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Application of manure from cattle administered antibiotics has sustained multi-year impacts on soil resistome and microbial community structure

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ABSTRACT

In agroecosystems, application of manure from livestock treated with antibiotics has the potential to spread antibiotic compounds, resistant bacteria, and antibiotic resistance genes (ARGs) to soil. Although environmental transmission of antibiotic resistance is a major human health concern, few studies have looked at long-term effects on soil microbial communities from applying manure from livestock administered antibiotics. We examined the impacts of three years of repeated manure additions from cattle under different antibiotic treatments on microbial community structure and ARG abundances. While manure additions altered both soil bacterial and fungal communities, manure from cattle administered antibiotics further altered soil bacterial communities, but not fungal, compared to manure from antibiotic-free cattle. Furthermore, addition of manure from antibiotic-free cattle resulted in increased abundances of several ARGs compared to soil with no manure inputs, but manure from cattle administered antibiotics did not change overall profiles of ARG abundances compared to manure from antibiotic-free cattle. Finally, although bacterial and fungal community structure and ARG abundances varied among years, manure treatment effects on each were persistent during the full three-year period. Taken together, our results suggest that manure and antibiotic impacts on soil microbial communities can persist for long periods of repeated manure application. Furthermore, soil management strategies for addressing the antibiotic resistance crisis should consider the broader context of manure management.

1. Introduction

Currently, 80% of total antibiotic usage in the US is in livestock production (Hollis and Ahmed, 2013), and global antibiotic use for livestock is projected to increase 67% from 2010 to 2030 as the demand for meat increases (Van Boeckel et al., 2015). When livestock are treated, antibiotic compounds, antibiotic resistance genes (ARGs), and resistant microorganisms can enter soils via land application of manure or direct excretion from grazing animals (Tasho and Cho, 2016). Anthropogenic inputs of antibiotics and ARGs are considered environmental contaminants that can contribute to the ongoing antimicrobial resistance (AMR) crisis (Pruden et al., 2006; Sanderson et al., 2016). Long term manure additions can increase detectable concentrations of both antibiotic compounds and ARGs in soil, including tetracycline (Fang et al., 2015; Tang et al., 2015) and cephalosporin (Wepking et al., 2017). In fact, a recent examination of archived soils from 1894 to 2010 suggests that the impacts of manure on soil AMR began after the 1940's, when widespread antibiotic usage began (Graham et al., 2016).

Beyond the risk of AMR transmission, manure inputs from cattle administered antibiotics can also alter the structure and function of soil microbial communities broadly. Exposure to antibiotics can increase microbial diversity (Grenni et al., 2018; Reichel et al., 2014), as well as alter overall community structure (Lin et al., 2016; Wepking et al., 2017). Additionally, antibiotics can impact the function of microbial communities, including impacts on nitrogen cycling in terms of changes in pools of N or in genes associated with N cycling (Ahmad et al., 2014; Grenni et al., 2018; Radl et al., 2015; Wepking et al., 2019) and respiration and carbon cycling (Hammer et al., 2016; Wepking et al, 2017,

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2019).

In light of concerns about environmental transmission of AMR, there are several factors that complicate our understanding of interactions among antibiotics, soil microbial communities, and AMR. Firstly, antibiotics are a well-known natural mechanism for competition among microorganisms (Waksman and Woodruff, 1940), and both antibiotic compounds and ARGs are naturally present in soils. Likewise, resistant bacteria and ARGs can be present even in manure from animals without a history of antibiotic exposure (Heuer et al., 2011). Nutrient availability can have a strong impact on ARG distributions in soil (Fierer et al., 2012; Zhao et al., 2017), and abundances of antibiotic resistant bacteria and ARGs can increase in soil after application of manure from antibiotic-free cattle (Udikovic-Kolic et al., 2014). Furthermore, multiple ARGs often exist on one plasmid (Herrick et al., 2014) and soil bacterial isolates have been found to be resistant to multiple antibiotics (Hu et al., 2017). Thus, multiple classes of ARGs can be co-selected following exposure to only one drug (Pal et al., 2015), complicating the factors that control AMR across the landscape. Given that previous studies sometimes add antibiotics directly to soil (Čermák et al., 2008; Lin et al., 2016) or artificially spike antibiotic compounds into manure (Blau et al., 2018; Jechalke et al., 2016; Zhang et al., 2017), rather than administering the antibiotic to the animal, such approaches may miss important interactions that occur in real-world scenarios.

A second challenge in understanding the role of agricultural soils in AMR transmission is that most studies focus on relatively short-term impacts, while only a few have spanned multiple years. For example, Zhang et al. (2017) found an initial increase in ARGs in soil one day after exposure to manure spiked with antibiotics, but the abundance of ARGs decreased over the next 130 days. Lin et al. (2016) found that the impacts of different antibiotics occurred at different times following exposure, but observations were limited to 20 days. However, it is not known if these impacts will persist in soil over long-term, repeated exposure. While a few studies have been conducted in fields that had manure applied over long periods, the antibiotics in the manure applied to the sites were not tightly controlled (Tang et al., 2015; Wepking et al., 2017). As a result, while previous work has been highly valuable in showing the potential for manure additions to impact AMR, it remains difficult to disentangle which factors related to soil, manure, or antibiotic exposure can be critical management controls or how such controls should be used within the context of temporal variability.

