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Examining the Effects of Time on Antibiotic Resistance Within Prairie Restorations

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Examining the Effects of Time on Antibiotic Resistance & Production Within Prairie Restorations

Abstract

Widespread antibiotic use in agriculture spreads antibiotic resistance (AR) genes throughout the ecosystem and can impact bacterial community composition and function. Restoring agricultural fields back to their native prairies has been shown to remediate other damaging effects of agriculture, and have the potential to remediate AR from the soil as well. In this study, our first aim was to determine if soil bacterial communities return to pre-agricultural conditions over time following restoration to native plant communities. We did this by assessing the soil characteristics, extracellular enzyme activities, bacterial community composition, and functional metagenomics across a chronosequence of seven restored prairies, one old-field, and one current agricultural plot. We used functional metagenomics to analyze the relative abundances of predicted pathways for antibiotic production and antibiotic resistance across the chronosequence. Though our chronosequence is limited in data points, soil bacterial communities appear to return to pre-agricultural conditions as soil characteristics, and community composition and function converge to similar properties as was found in the old field by 30 years since initial restoration. However, more replicates are needed to draw any definitive conclusions. Production of antibiotics frequently used in agriculture, such as streptomycin and tetracycline, decrease with age of restoration, while pathways for production of antibiotics that are traditionally produced by fungi, such as penicillin or cephalosporin, increases with age of restoration. Correspondingly, ß-lactam resistance and caprolactam degradation follow the same trend as penicillin or cephalosporin production. Our results indicate that prairie restoration may be a useful strategy for addressing the consequences of agricultural antibiotic use.

Introduction

Current agricultural practices can have devastating effects on our ecosystems. Biological diversity such as diversity in plants, animals, and insects decreases significantly from that of native grasslands[1]. Moreover, the loss of native plants can cause increased soil erosion as well as decreased soil health and air quality caused by an increased in greenhouse gas emissions[2]. The loss of animal diversity results in fewer pollinators[3], and fewer organisms acting as natural pest control which creates an increased need for pesticides[2]. As plant productivity decreases and pests increase, more and more fertilizers, pesticides, and other additives are added into the ecosystem in the pursuit of production. Through soil runoff, native water systems are polluted and spread pollutants far beyond the local environment as they connect to larger rivers, lakes, and oceans[4]. Within the soil, constant fertilizer application and removal of biomass alters microbial respiration as soil nitrogen and carbon content change drastically[5–8]. This change in soil characteristics inevitably leads to a shift in soil microbial communities.

Every year, over 63,000 tons of antibiotics are used in the agricultural and animal husbandry industries[9]. They are used for veterinary treatment, disease prevention, and as growth enhancers[10]. The widespread use of antibiotics has led to an increase in antibiotic resistant (AR) bacteria within the environment as AR genes are transferred by agricultural bacteria to environmental bacteria[9,11–14]. In some cases, antibiotics will select for resistant bacteria as only those with AR mechanisms will survive to proliferate and pass their AR genes down to future generations. Bacteria can also transfer genes among the community via horizontal gene transfer, particularly through resistance plasmids[15–17]. Moreover, once in the environment, AR bacteria can be transferred to humans, making many diseases more difficult to treat with current antibiotics[10,11,18,19].

Manure fertilizer serves as a major reservoir of AR genes as 40-95% of administered agricultural antibiotics are subsequently excreted, and then disseminated into the environment[11,13,19–21]. Once in the soil, these genes are spread widely via water runoff[10], and migratory animals, such as birds, that can distribute AR genes over regional and global distances[22]. Therefore, introducing antibiotics into the soil microbiome through agriculture and animal husbandry has consequences that reach far beyond the local environment.

It is clear that common agricultural practices, such as continual disturbance and fertilizer application, affect the soil microbiome by changing the form and availability of C and N within the soil[23–27]. However, Wepking et al. posit that the presence of AR genes affects the metabolic efficiency of bacterial communities found within agricultural soils[12,21]. In their research, soils exposed to manure from antibiotic treated cattle exhibited not only greater levels of AR genes, but also decreased carbon use efficiency as well as altered nitrogen cycling when compared to soils exposed to manure without antibiotic exposure. This suggests that the presence of AR genes may change the overall function of a bacterial community.

