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Research Article

Taxonomic and functional restoration of tallgrass prairie soil microbial communities in comparison to remnant and agricultural soils

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Abstract

Restoring ecosystems requires the re-establishment of diverse soil microbial communities that drive critical ecosystem functions. In grasslands, restoration and management require the application of disturbances like fire and grazing. Disturbances can shape microbial taxonomic composition and potentially functional composition as well. We characterized taxonomic and functional gene composition of soil communities using whole genome shotgun metagenomic sequencing to determine how restored soil communities differed from pre-restoration agricultural soils and original remnant soils, how management affects soil microbes, and whether restoration and management affect the number of microbial genes associated with carbohydrate degradation. We found distinct differences in both taxonomic and functional diversity and composition among restored, remnant, and agricultural soils. Remnant soils had low taxonomic and functional richness and diversity, as well as distinct composition, indicating that restoration of agricultural soils does not re-create soil microbial communities that match remnants. Prescribed fire management increased functional diversity, which also was higher in more recently planted restorations. Finally, restored and post-fire soils included high abundances of genes encoding cellulose-degrading enzymes, so restorations and their ongoing management can potentially support functions important in carbon cycling.

Keywords: carbon cycle; ecosystem restoration; grassland; metagenomics; prescribed fire; soil microbiome

Introduction

Ecosystem restoration practices seek to repair damaged ecosystems by removing and reversing the drivers of ecological degradation that reduce biodiversity and ecosystem functions, including the functions that benefit people as ecosystem services (Suding 2011, Aronson and Alexander 2013). This is an important additional strategy beyond the preservation or sustainable use of undegraded habitats. There is now broad recognition that, in order to achieve these goals, restoration planning and assessment need to include the diverse soil microbial communities (Kardol and Wardle 2010, Yang et al. 2019, Hart et al. 2020, Armbruster et al. 2021) that underlie critical ecosystem functions like nutrient cycling and carbon sequestration (Bardgett and van der Putten 2014, Conant et al. 2017, de Menezes et al. 2017, Mackelprang et al. 2018).

Grassland ecosystems are particularly important targets of restoration worldwide (Török et al. 2021). Covering over a quarter of the global terrestrial landscape and storing one-fifth of the world's soil carbon stocks (Ramankutty et al. 2008, Jansson and Hofmockel 2020), grasslands are an essential natural resource for carbon sequestration (Jones and Donnelly 2004, Lal 2004, Bengtsson et al. 2019). Thus, protection and restoration of grasslands are critical for mitigating the impacts of climate change (Dass et al. 2018, Docherty and Gutknecht 2019). In North America, prior to European colonization the eastern portion of the Great Plain consisted largely of tallgrass prairie habitat shaped by Indigenous stewardship and natural disturbances that included frequent fire and grazing (Kimmerer and Lake 2001, Anderson 2006, McClain et al. 2021). More than 90% of tallgrass prairie has been converted to other land uses, especially agriculture, with remnant prairie existing mostly as small isolated fragments (Samson and Knopf 1994, 1996). Beginning in the 20th century, restoration activities aiming to re-convert agricultural areas into prairie ecosystems have become widespread, commonly through reseeding native plant species and reinstituting disturbances through prescribed fire and other management activities.

Conversion to agriculture has profound effects on soil communities and function. Tillage and long-term fertilization alter soil biodiversity (Coolon et al. 2013, Dai et al. 2018, House and Bever 2018) and physicochemical conditions (Post and Kwon 2000), resulting in soil ecosystems that are significantly different from remnant soils (Fierer et al. 2013). Restoration activities attempt to re-create the conditions in remnant references (McDonald et al. 2016, Gann et al. 2019), but this is challenging and restored soil communities may still differ from remnants decades after the cessation of agricultural disturbances and the re-establishment of native vegetation and historical disturbance regimes (Barber et al. 2017a, Mackelprang et al. 2018, Barber et al. 2023). However, it is less clear if these compositional differences also lead to differences in the functional potential of soil microbial communities, or if functional redundancy among taxa allows similar functions and processes to occur under different microbial compositions. Addi-

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tionally, there is turnover in community composition as different taxa increase or decrease in abundance across a growing season (Barber et al. 2023). These phenological community changes might result in different taxonomic or functional patterns among agricultural, restored, and remnant sites.

The shift from frequent disturbance in agricultural fields to little or no physical disturbance in restored prairie is likely to drive differences observed between these soil conditions. Frequent physical disruption from tillage, pulses of fertilizer, and different crops may drive greater variability among agricultural soils through space and time than among restored soils with infrequent disturbance and primarily perennial plants. This stability in restored (and remnant) prairies may also favor some fungi over bacteria, a pattern which has been observed following agricultural abandonment (van der Wal et al. 2006, Jesus et al. 2016, Mackelprang et al. 2018). Similarly, higher C: N ratio and soil organic matter content both can positively correlate with fungal relative abundance (van der Heijden et al. 2008, Fierer et al. 2009). Within fungi, arbuscular mycorrhizal fungi in particular may benefit from the establishment of perennial grasses and other mycorrhiza-dependent species (Wilson and Hartnett 1998, Mackelprang et al. 2018). If these community changes are associated with differences in function as well, the high plant species diversity, plant biomass, and litter production that results from aboveground growth each year might select for higher abundance and diversity of carbohydrate-degrading genes in soil communities (Guo et al. 2018).