To address these knowledge gaps, we analyzed bacterial and fungal community structure and abundances of multiple ARGs during a three-year grassland manure-addition experiment. The work is an extension of early results from these same experiments previously reported by Wepking et al. (2019). The goal for this component of the study was to describe multi-year impacts to soil microbial communities of repeated and controlled additions of manure alone and manure from animals administered different antibiotics. The specific objectives of this work were to 1) identify long-term impacts of repeated manure additions from cattle under different antibiotic treatments on soil microbial community structure and ARG abundances and 2) identify potential co-occurrences among ARGs, as previous studies have suggested (Hammer et al., 2016; Herrick et al., 2014).

2. Methods

2.1. Study design

The samples and data collected for this study were from long-term experimental manure additions to grassland plots conducted between 2014 and 17 at the Virginia Tech Kentland Research Farm in Blacksburg, VA (N37.199490, W-80.584659). The plots were situated on grasslands previously managed by occasional mowing. The location was specifically selected to have no known history of grazing or application of manure or other inputs. The soils in experimental plots were composed of Unison and Braddock cobbly soil series. Experimental details, including manure collection methods, and soil nutrient data from the first year of the experiment, were first described by Wepking et al. (2019). For this study, which aims to detail long-term changes in soil microbiota and ARG abundances, monthly manure amendments were continued for a full three-year period. The experimental site was divided into six replicate blocks with four treatment plots in each block. The four treatments included a control treatment with no manure (NM), a control treatment with manure from cattle administered no antibiotics (CON), manure from cattle administered cephapirin benzathine (CEPH), and manure from cattle administered pirlimycin hydrochloride (PIR). Both cephapirin benzathine and pirlimycin hydrochloride were chosen because they are commonly used to treat mastitis in dairy cattle. However, they differ in mode of action; cephapirin is a first-generation cephalosporin that causes cell lysis, and pirlimycin hydrochloride is a lincosamide that inhibits protein synthesis.

Manure for spreading on the treatment plots was collected once per year from cattle two to three days after administration of antibiotics, homogenized, and preserved at −20 °C until use. Frozen manure was then thawed as needed immediately prior to monthly application (see Wepking et al., 2019 for details). The concentrations of antibiotics, C, and N in manure from the first year is reported in Wepking et al. (2019). Briefly, cephapirin was below detection (<0.36 ng g manure^{-1}) in manure collected from cattle administered cephapirin. Pirlimycin was present at 149 ± 3.4 ng g manure^{-1} in manure collected from cattle administered pirlimycin. The carbon content in CEPH manure was slightly lower (49.0%) than CON (49.9%) and PIR (49.6%), while the nitrogen content was not statistically different among CON, CEPH, and PIR manures (3.3%, 3.4%, and 3.5%, respectively). Collecting manure from cattle after administration of antibiotics, rather than spiking manure with a known concentration of antibiotic was chosen to mimic realistic inputs that also include resistant bacteria and ARGs that may be in manure as a result of antibiotic treatment. While the concentrations of antibiotics in manure could not be controlled through this approach, as there is variability in the amount excreted by animals, the doses of antibiotics the cattle received were controlled. Thus, although there were low and variable concentrations of antibiotics in the applied manure, the experimental design better captures the real-world effects that active antibiotics, degraded metabolites, resistant bacteria, and ARGS can have on soil microbial communities.

Manure was applied to the treatments monthly at 648 g/m^2 for three years, beginning in Nov. 2014. To identify long-term changes in soil microbial communities, soil samples from each plot were first collected eight months after the first manure application (May 2015) and then one and two years later in May (2016–17), for a total of 72 samples. Soil samples were collected to a depth of 10 cm in a 0.05 m^2 monolith cut from each plot. After plant roots were removed, the soil was sieved through 4.75-mm mesh and homogenized. A soil subsample (~2–3 g) was collected into a Whirl-Pack bag and stored at −80 °C for microbial analysis.

2.2. Microbial community structure

The structure of soil microbial communities were analyzed via marker gene amplicon sequencing. Total DNA was extracted from soil samples using Qiagen PowerSoil DNA extraction kits (Qiagen, Hilden, Germany). The V4 region of the 16S rRNA gene and the internal transcribed spacer region between the 18S and the 5.8S rRNA gene (ITS1) were amplified to characterize the structure of bacterial/archaeal and fungal components of the community, respectively, following Earth Microbiome protocols (Caporaso et al., 2012). Each sample was amplified in triplicate via polymerase chain reaction (PCR) on conventional thermal cyclers (Bio-Rad, Hercules, CA, U.S.A.). Amplification primers were 515F (5′-GGA CTA CNV GGG TWT CTA AT-3′) (Parada et al., 2016) and 806RB (5′-GTG YCA GCM GCC GCG GTA A-3′) (Apprill et al., 2015) for bacteria and ITS1-F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) (Gardes and Bruns, 1993) and ITS2 (5′-GCT GCG TTC TTC ATC GAT

GC-3′) (White et al., 1990). After amplification, PCR triplicates were pooled and visualized on agarose gels with negative controls for each sample to verify no contamination was present. PCR products were purified with QIAquick PCR purification kits. Amplicons were sequenced on the Illumina MiSeq platform with 300-bp single reads at the Biocomplexity Institute at Virginia Tech. Raw sequence reads are archived under BioProject ID PRJNA632833.