Ecological restoration could play an important role in remediating AR genes from the environment. Traditional practices of restoration - including re-introducing native plants and prescribed fire into the landscape and removal of invasive species - improve previously cultivated ecosystems in a variety of other ways. First, plant diversity, pest-reducing insects, birds, and pollinators all increase significantly following restoration[28]. In turn, this decreases erosion, the need for pesticide use, and ultimately increases plant productivity in the ecosystem[1,2].

Restoration also affects soil bacterial community structure and function, though the effect is slower and more site-dependent than with plant communities. For example, after nearly 30 years of restoration in the grasslands of Illinois, Barber et al. observed C content increase with age, and

class-level diversity, richness, and evenness decrease with age, as well as significant changes in taxonomic abundance in which restorations began to resemble remnant prairies that have never been used for agricultural purposes[23]. However, in prairies near Manhattan, KS, Jangid et al. observed there were no restorations resembling a remnant[29]. These changes in community composition could be related to different soil physiochemical properties such as pH, soil texture, and soil organic matter[29]. Both studies observed that intermediate communities developed within the chronosequence, which were distinct from the communities in both the cultivated and remnant fields.

Through long-term antibiotic use, fertilizer application, monocropping, and biomass removal, agricultural practices significantly change the soil microbiome[6–8,12,21]. This is evidenced by the differences seen in the microbial composition of restored prairies compared remnant prairies.[24,29,30]. For example, remnant soil microbial communities tend to harbor more oligotrophic taxa, such as those within the phylum Verrucomicrobia, which are adapted for slow growth on recalcitrant carbon substrates[24], while bacteria in current agricultural plots exhibit copiotrophic life strategies, utilizing labile carbon substrates and performing free living nitrogen fixation[23,29]. Furthermore, the presence of AR genes may exacerbate these differences through altering metabolic efficiency[12,21]. Despite widespread antibiotic use within agriculture, to our knowledge the impacts of past antibiotic use on prairie restoration in regards to community composition and function has gone relatively unstudied.

To identify how past antibiotic use might affect community function and composition within prairie restorations, we analyzed bacterial communities across a chronosequence of seven restored prairies ranging in age from 2-30 years old as well one old-field and one current agricultural plot. All sites were located one the same property owned by the Edward Lowe

Foundation in Cassopolis, MI. We addressed three questions: 1) Do soil bacterial communities return to pre-agricultural conditions over time? 2) Do indicators for antibiotic *production* change over time? 3) Do indicators for antibiotic *resistance* change over time? Our results demonstrate that the time since restoration has a significant effect on community composition, extracellular enzyme activity and predicted pathways for AR production and resistance.

Methods

Soil Sample Collection

We collected eight soil samples on July 15, 2019 from the Edward Lowe Foundation (ELF) in Cassopolis, MI (Table 1). Seven of the samples were from restored prairies ranging in age of restoration from 30 years to 2 years. We took the eighth sample from a current agricultural plot that was planted with corn at the time of sampling. We used previously-collected soil from an old field at ELF for our final sample. All soils are classified as Alfisols. We collected three 10-cm deep cores at each site and homogenized together in a Ziploc bag. We used cleaned corers between sites to prevent cross-contamination between samples. Immediately after collection, we froze a subset of the soil samples at -80 C for DNA extraction. We sieved the remaining soil samples through a 2-mm sieve to measure other soil characteristics described below.

Table 1: Location of each soil collection site at the Edward Lowe Foundation in Decatur, Michigan

Year	Latitude	Longitude
Current Agricultural Plot	41.95437°	85.97748°
1990	41.95542°	85.99345°
2001	41.95453°	85.98952°
2002	41.96255°	85.98048°
2008	41.95525°	85.98567°
2014	41.95407°	85.97675°
2017a	41.95465°	85.97697°
2017b	41.95523°	85.97798°

