. Discussion

The quantification of manure associated resistance genes and bacteria within runoff and soil core samples from paired plot treatments was used to evaluate the effectiveness of CP 43 prairie strip installments to mitigate the impact of field applied swine manure slurry on downstream waters and soils. While previous reports have identified the ability of vegetated filter strips to remove manure associated bacteria in runoff water from agricultural fields (Collins et al., 2004; Coyne et al., 1998; Fox et al., 2011; Sullivan et al., 2007), far fewer have characterized their impact on manure associated resistance genes (Flater et al., 2022; Joy et al., 2013) and, to our knowledge, none have tracked the transport of manure associated bacteria through their soil profiles.

The application of swine manure to a field plot impacts runoff water, runoff sediment, and recipient soil communities through the increased presence and abundance of manure associated resistance genes and manure associated bacteria, emphasizing the potential risk of AMR movement from manured fields. Our results build upon those reported in a previous investigation of similar methodology in which poultry litter was used as the manure source (Flater et al., 2022). While our findings support those seen with poultry litter, that AMR contaminants are significantly reduced from manured runoff that first passes through a prairie strip, novel findings related to the identity, transport, and reduction level of our respective manure indicators were also observed.

Genes detected within runoff were evaluated to try and classify their origin as being predominantly from the manure amendment (Fig. 2). Of the 27 resistance genes investigated in this study, four were detected in runoff samples from the control, Strip + No Manure treatment plots. All genes identified in the runoff from control, Strip + No Manure treatment plots were associated with MGEs, and included one integrase, two insertion sequences, and a transposase. Detection of these genes was unsurprising as MGEs have been proposed as a marker of anthropogenic influence, so their potential presence within the background soils of an active farm was predictable (Gillings et al., 2015; Willms et al., 2020).

Of the resistance genes analyzed, the gene *tetM*, a gene associated with tetracycline resistance, was of particular interest due to its exceptionally high relative abundance in the swine manure, which was more than double the next most abundant gene. This high presence of the *tetM* gene paralleled the use of tetracycline antibiotics on the farm where our manure was sourced. Widespread in both Gram-positive bacteria and Gram-negative bacteria, *tetM* is a ribosomal protection protein with at least 42 identified host genera and a known association to the *Tn916/Tn1545* family of conjugative transposons (Roberts, 2005). Our experiment, combined with previous research, identifies *tetM* as a particularly valuable gene for monitoring efforts due to its detection in swine, poultry, and dairy manures, its increased presence and resiliency in water and soil microbiomes following exposure to manure, and its potential genetic mobility (Alt et al., 2021; Flater et al., 2022; Muurinen et al., 2017; Neher et al., 2020b; Tamminen et al., 2011; Wang et al., 2017; Wu et al., 2010).

As was expected, the most abundant resistance genes identified in the swine manure also tended to be the most abundant resistance genes present in the manure impacted runoff samples (Fig. 4). However, differences in the overall composition of the resistance genes were observed between the two runoff matrices. For example, when considering the manure associated resistance genes, both *tet(44)* and *tet(L)* were absent from all of the manure impacted runoff sediment samples. As well, the average relative abundance of *tetM* was three-fold higher in the No Strip + Manure runoff water when compared to the No Strip + Manure runoff sediment. Although differences between the runoff matrices could have been the result of inhibitors and humic substances influencing the performance of the PCR assays, it could also be hypothesized that the bacterial hosts of *tet(44)*, *tet(L)*, and *tet(M)* are not as strongly associated with the solid phase of swine manure slurries.

Both chemical and microbial factors could contribute to a specialization of resistance genes within the aqueous or solid phase of swine manure. Research by Zhou et al. (2017) reported strong negative correlations between major tetracycline resistance genes, including *tetM*, and soil organic matter, indicating that these genes may be more likely housed by bacteria present in manure slurry as free microbes, unattached to manure particles. Similarly, previous studies investigating the suspended and attached microbiomes found in sewer overflow, urban recreational water, and manure-polluted freshwater-sediment microcosms all reported distinct microbial compositions associated with these respective phases (Eramo et al., 2017; Fang et al., 2018; Xiong et al., 2015). The presence of residual antibiotics can often provide a selection pressure for the maintenance of resistance genes, however the bioavailability of these antibiotics within a manure's aqueous and solid phases is likely to differ. Since tetracycline has a high soil adsorption coefficient, the solid phase of settled manure could have less tetracycline in a bioavailable form when compared to the aqueous phase, ultimately resulting in a reduced selection pressure for tetracycline resistance (Chen et al., 2015). Future research should investigate whether slurry manures with both liquid and solid phases have unique resistomes associated with these phases, as those genes associated with the liquid phase could have an increased risk of transportation.