Raw sequence reads were processed with USEARCH (Edgar, 2010) and QIIME version 1.8 (Caporaso et al., 2010). Sequences less than 240 bp or greater than 1 expected error were discarded. Sequences passing quality filtering were then clustered into operational taxonomic units (OTUs) at 97% similarity. Chimeric sequences were identified and removed using UCHIME (Edgar et al., 2011). Taxonomy was assigned using the RDP classifier (Wang et al., 2007) with GreenGenes 13.8 (DeSantis et al., 2006) and UNITE 6.97 (Kõljalg et al., 2013) reference databases for bacteria and fungi, respectively.

Quantitative PCR (qPCR) was used to obtain absolute abundances of total bacteria in each sample using the same DNA extracts as described above. Total abundances of bacteria were quantified using a standard curve of plasmids containing a known copy number as described previously (Fierer et al., 2005). Bacterial primers for qPCR were EUB518 (5′-ATT ACC GCG GCT GCT GG-3′) and EUB338 (5′-ACT CCT ACG GGA GGC AGC AG-3′). Samples were amplified in triplicate, and the mean value for each sample was used for statistical analysis. To ensure data quality, values were only accepted when the standard curve from the same plate had an $R^2 > 0.95$, and an efficiency of 0.9–1.1.

2.3. Antibiotic resistance gene abundances

To quantify absolute abundances of ARGs, we used microfluidic qPCR (mfqPCR) to simultaneously quantify the abundances of 47 different genes in each sample. The gene targets are summarized in Table S1, with details and protocols outlined in Ahmed et al. (2018) and Sandberg et al. (2018). The targeted genes include tetracycline, sulfonamide, β-lactamase, and lincosamide resistance genes, along with metal resistance and integrase genes. By using this broad panel of genes, we could identify patterns among genes related to the antibiotics applied in this study, but also to other ARGs that may be present in the soil or manure. Furthermore, we could better examine co-occurrences among multiple genes related to AMR to address our third objective.

2.4. Statistical analysis

To identify significant changes in bacterial and fungal communities, the unrarefied matrices of OTU relative abundances by sample were analyzed in R (version 3.5.1; R Core Team, 2018) using *vegan* (version 2.5–6; Oksanen et al., 2019), *phyloseq* (version 3.9; McMurdie and Holmes, 2013) and *DESeq2* (version 3.9; Love et al., 2014). Variance stabilizing transformations ("varianceStabilizingTransformation" in the package *DESeq2*) were used to achieve homoscedastic variance among samples before further analysis (McMurdie and Holmes, 2014). Patterns in microbial communities among treatments and years were visualized with Principle Coordinates Analysis (PCoA) of Bray-Curtis distance matrices. Differences in overall microbial community structure were tested using PERMANOVA with the function "adonis2" in the package *vegan*. The main effect was treatment with year as an uncontrolled covariate. Post-hoc pairwise comparisons were analyzed with "pairwise.adonis" in the package *pairwiseAdonis* (version 0.01; Arbizu, 2017). Taxa that were differentially abundant, or responsive, to treatment or year were determined using the function "DESeq" in the package *DESeq2*. Taxa were deemed responsive if the *P*-value was <0.05 and the $\log_2$ fold change was greater than 1.

To compare ARG abundances across samples, PCoA on Bray-Curtis distance matrices were used to visualize patterns in ARG profiles. Differences among profiles (i.e., abundances of all measured ARGs considered together) were analyzed similarly to methods described above using "adonis2" and "pairwise.adonis". Differences in abundances of individual genes among treatments and years were analyzed with Mann-Whitney U tests with false discovery rate (FDR)-adjusted p-values to correct for multiple comparisons. Jaccard's similarity matrix was used to test for co-occurrence of ARGs. Only genes that were detected in at least 20% of all samples were examined for correlations. For all analyses, $p < 0.05$ was considered significant; $p < 0.1$ was considered marginally significant for some important results.

3. Results

The overarching goal of this study was to determine how multiple aspects of the soil microbial community responded to manure from cattle under different antibiotic regimes over a three-year period. Thus, for the purpose of analysis, the main effect was manure (NM, CON, CEPH, and PIR), with year as an uncontrolled covariate. Primary response variables included bacterial community structure, fungal community structure, and ARG abundance profiles. Interactions between time and year were included in additional statistical models for bacterial and fungal community structures as well as ARGs. However, none of the interactions were significant ($p > 0.05$).