Soil Characteristics

We measured soil physiochemical characteristics including: pH, percent soil moisture, percent soil organic matter (SOM), ammonium and nitrate concentrations, and extracellular enzyme potential activities of beta-glucosidase, cellobiohydrolase, N-acetylglucosaminidase, and xylosidase. We determined soil pH by using an Accumet Research AR25 Dual Channel pH/Ion Meter in a slurry of 5g soil sample with 20 mL of ultrapure water. We determined percent soil moisture by incubating 9g soil samples at 60°C for 1 week and calculating the mass loss before and after drying. We determined percent SOM by placing 5g of oven dried sample in a Fisher Isotemp Muffle Furnace Model 184A ashing oven for 2 hours at 360°C and determining the mass loss before and after ashing. We conducted extractions with 10 g soil and 50 mL of 2M KCl prior to running the ammonium and nitrate assays for each soil sample, using a 96-well plate method described by Ringuet et al[31]. The extracellular enzyme activities of β-glucosidase, cellobiohydrolase, N-acetylglucosaminidase, and xylosidase were examined by assaying based on the procedures in previous work[32,33]. Briefly, this method uses fluorescently labeled substrates to determine potential activity rates of enzymes associated with mobilization of glucose (labile carbon substrate), cellulose (recalcitrant carbon substrate), N-acetylglucosamine (organic nitrogen), and xylose (recalcitrant carbon substrate), respectively[32–34].

Microbial Community Composition and Predicted Metagenomic Function

We extracted DNA using the a PowerSoil DNA extraction kit (Qiagen, Inc., Hilden, Germany). We included a negative control extraction to correct for any contaminant sequences introduced during extraction or sequencing. Following DNA extraction, we sent the extracts to the

Michigan State University Genomics Core Facility for bacterial 16S rRNA-gene amplicon preparation using 515F/806R primers and paired-end Mi-Seq sequencing (Illumina, San Diego, California, USA) according to Caporaso et al[35]. Once sequence results were demultiplexed and returned, we merged forward and reverse reads using Pandaseq (version 2.8)[36], removing any sequences shorter than 245 bp and longer than 275 bp. The average length of sequences in the dataset was 253.0891 +/- 0.7466 bp. We removed chimeric sequences using the vsearch algorithm (version 2.4.3)[37]. Following chimera removal, a total of 560,409 high-quality sequence reads were present in the dataset. We assigned OTUs using an open-reference procedure with the Silva version 128 reference database[38]. We removed any OTUs that were identified as Archaea, mitochondria, chloroplast or within the genus *Ralstonia* were removed from the dataset prior to analysis. We examined the DNA extraction control to determine whether any potential contaminant sequences were introduced into the samples during extraction, amplicon preparation or sequencing. We were left with 543,249 sequences upon removal of all sequences associated with Archaea, mitochondria, chloroplast, *Ralstonia*, and the negative control. Once we completed these clean-up steps, the total number of sequences per sample ranged from 47,701 – 72,063; we did not rarefy the data prior to statistical analyses. We then used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)[39] to analyze the predicted metagenomics pathways using the 16S rRNA sequence data, as described in Docherty and Gutknecht[34].

Statistical Analysis

For all univariate measurements, we used regression analysis to determine whether there was a change in the variable over time-since-restoration. Using unweighted unifrac distance metrics, we assessed for similarities between communities from each sample, and then used

principle coordinates analysis to visualize community similarity. We used PICRUSt to examine eight different functional metagenomic pathways between communities. These eight pathways were split into three categories: general function, antibiotic production, and antibiotic resistance. Under general function, we assessed the relative abundances of predicted pathways for cell division and replication, recombination, and repair proteins. We chose these as they provide insight into the type of life history characteristics that bacteria favor at each point in the chronosequence. The predicted antibiotic production pathways we examined were for penicillin or cephalosporin biosynthesis, streptomycin biosynthesis, tetracycline biosynthesis, and vancomycin biosynthesis. The predicted antibiotic resistance pathways we assessed were for caprolactam degradation and ß-lactam resistance.