Overall, prairie strips significantly reduced the total relative abundance of resistance genes (Fig. 3) and the individual relative abundance of manure associated resistance genes (Fig. 4) in both runoff water and runoff sediment. Although one Strip + Manure treatment plot, plot 4, did demonstrate less reduction than its replicate counterparts, its inclusion in statistical testing did not affect these results. While this level of variation among replicate plots can be common in field-scale experiments, the differences associated with plot 4 could have been caused by the plot's higher runoff flow rate. The average runoff flow rate from plot 4 (1.2×10^{-5} $m^3 s^{-1}$) was 1.2–9.3-fold higher than the other average runoff flow rates for all other plots (Supplemental Fig. S1), indicating that the prairie strips have reduction limitations linked to runoff flow rate. To address the relationship between runoff flow rate and contaminant reduction, a current effort of the STRIPS team includes the creation of a design tool that will calculate the necessary prairie strip width to achieve a certain level of contaminant reduction (Craig, 2021). This calculation will be based on user defined variables, including incoming contaminant concentration, runoff flow rate, and either a desired output contaminant concentration or established water quality standard.

By the end of our experiment, most manure associated bacteria had disappeared from the soil, with only a few bacterial orders lingering (Fig. 5). In particular, the orders Clostridiales and Bacteroidales were highly enriched throughout our sampling timeline and were largely representative of the manure associated bacteria still present in the soil on day 153. These bacterial orders, as well as the specific, respective bacterial genera *Clostridium* sensu stricto 1 and *Bacteroides*, are of particular interest as they can contain human pathogens, they have previously been identified as being swine manure derived, they are highly transportable with downstream waters, and they are persistent in recipient soil communities (Ding et al., 2014; Leclercq et al., 2016; Mwaikono et al., 2016; Rieke et al., 2018a). For example, an experiment by Rieke et al. (2018b), analyzing the impact of swine manure on soil bacterial communities over time, defined both Clostridiales and Bacteroidales as manure stimulated bacterial orders, while *Clostridium* sensu stricto was classified as a manure derived bacterial genus; all remained at elevated abundances in recipient soils 108 days after manure application.

Based on the physical characteristics of the swine manure, transport of manure associated bacteria horizontally from the crop soil into the prairie strip soil was expected. As swine manure is often applied in a

slurry or liquid form, it is often more readily transported via runoff and its integration into the soil matrix is expedited (Fahrenfeld et al., 2014; Thurston-Enriquez et al., 2005). As well, it was expected that the prairie strip soils would show an increased transport of manure vertically into the soil profile when compared to the crop soils because vegetative filter strips are known to increase the infiltration of runoff water and bolster the water holding capacity of soils by reducing flow velocity, promoting ponding, and increasing soil porosity (Lin et al., 2011; Rachman et al., 2004; Udawatta et al., 2008b). This deeper movement of manure into the prairie strip soil profile was generally supported as manure associated bacteria were present at a higher relative abundance in the 0–5 cm depth when compared to the 5–15 cm depth in crop soils but were often present at an equal or higher relative abundance at the 5–15 cm depth when compared to the 0–5 cm depth in prairie strip soils. Previous research has also demonstrated that the soil profile created by prairie strip installments offers increased infiltration and deeper water movement when compared to the soil profile created by a crop rotation of corn and soybeans (Hernandez-Santana et al., 2013; Udawatta et al., 2008a). Therefore, the exploration of a second level of subsurface mitigation potentially offered by the microbial community associated with the growth of prairie strips is also warranted.

5. Conclusion

During this study, we describe the reduction, or complete removal, of swine manure associated resistance genes in runoff that has first passed through CP 43 prairie strip installations and begin to characterize the fate of manure associated bacteria within the prairie strip soils. Our results frame prairie strips as best management practice capable of linking improved environmental quality to benefits for human health, ultimately supporting a more sustainable agricultural system. Our work is relevant to stakeholders, including farmers and landowners, with interest in incorporating CP 43 prairie strips as part of an effective manure management plan. Opportunities for future work include 1) establishing explicit resistance gene targets for water monitoring and assessment by governmental agencies, 2) identifying whether certain resistance genes are more likely to be mobilized from specific manure sources, putting them at a higher risk for downstream transport, and 3) further disentangling the movement of contaminants through prairie strip soils by extracting soil from greater depths (i.e., greater than 15 cm) and investigating the specific movement of manure associated contaminants through macropores, rather than bulk soil.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

All data and code are available via GitHub or through the NCBI SRA, links to both sources are provided within the manuscript text.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.agee.2023.108469.

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