3.1. Impacts of manure and antibiotics on microbial community structure

Both bacterial ($F_{3,68} = 1.81$, $P = 0.001$; Fig. 1A) and fungal ($F_{3,71} = 2.90$, $P = 0.001$; Fig. 1B) community structure were impacted by manure treatments across all three years. The addition of manure from antibiotic-free cattle (CON) resulted in significantly altered community structure for both bacteria ($F_{1,44} = 1.58$, $P = 0.02$) and fungi ($F_{1,44} = 2.87$, $P = 0.01$) compared to no manure controls (NM). Sixteen bacterial OTUs were differentially abundant between the NM and CON treatments (Fig. 1C). Apart from one OTU in the phylum Latescibacteria (identified as WS3 in GreenGenes, but since renamed; Lin and Pan, 2015) that were relatively more abundant in CON, all other responsive OTUs decreased in the CON samples. Most responsive OTUs belonged to Proteobacteria or Bacteroidetes. Those that could be identified to genus included *Flavobacterium* in the phylum Bacteroidetes, *Cellvibrio, Devosia,* and *Methylotenera* in the phylum Proteobacteria, and *Luteolibacter* in the phylum Verrucomicrobia. For fungi, there were 44 responsive OTUs between NM and CON, mostly from Ascomycota (Fig. 1D). Among those that could be identified at the genus level, *Preussia*, *Phialophora*, *Scedosporium*, and *Chaetomium*, in the phylum Ascomycota, and *Agrocybe* and *Panaeolus* in the phylum Basidiomycota were all relatively more abundant in CON. In contrast, *Paraglomus* in the phylum Glomeromycota and *Psilocybe* in the phylum Basidiomycota were relatively more abundant in NM.

Manure from cattle treated with antibiotics also caused significant shifts in bacterial community structure compared to manure from antibiotic-free cattle across all three years. Specifically, bacterial community structure in both the CEPH and PIR treatments were different from those in CON ($F_{1,44} = 2.29$, $P = 0.003$ and $F_{1,44} = 3.05$, $P = 0.003$ for CEPH and PIR, respectively). Importantly, the soil bacterial communities of CEPH and PIR shifted in the opposite direction from NM treatments in ordination space compared to those in the CON treatment (Fig. 1A). Similar to changes seen between NM and CON, most responsive OTUs that were differentially abundant between CON and CEPH and PIR belonged to the phyla Proteobacteria or Bacteroidetes. Additionally, most responsive OTUs among the treatments were increased in the antibiotic treatments compared to CON, although there were a few exceptions (Fig. 1C). For example, *Nitrospira* was relatively more abundant in CON than PIR. Although CEPH and PIR did not have significantly different bacterial communities overall ($F_{1,44} = 1.31$, $P = 0.08$), individual OTU differences from CON were not the same in each of these treatments. More bacterial OTUs were different between PIR and CON (100) than CEPH and CON (39). Of the 39 OTUs that were different between CEPH and CON, 23 were also different between PIR