Results & Discussion

Changes in Soil Characteristics Over Restoration Chronosequence

Soil characteristics changed over time since restoration. Soil pH decreases over time since restoration ($R^2 = 0.2377$), while the percent SOM increases ($R^2 = 0.1645$). An increase in SOM is a sign that more biomass is stored within the soil, which may be of plant or microbial origin. Since agricultural fields undergo too much disturbance and biomass removal for significant accumulation of SOM, this is viewed as a positive change brought about by restoration and ceasing agricultural activity. Corresponding to this change in SOM, enzymatic activities of cellobiohydrolase ($R^2 = 0.1345$), N-acetylglucosaminidase ($R^2 = 0.4643$), and xylosidase ($R^2 = 0.2893$) all increased with respect to time since restoration (Figure 1). Cellobiohydrolase is an extracellular enzyme released by microorganisms to mobilize carbon from cellulose, which is the

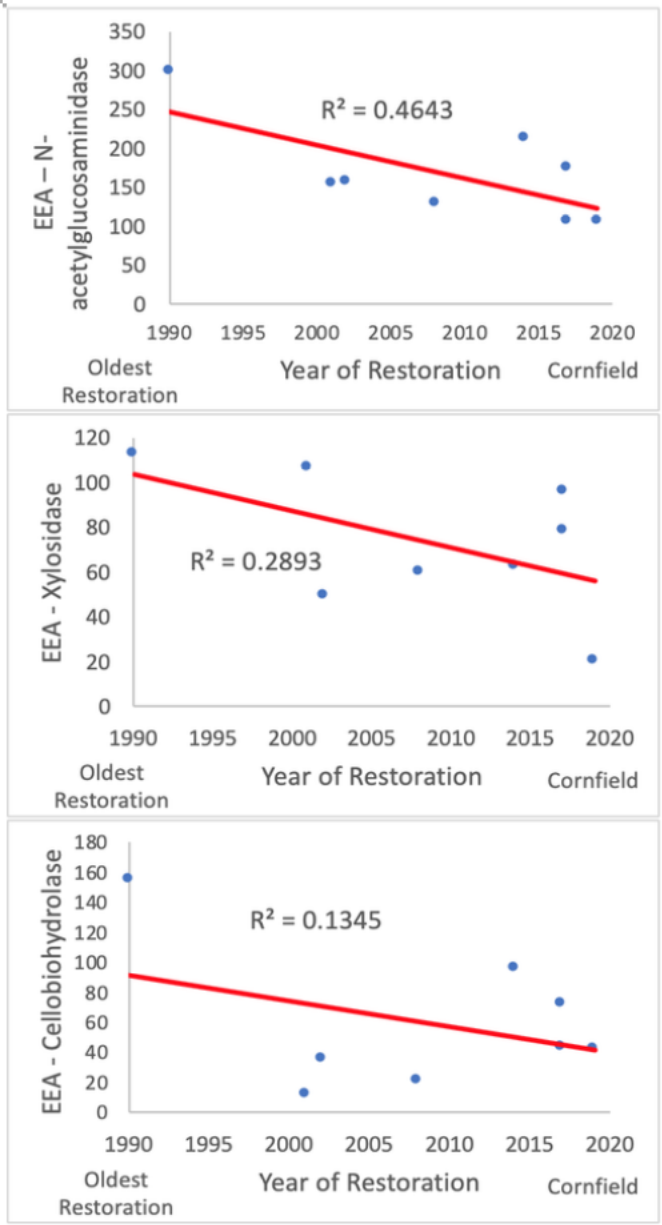

Figure 1: Potential activity of N-acetylglucosaminidase, potential activity of xylosidase, and potential activity of cellobiohydrolase all increase with respect to time since restoration.

main component of plant biomass. Higher cellobiohydrolase activity supports the notion that more plant matter is being stored in the soil and selecting for a microbial community that degrades cellulose. Likewise, xylosidase oxidizes xylose, a material found in woody plant matter and the main component of hemicellulose; thus, carbon is potentially being mobilized from multiple organic sources in older restorations. Similarly, increases in N-acetylglucosaminidase indicate an increase in microbial nitrogen mobilization from organically-bound forms. Without constant fertilizer application, the microbial communities must adapt to acquire nitrogen in less available forms, such as organic nitrogen, instead of nitrate. We did not observe any changes in in % soil moisture (R^2 = 0.0018), ammonium and nitrate content (R^2 = 0.0122 and R^2 = 0.0.349), or the enzymatic activity of ß-glucosidase (R^2 = 0.069).