The diversity and complexity of soil microbial communities necessitates molecular methodologies to understand taxonomic and functional community structure. Techniques based on targeted amplicon sequencing provide some information on communities, but inferring potential function from these data is challenging. In this study, we used whole genome shotgun metagenomic sequencing to simultaneously characterize taxonomic and functional composition of soil communities. We compared agricultural, restored, or remnant tallgrass prairie soils sampled at three times during a growing season to answer three questions: (i) How does the taxonomic composition differ among soil microbial communities, and are these differences accompanied by similar variation in functional gene composition? (ii) Does prescribed fire affect soil microbial community composition? (iii) Does restoration increase the number of microbial genes associated with carbohydrate degradation compared to agricultural soils?

Methods

Sites and soil collection

We identified 10 sites within or adjacent to Nachusa Grasslands, a reserve and ecosystem restoration project owned and operated by The Nature Conservancy in north-central Illinois, United States. Nachusa consists mostly of restored and remnant (i.e. never plowed for agriculture) tallgrass prairie, with some wetland and oak savanna habitats present as well. We chose two remnants and six restorations, as well as two agricultural field adjacent to the reserve (Table 1). Using sites that are located in the same area reduces the effects of soil variation, which can influence soil communities, especially in the early years after restoration establishment (Jesus et al. 2016). All ten sites are part of a larger multitrophic monitoring program (Barber et al. 2017a, Guiden et al. 2021, Barber et al. 2023). The restorations were formerly cropland that was seeded with native prairie plant species and, along with the remnants, receive the same management protocols including regular herbicide control of invasive plants and prescribed fire applied approximately every other year (agricultural fields are not burned, while all restored and remnant sites receive prescribed fire). Restorations ranged from 4 to 30 years since seeding, with plant taxonomic diversity generally higher in young restorations but declining over time because not all planted species persist. Plant species in restorations ranged from 31 (in the oldest restoration) to 43 (in the second youngest restoration); species richness in remnants was comparable to the oldest restoration (31–32 species). Soils at all sites are fine-scale mix of sandy and silty loams. Re-introduced American bison (*Bison bison*) have access to five of the six restorations, one of the remnants, and none of the agricultural fields. For more details on site history and management, see Hansen and Gibson (2014) and Bach and Kleiman (2021). We sampled soil from each of the ten sites on 2 May, 8 August, and 13 October 2017 representing early, peak, and late time points in the growing season (spring, summer, and autumn, respectively). Approximately 3 g of soil from the top 5 cm was collected at five points within a 1 ha area, pooled and mixed, and stored at −80°C. Any loose leaf litter, which is usually present in sites that were not burned the previous dormant season but absent in sites that were, was moved aside before sampling soil.

Sequencing and processing

For all 30 samples, we extracted DNA from 0.2 g of soil with the DNeasy PowerSoil DNA Isolation Kit (Qiagen, Venlo, Netherlands). Whole genome shotgun metagenomic sequencing was performed at the U.S. Department of Energy's Joint Genome Institute on the Illumina NovaSeq S4 platform using 300 bp paired-end reads. All genomes are publicly available at JGI IMG under Study ID Gs0144357. Pre-processing, correction, assembly, and annotation was completed by JGI: (i) Reads were pre-processed and corrected using bbduk and bbcms, respectively, from BBTools (Bushnell 2014); (ii) Trimmed and corrected reads were then assembled using metaSPAdes 3.13.0 (Nurk et al. 2017) and mapped using bbmap from BBTools; (iii) Finally, reads were annotated with the IMG Annotation Pipeline v.5.0.3 (Huntemann et al. 2015, Chen et al. 2019) for taxonomic classification and the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) database (Kanehisa et al. 2016) for functional annotation. Importantly, functional annotation indicates potential function rather than measures of actual function in soils. Although we use terms like "functional diversity" and "functional composition," these should be interpreted as descriptors of functional potential. Prior to statistical analyses, features present in 10% or less of all samples were removed to reduce data sparsity. To create taxonomic and functional community feature tables, we excluded all sequences assigned to lineage or functional ortholog with under 55% identity or e-values below 0.0001 and summed the remaining assigned sequences within each sample.