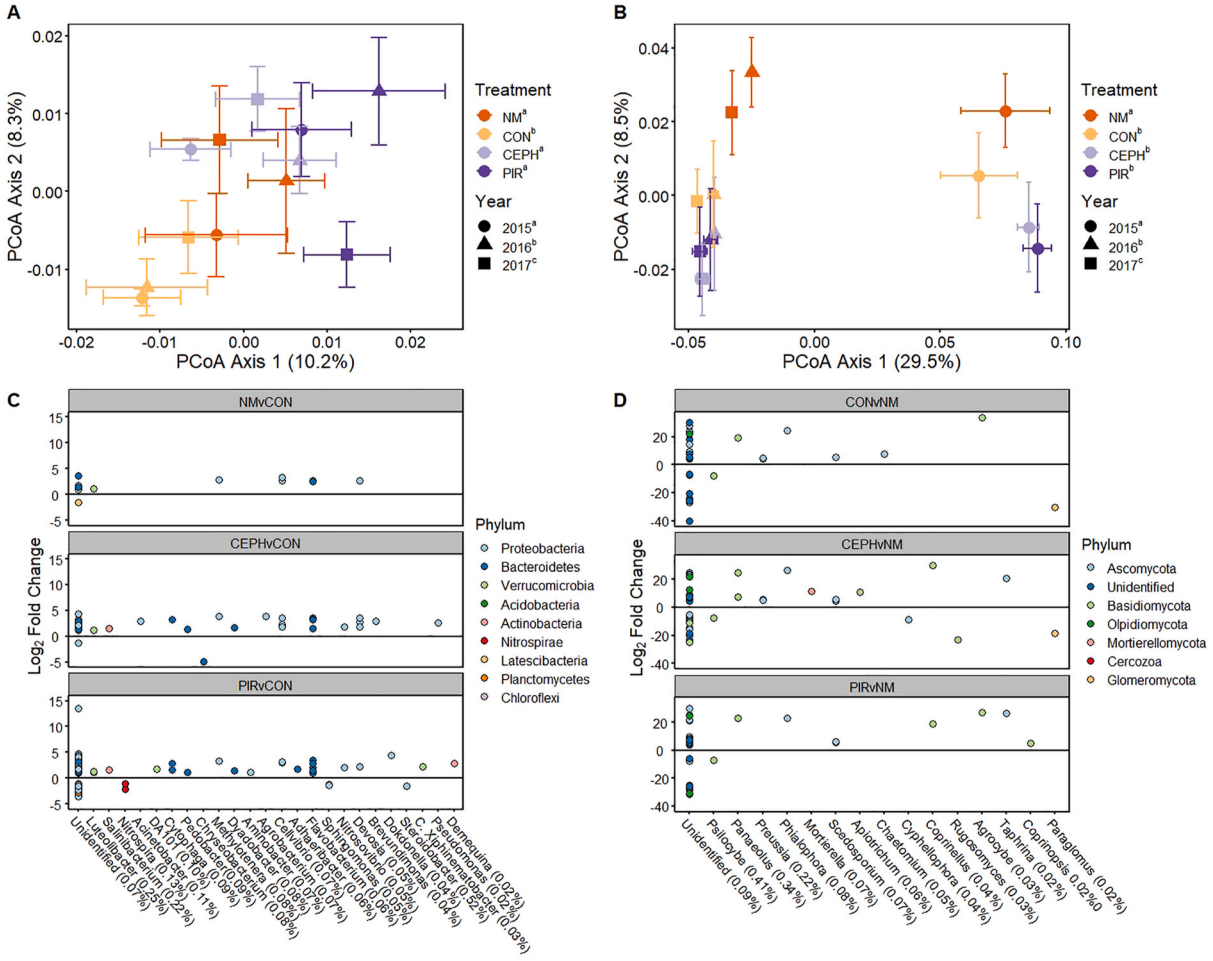

Fig. 1. Upper plots are principal coordinate analyses visualizing similarities among treatments and years of soil bacterial/16S (A) and fungal/ITS (B) communities. Points represent centroids for each treatment and year combination. Error bars represent standard error. Superscript letters in the legend indicate significant differences based on PERMANOVA among treatments and years. Lower plots represent fold changes between treatment pairs that significantly varied between treatment pairs for bacteria (C) and fungi (D). Percent values in brackets show the mean relative abundance of each genus across all samples. Each point is one OTU, colored by phylum and grouped by genus.

and CON.

In addition to the main treatment effects, bacterial community structure also varied among years ($F_{2,68}$ = 2.20, P = 0.001), with different communities in each year (all $P < 0.05$; Fig. 1A). Year explained a similar amount of variance as treatment (R^2 = 0.060 and 0.075, respectively), but there were fewer responsive bacterial OTUs among years compared to manure treatments (Figure S1). Most responsive bacterial OTUs belonged to Proteobacteria and most differential abundance changes were relatively small.

In contrast to bacteria, there were no differences across all three years in fungal community structure among treatments using control manure or manure from cattle administered antibiotics. Although CEPH and PIR had different fungal communities compared to NM ($P < 0.05$), CEPH and PIR fungal communities were not different from CON communities ($P = 0.35$ and 0.44 for CEPH and PIR, respectively), indicating that changes were primarily a result of manure additions, regardless of antibiotics. Fungal communities also varied among years ($F_{3,71} = 15.76$, $P = 0.001$) and, again, all years were different from one another (all $P < 0.05$; Fig. 1B). In contrast to bacteria, year explained a much larger portion of variance than treatment ($R^2 = 0.297$ and 0.082, respectively). Most of the responsive fungal OTUs differed in 2015 and years primarily belonged to the phyla Ascomycota and Basidiomycota (Figure S2).

3.2. Impacts of manure treatments on ARGs

Of the 47 ARGs in the mfqPCR array, 33 were detected in at least one sample, and 18 were detected in at least 20% of all samples. The most ubiquitous genes were *intI1*, bla_{SHV}, *tet*(W), and *floR*, found in 97%, 96%, 81%, and 80% of samples, respectively. The ARGs with the greatest abundance across all samples were bla_{SHV}, *intI1*, *floR*, and *mexB*, with 7.6 ± 1.7, 6.2 ± 1.2, 4.4 ± 2.4, and 4.3 ± 3.3 log copies/g soil, respectively (mean ± standard deviation). Because the use of mfq-PCR provided data on many ARGs, we used two approaches for evaluating changes in ARG abundances. Firstly, we compared ARG profiles (*i.e.*, relative abundance changes among all ARGs present in each sample) among treatments and years using PCoA and PERMANOVA. However, a lack of change in overall profiles of ARG abundances using this approach does not mean that no biologically important changes in individual ARGs occurred. Thus, we then followed up this first approach by determining changes in abundances of all ARGs individually using Mann-Whitney U tests.

When considering absolute ARG abundances (copies g^{-1} soil), overall profiles of ARG abundance varied among treatments ($R^2 = 0.53$, $F_{3,71} = 26.6$, $P = 0.001$, Fig. 2A), but differences among years were only marginally significant (R^2 = 0.02, $F_{2,71}$ = 2.80, P = 0.069, Fig. 2A). Among treatments, the only pairwise significant differences were between NM and the other three treatments (CON, CEPH, and PIR; all $P <$