Bacterial Community Changes Over Restoration Chronosequence

We analyzed bacterial community similarity across the nine sites within our chronosequence using unweighted unifrac distance metrics (Figure 2). The communities in the most recent restorations (2017) are most similar to the community in the active cornfield. Similarly, the community in the oldest restored prairie (1990) is most similar to the old field that was never plowed or fertilized in traditional row-crop management. Between these two extremes of the chronosequence, there are different transitional communities associated with the prairies. There is a rough pattern indicating that prairies restored longer are more similar to the oldest restored prairie and the old field (e.g. 2001). However, some newer prairies also harbor a bacterial community that is similar to this group (e.g. 2014), while some older prairies are more similar to the current cropland (e.g. 2002, 2008). These sites did not closely match either the old field/1990 restoration group or the cornfield, which was expected based on the literature[23,24,29].

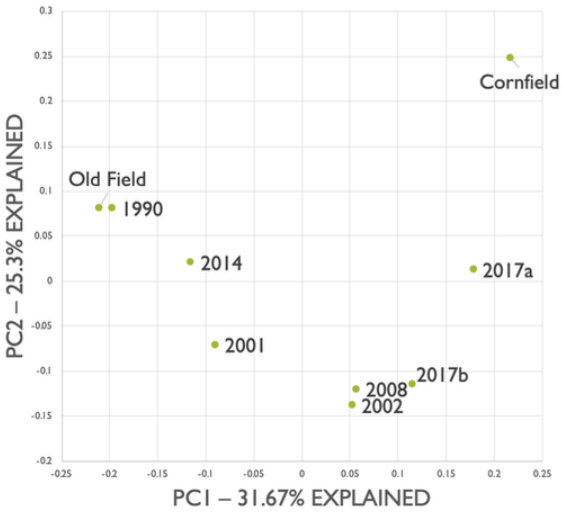

Figure 2: Principal coordinates analysis (PCoA) ordination showing bacterial community similarity based on unweighted unifrac distances. The bacterial community in the newer restored prairies (2017) are most similar to the active cornfield while community in the oldest restored prairie (1990) is most similar to the old field.

Overall, the predominant phylum at each site was *Proteobacteria* (26.8% - 38.3%), with *Acidobacteria* (14.6% - 22.4%), *Bacteroidetes* (6.9% - 10.3%), and *Verrucomicrobia* (6.7% - 15.7%) all at the next highest percentages of the community (Figure 3). As seen previously in the literature[23–25,29], *Verrucomicrobia* prevalence increased over time since restoration ($R^2 = 0.2424$). This is most likely due to its oligotrophic nature, as *Verrucomicrobia* prevalence decreases in soils amended with fertilizers and thrives in soils with high levels of organic matter[7]. Furthermore, *Verrucomicrobia* possess functional pathways that support the slow growth in limited nutrient environments that is characteristic of oligotrophs[24]. Unfortunately, other distinguishing characteristics of *Verrucomicrobia* are not well understood as it is not readily culturable. *Gemmatimonadetes* decreased over time since restoration ($R^2 = 0.2942$). *Gemmatimonadetes* is a