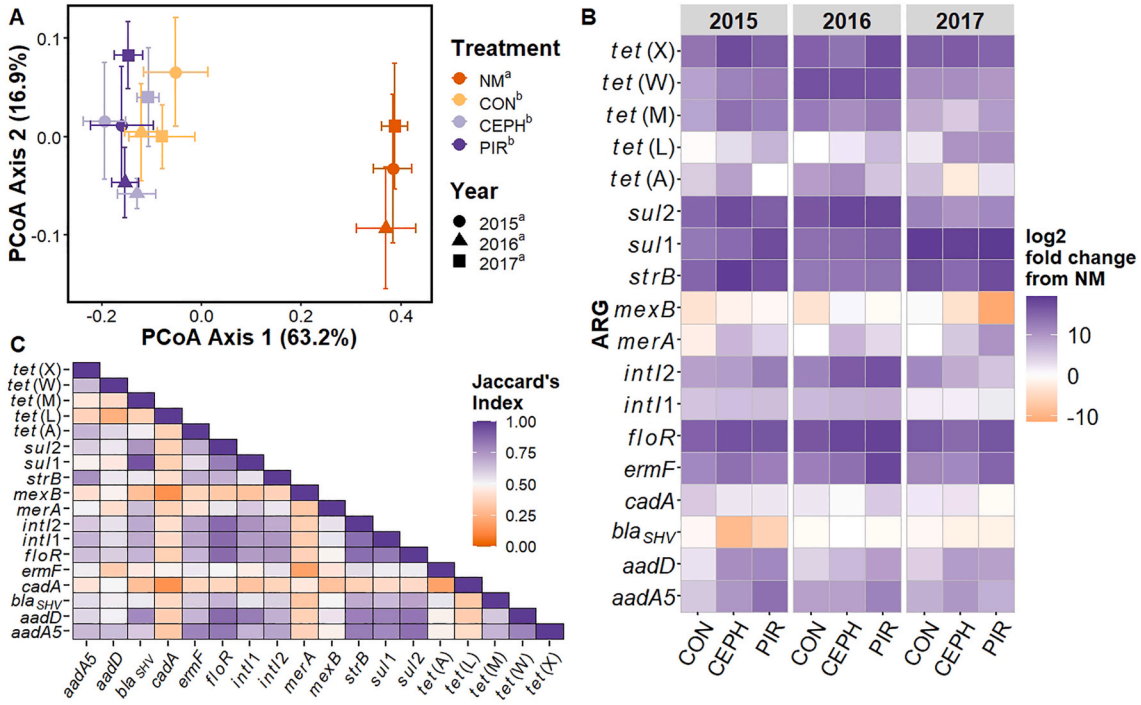

Fig. 2. Principle coordinate analysis visualizing similarities among treatments and years in ARGs quantified via mfqPCR (A). Points represent centroids for each treatment and year combination. Error bars represent standard error. Superscript letters in the legend indicate significant differences based on PERMANOVA among treatments and years. Log_2 fold change in absolute abundance (copies g^{-1} soil) of CON, CEPH, and PIR to NM in genes that were observed in >20% of all samples in each treatment and year (B), and co-occurrence of genes with Jaccard's Index across all samples (C). P-values from all pairwise comparisons by treatment are shown in Table S2.

0.05). Generally, individual ARGs were more abundant in plots with any manure applied, regardless of antibiotic exposure (Fig. 2B). Raw mean ARG abundances (*i.e.*, not corrected for fold change) and *P*-values from Mann-Whitney U tests are shown in Table S2. Eleven genes – including integrase genes and tetracycline, sulfonamide, and aminoglycoside resistance genes – had greater abundances in all three manure treatments (CON, CEPH, and PIR) compared to NM (all $P < 0.05$) based on Mann-Whitney U tests. Additionally, the tetracycline resistance gene *tet* (A) had greater abundance in CON than NM, *aadD* and *tet*(L) had greater abundance in both CEPH and PIR compared to NM, and PIR had a greater abundance of *merA*, but lower abundance of bla_{SHV} compared to NM. The lack of significant change in total ARG profile does not preclude biologically important changes in individual genes. However, when individual genes were analyzed among CON, CEPH, and PIR, the only additional significant difference detected was a higher abundance of *tet* (L) in PIR compared to CON.

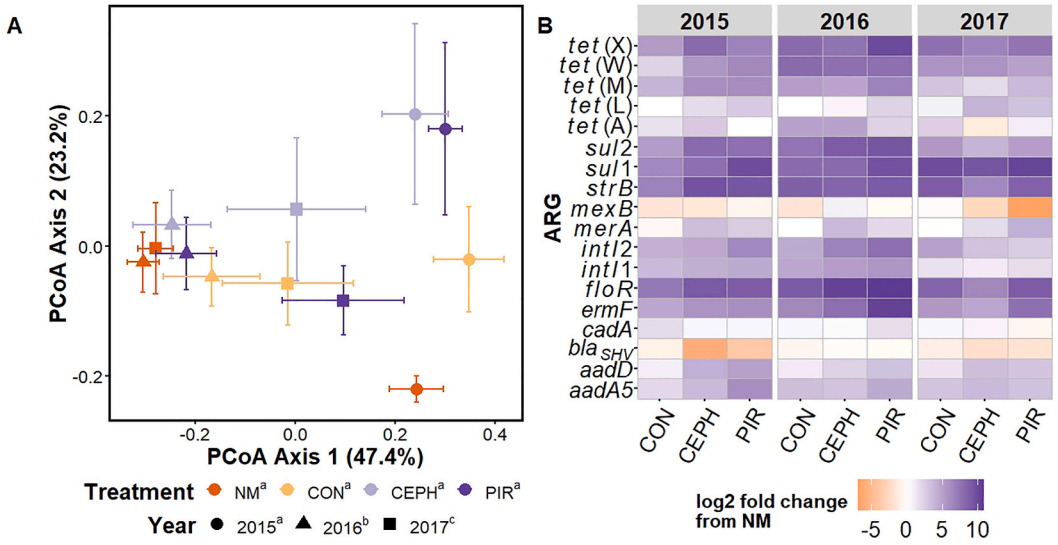

Fig. 3. Principle coordinate analysis visualizing similarities among treatments and years in overall ARG profiles quantified via mfqPCR, normalized to 16S (bacterial) abundance (A). Points represent centroids for each treatment and year combination. Error bars represent standard error. Superscript letters in the legend indicate significant differences based on PERMANOVA among years and treatment. Log_2 fold change of ARG copies per 1×10^7 16S copies of CON, CEPH, and PIR to NM in genes that were observed in >20% of all samples in each treatment and year (B). P-values from all pairwise comparisons by treatment are shown in Table S3.