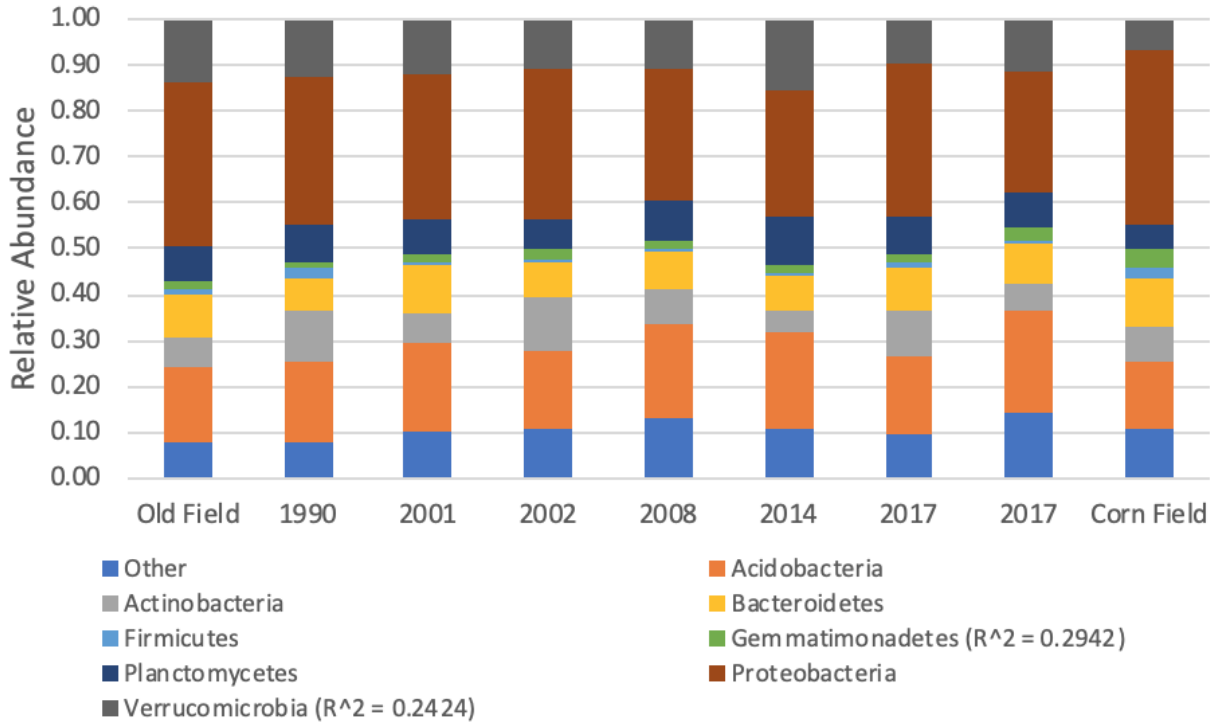

Figure 3: Microbial community compositions are shown to change over time as indicated by relative abundances. Specifically, *Gemmatimonadetes* decreases with time since restoration and *Verrucomicrobia* increases with time since restoration.

common soil bacteria exhibiting copiotrophic life strategy patterns[40]. This may be one reason why *Gemmatimonadetes* thrives in newer restorations compared to older ones.

While the bacterial community in the oldest prairie resembled the old field and the bacterial communities in the newest restored prairies (2017) resembled the active cornfield, it is important to consider the reasons for variation among the transitional communities from the prairies restored between 2001-2014. Each prairie was restored in a different year, and the precipitation and temperature patterns in the year of restoration can be significant predictors of restoration success[41]. Additionally, though all prairies contained similar plant communities, not all the prairies were seeded with the same original seed mix and it is likely they have undergone varying management practices over the years. While all soils are Alfisols, each individual restoration could also have different soil characteristics depending on slope and location. This variation in soil characteristics could cause variation among bacterial communities. Lastly, more replicates are needed. Our data shows that these trends may exist; however, more data points are needed in order to make a more definitive conclusion.

Table 2: We used functional metagenomic data to assess for change over time. Regressions show a change in functions related to general cellular functioning, antibiotic production, and antibiotic resistance all over time.

Cellular Function	R^2	Trend with Time Since Restoration
Cell Division	0.3068	Decrease
Replication, recombination, and repair proteins	0.2241	Decrease
Biosynthesis of vancomycin group antibiotics	0.0055	NA
Tetracycline biosynthesis	0.2692	Decrease
Streptomycin Biosynthesis	0.2521	Decrease
Penicillin and cephalosporin biosynthesis	0.1421	Increase
Beta-lactam Resistance	0.2032	Increase
Caprolactam Degradation	0.3024	Increase

Analysis of functional metagenomics pathways indicates that general bacterial functions changed over time since restoration (Table 2). Both cell division and replication, recombination, and repair proteins decrease with age (R^2 = 0.3068 and R^2 = 0.2241), suggesting

slower bacterial growth with increasing time since restoration. This could be due to reduced disturbance with the cessation of agricultural tilling. Slower growth is associated with oligotrophic life strategies, which is commonly found in bacterial communities within native tallgrass prairies[24]. These data suggest that restored prairies begin to harbor bacterial communities similar to remnants as time goes on.