Total 16S rRNA gene abundances did not differ among years ($F_{2,71} = 1.68$, $P = 0.19$) or treatments ($F_{3,71} = 0.65$, $P = 0.58$. However, when we normalized ARG abundances to 16S rRNA gene abundances, there were some changes in the importance of factors affecting ARGs. The effect of manure treatments on normalized ARG profiles was marginally significant ($R^2 = 0.07$, $F_{3,71} = 1.83$, $P = 0.078$; Fig. 3), but no pairwise comparisons of treatments yielded FDR-adjusted *P*-values < 0.05. Conversely, year explained a greater portion of the variance in normalized ARG profiles ($R^2 = 0.10$, $F_{2,71} = 8.44$, $P = 0.001$, Fig. 3), where ARG profiles each year were statistically different ($P < 0.05$). When differences in individual genes were analyzed among treatments, the results were nearly the same as absolute ARG abundances, with the exception that bla_{SHV} was not significantly different between PIR and NM, and *tet*(L) was not significantly different between PIR and CON. (Fig. 3B, Table S3).

Finally, because microfluidic qPCR estimates abundances of several genes simultaneously, we were also able to examine co-occurrence among ARGs. We quantified co-occurrence of all possible ARG pairs based on the presence or absence of genes across all samples, using Jaccard's Index. Several genes, including *strB*, *sul1*, *sul2*, *florR*, and *tet*(W) tended to occur in the same samples (Jaccard's Index ≥ 0.80, Fig. 2C). Additionally, *intI1* tended to co-occur with *floR* and *tet*(W). Meanwhile, several genes, including *cadA*, *merA*, *tet*(A), and *tet*(L), had consistently low pairwise Jaccard values, suggesting they do not tend to co-occur with other ARGs in soil.

4. Discussion

Two key aspects of this study were 1) the controlled, continuous addition of manure from cattle under multiple antibiotic regimes for three years, and 2) the amount of data simultaneously collected describing different components of the soil microbiome, including the structure of bacterial and fungal communities and numerous ARG abundances. As a result of this design, we were able to control for multiple critical components of the AMR puzzle, including the effects of applying manure from cattle with no antibiotics administered, the effects of applying manure from cattle administered different antibiotic types, and the temporal variability that occurs in key response variables over multiple years.

4.1. Impacts of manure and antibiotics on microbial community structure

A first key outcome of this work is that relative impacts of manure from cattle under different antibiotic regimes are important in structuring the soil microbiome, but the responses vary among components of the microbial community. Similar to previous studies, manure additions altered bacterial community structure (Blau et al., 2018; Han et al., 2018; Udikovic-Kolic et al., 2014). Nutrient and organic matter inputs were likely an important factor in manure effects on both soil bacteria and fungi. For example, bacterial OTUs that increased in CON plots were mostly in Proteobacteria and Bacteroidetes, which are typically considered copiotrophic (Fierer et al., 2007; Sauvadet et al., 2019). Likewise, soil fungal community composition is known to be sensitive to C:N ratios (Lauber et al., 2008) and organic inputs (Sauvadet et al., 2019). Fungal genera that increased in manure treatments included *Mortierella* and *Taphrinia*, which are also copiotrophic (Sauvadet et al., 2019), and *Panaeolus* and *Preussia*, which are commonly associated with manure (Doveri, 2010; Sarrocco, 2016; Wepking et al., 2017).

In contrast to manure from antibiotic-free cattle, manure from cattle under two specific antibiotic treatments (CEPH and PIR) shifted soil bacterial communities in the opposite direction from NM than did manure from cattle without antibiotics (Fig. 1A). This suggests that the effect of antibiotic-free manure and manure from cattle administered antibiotics on bacterial community structure are distinctly different. Previous studies have also found shifts in microbial community composition between soils amended with manure or manure and antibiotics (Blau et al., 2018; Pankow, 2017). Among responsive taxa, two bacterial genera that had higher relative abundances in both antibiotic treatments matched those previously shown to have increased relative abundance following antibiotic exposure, including *Leuteolibacter* (Wepking et al., 2019) and *Devosia* (Hassan et al., 2015). Other genera that increased in both antibiotic treatments have been shown to be resistant to other various chemical disturbances, including *Salinibacterium* (Igun et al., 2019; Otlewska et al., 2017) and *Cellvibrio* (Filimon et al., 2014). Additionally, we identified several bacterial genera that were relatively more abundant in PIR compared to CON, including uncultured *Verrucomicrobia* Da101, *Adhaeribacter*, *Dokdonella*, *Candidatus Xiphinematobacter*, and *Demequina*, which have not been previously documented to respond to antibiotic exposure.

Underlying manure and antibiotic treatment effects, it is important to note that we observed significant temporal variation in both bacterial and fungal community structure from year to year across all treatments. Although this study was not designed to identify environmental drivers of temporal variability in microbial communities, weather conditions are one obvious possibility. The local weather station recorded lower total rainfall in the week prior to sampling in 2015 (3 mm) than in 2016 (24 mm) and 2017 (34 mm), coinciding with a large shift in fungal community structure in 2015, and soil moisture is known to impact soil microbial community structure (Kaisermann et al., 2015; Lupatini et al., 2019).

4.2. Impacts of manure and antibiotics on ARGs

Another important finding of this work is that the overall ARG profiles observed in soil differed primarily with the application of manure, regardless of whether cattle were administered antibiotics. This was unexpected, given that previous work has reported higher ARG abundances in soils amended with manure and antibiotics, compared to manure without antibiotics (Zhang et al., 2017). However, increased ARG abundances have also been observed in manure from animals not treated with antibiotics (Heuer et al., 2011; Heuer and Smalla, 2007; Udikovic-Kolic et al., 2014). It is also important to note that this result may be specific to our study conditions and additional experimental investigation is required to determine if this is a common effect. If manure from antibiotic-free cattle consistently has large impacts on ARG abundances, this is a critical point to consider in managing AMR. It is also important to note that although ARG abundances were typically lower in the no-manure control soils, there were still detectable quantities of ARGs. Antibiotics and resistance genes are commonly found naturally in soils (Fletcher, 2015; Nesme and Simonet, 2015), thus it is important to remember that we cannot expect ARGs to be absent from any given soil, even in undisturbed ecosystems.

The use of mfqPCR allowed simultaneous examination of a wide range of ARGs and many genes varied in ways that were unexpected based on the antibiotic treatments applied. Of the 15 genes that increased in abundance in at least one of the manure treatments compared to NM, only two (bla_{SHV} and *ermF*) are known to confer resistance to the antibiotics given to cattle in our study. Furthermore, *ermF* confers resistance to lincosamides such as pirlimycin, but was actually more abundant in all three manure treatments compared to NM. The response of bla_{SHV}, which confers resistance to β-lactams such as cephapirin, was also counterintuitive; it was more abundant in NM compared to PIR, but the abundance was not different between NM and CEPH. One possible explanation for increases in unexpected classes of ARGs is co-selection, which can occur when multiple ARGs are found on a single plasmid (Herrick et al., 2014). In such a case, exposure to one antibiotic can increase resistance to multiple classes of antibiotics (Hammer et al., 2016; Herrick et al., 2014). This would also help explain our observation that several genes tended to occur together. For example, the Jaccard similarity values from our data suggested groupings of *strB*, *sul1*, *sul2*, and *tet*(W), as well as *intI1* with *floR* and *tet*(W). Some of these relationships agree with previously published evidence

for co-occurrence of *sul1*, *sul2*, and *intI1* (Pal et al., 2015).

While absolute abundance of ARGs in soil can be indicators of the overall amount of AMR, such changes can be difficult to interpret. ARG abundances can be problematic because they can change due to an increase in total bacteria or because of selection for individual bacteria that are more likely to carry one or more copies of a particular ARG (Udikovic-Kolic et al., 2014). In the latter case, analyzing ARG abundance normalized to total 16S rRNA gene abundance can be a better indicator of the relative number of bacteria that may carry a resistance gene. For example, we found that treatment explained a large portion of the variance in ARG profiles g^{-1} soil, but explained little variance for overall ARG profiles normalized to 16S rRNA gene abundance. Thus, the patterns among ARG profiles appear to be related to changes in overall bacterial abundance, but this was further complicated in our study as we did not detect significant changes in 16S rRNA gene abundances among treatments.

Despite no significant differences in ARG profiles normalized to 16S rRNA abundance among treatments, examining the normalized abundance changes in individual ARGs showed many significant results. In fact, most of the pairwise comparisons that were statistically significant when comparing absolute ARG abundances were also significant when comparing normalized ARG abundances. When coupled with the fact that 16S copy numbers were not significantly different among treatments, these results suggest that the relative increases in ARG abundances with manure application were the result of selection for AMR and not simply due to increased bacterial abundance. Regardless, increased bacterial numbers and selection for more resistant bacteria can both clearly be important pieces of ARG abundance and the AMR puzzle. Additional research establishing whether human health or transmission risks are more clearly associated with one change or the other will be necessary to determine how best to monitor and manage AMR in soil systems.

5. Conclusions

Adapting soil and manure management practices to address the AMR crisis will require a nuanced understanding and approach. Responses of the soil microbiome to manure from cattle treated with antibiotics clearly vary by microbial taxa and over time. We found marked differences in the responses of fungal and bacterial communities in soil following application of manure from under different antibiotic regimes. Specifically, manure from cattle treated with antibiotics affected bacterial community structure compared to both manure from antibiotic-free cattle and no manure additions. In contrast, fungal community structure was affected only by addition of any manure, regardless of whether cattle were given antibiotics. Critically, we also found that manure inputs from antibiotic-free cattle increased ARG abundances compared to soil without manure amendments, yet there were almost no differences between manure inputs from cattle that received antibiotics and those that did not. And finally, we observed that these changes are detectable over multiple years with repeated manure additions, despite variation in microbial community structure over time. Clearly, a better understanding is required of how factors other than antibiotic usage alone influence soil AMR over longer time scales. In particular, future work should 1) further examine the potential influence of microbial-C substrate availability, nutrient status, and environmental factors on microbial community responses to antibiotics and manure, and 2) allow for more rigorous examination of possible interactions among multiple controlling factors.

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.

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Appendix A. Supplementary data

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