Antibiotics Production and Resistance Changes Over Restoration Chronosequence

Using predicted functional metagenomics pathways, we examined several pathways for antibiotic production (Table 2). Production of streptomycin and tetracycline decreased over time since restoration ($R^2 = 0.2521$ and $R^2 = 0.2692$), but vancomycin production did not change over time ($R^2 = 0.0055$). Streptomycin and tetracycline are used broadly in agriculture[14], and are also produced by common soil bacteria within the phylum *Actinobacteria*. *Actinobacteria* relative abundances did not change over time, suggesting decrease in production was either caused by a change in the relative abundances of taxonomic groups within *Actinobacteria*, or was caused by a shift in functional response. Conversely, pathways indicating production of penicillin/cephalosporin, which are both β-lactam antibiotics, increased over time since restoration ($R^2 = 0.1421$). This class of antibiotics is produced by *Penicillium* fungi which is commonly found in soils[42,43]. Increased β-lactam production over time coupled with decreased streptomycin and actinomycin production over time indicates a functional shift in the soil community, where older restorations contain more fungi, while newer restorations contain more bacteria. This shift from bacterial-dominated to fungal-dominated communities in soils is a commonly observed trend in restoration chronosequence studies[44,45].

This observation is further supported by our observations of pathways for antibiotic resistance. ß-lactam resistance and caprolactam degradation both increased with time since restoration (R^2 = 0.2032 and R^2 = 0.3024). This suggests that soil communities respond to increased ß-lactam production with increased ß-lactam resistance, further supporting the idea of a bacterially-dominated community in more recent restorations and a fungal-dominated community in older restorations[46–49]. Antibiotic resistance and production appear to change over time since restoration. Biosynthesis of antibiotics commonly used in agriculture decreases over time while biosynthesis for antibiotics formed by fungi increased over time. This is significant as remnants and older restorations are known to harbor a larger fungal community compared to that of an agricultural plot or young restoration[46]. Similarly, a corresponding increase in resistance to fungal antibiotics was observed. This could be an indication that microbial communities within prairie restorations are converging on that of a remnant prairie.

Future Directions

This study provides evidence that restoration can influence antibiotic production and resistance profiles over time. More research is necessary to determine whether the predicted metagenomics pathway data we describe translates to active antibiotic production and resistance. Further data points along this chronosequence, including sites from a variety of locations are required to determine whether the trends we described are broadly applicable and statistically replicable. Specifically, our study provides useful targets for future analysis. Antibiotic resistance genes and chemical production of streptomycin, tetracycline, penicillin and cephalosporin are useful targets, since our results indicate that the production pathways for these antibiotics change over time-since-restoration. Quantitative PCR assays can be used to verify the abundance and

activity of antibiotic production and resistance genes for these targets[50]. Finally, calculating fungal to bacterial ratios using a technique such as phospholipid fatty acid analysis (PLFA) can help identify whether there is a shift in microbial biomass with restoration, by using a single assay that can compare the two groups.

Conclusions

This study aimed to determine if restoration practices can return bacterial communities to pre-agricultural conditions over time, and if indicators for antibiotic production and resistance change over time. Bacterial communities appear to converge on that of the remnant over time since restoration. Percent SOM increases, which can indicate prairies are recovering from agriculture as this suggests more plant material is being stored within the soil. Similarly, there is an increase in potential activity of cellobiohydrolase, xylosidase, and N-acetylglucosaminidase, indicating higher rates of carbon and nitrogen mobilization. An increase in oligotrophs and a decrease in copiotrophs is exhibited, and this is supported by a decrease over time in functional metagenomic pathways for both cell division, and proteins responsible for replication, recombination, and repair. Streptomycin and tetracycline biosynthesis decrease over time since restoration. These are both produced by *Actinobacteria*, yet there is no change in *Actinobacteria* prevalence over time, suggesting a shift in function instead. Penicillin/cephalosporin biosynthesis increases over time since restoration, suggesting more fungal dominance as time goes on. ß-lactam resistance and caprolactam degradation increase over time as well, which is not surprising as antibiotic presence will select for its corresponding resistance. Overall, bacterial communities appear to converge on remnants in community composition and function as related to antibiotic use. However, further research is necessary to fully support these claims.

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