We calculated rarefied richness, rarefied Shannon diversity, and rarefied Pielou's evenness for each sample using rrarefy(), specnumber(), and diversity() in the "vegan" package of R (Oksanen et al. 2013) for either assigned lineages (OTUs) or functional orthologs (KO identities), rarefied to the level of the sample with the lowest count. For OTUs, we restricted analyses to archaea, bacteria, and fungi. To compare taxonomic and functional composition among samples, we used the same community feature tables as above and applied a centered log-ratio (clr) transformation to account for the compositional nature of the data. From the transformed value, we then calculated pairwise Euclidean distances

Table 1. Sampled sites with type, age (years since planting in 2017 when sampling occurred) for restorations, and fire (site burned in the previous dormant season). All sites were sampled in May, August, and October.

Site name	Site type	Restoration age	Fire
HF	restoration	4	Y
HN	restoration	5	Y
CCW	restoration	9	N
HLP	restoration	17	N
WH	restoration	25	Y
MU	restoration	30	N
SOY	agriculture	–	–
AG	agriculture	–	–
MR	remnant	–	Y
TCR	remnant	–	N

using vegdist() in the "vegan" package to estimate dissimilarity (beta diversity) among each pair of samples.

Taxonomic and functional alpha and beta diversity

To address whether there are taxonomic or functional differences in microbial communities among site types (Question 1), we used the three alpha diversity measures (richness, diversity, and evenness) and the taxonomic and functional distances calculated from clr-transformed data. For each of the three alpha diversity measures, we analyzed differences among the three site types (agricultural fields, restorations, and remnants) using linear mixed models with site type and season as fixed factors and site as a random factor using lmer() in the "lme4" package (Bates et al. 2015). Fixed factors were evaluated using type II Wald χ^2 tests with Anova() in the "car" package (Fox and Weisberg 2019), and post-hoc Tukey tests were performed with emmeans() in the "emmeans" package (Lenth 2021). Next, we analyzed both taxonomic and functional distances with perMANOVA, using adonis() in the "vegan" package to determine if composition differed among the three site types, including site type and season as factors. We visualized these differences with nonmetric multidimensional scaling (NMDS).

To identify the taxa and functional orthologs driving differences in taxonomic and functional composition, respectively, we performed indicator species analysis using volcano plots to visualize differential representation among site types. We used t-tests to identify the taxa and functional orthologs that differed significantly in relative abundance between each treatment comparison. To account for multiple comparisons, we adjusted the p-values for false discovery rate using the p.adjust() function in the "stats" package, specifying method as "fdr." We then calculated the log2 fold change in relative abundance between each treatment comparison and used log2 fold change ≥ 2 or ≤ −2 and $P_{adj} < 0.05$ as significance thresholds to identify taxa and orthologs significantly over- or underrepresented in (i) restored vs. remnant sites, (ii) restored vs. agricultural sites, and (iii) remnant vs. agricultural sites. We examined these indicator taxa to determine taxonomic groups that frequently characterized one site type or distinguished it from other types.

Fire effects and restoration characteristics

Next, we determined if restoration characteristics, including recent fire, influenced microbial communities (Question 2). To analyze alpha diversity, we again used linear mixed models, with restoration age, prescribed fire, and season as fixed factors and site ID as a random factor. Age was the number of growing seasons since the site was seeded, and fire was a binary variable indicating whether the site was burned in the preceding dormant season. Fixed factors were evaluated as described above. Then we analyzed the composition of restorations only, again with perMANOVA, using restoration age, prescribed fire, and season as factors and visualized results with NMDS. To determine which taxa and functional orthologs drove fire impacts on diversity and composition, we repeated the indicator species analysis as above, but just comparing burned vs. unburned restored sites.

Carbohydrate degradation and nitrogen cycling

We used functional ortholog annotations to determine if the abundance of genes involved in carbohydrate degradation changed following restoration or with site characteristics (Question 4). We searched KEGG orthology for cellulose-related enzymes (beta-glucosidase, beta-cellobiosidase, endoglucanase, cellulase), which were mainly located within 09101 (Carbohydrate metabolism), pathway 00500 (Starch and sucrose metabolism). For each identified ortholog, we divided the count of sequences identified in each sample by the total number of categorized ortholog counts in the sample, and expressed the counts as gene counts per 10^6 sequences. Total cellulose-degrading genes was analyzed using linear mixed effect models following the same approach described for alpha diversity measures above, except we included agricultural sites in the site characteristics analysis, with age equal to zero, because it did not differ from restoration in the site type model.

Because we identified several indicator taxa that may play a role in nitrogen cycling (see Results), we also carried out a *post hoc* analysis of genes involved in nitrification (00910 Nitrogen metabolism, Modules M00528 and M00804) and nitrogen fixation (M00175). As with the carbohydrate degradation gene analysis, we standardized and summed all orthologs associated with these N pathways, although patterns generally followed the abundance of the two most common orthologs, the two subunits of nitrate reductase/nitrite oxidoreductase. We analyzed standardized gene counts for differences among site types or restoration characteristics, as described for carbohydrate degradation genes.

Results

Taxonomic and functional alpha and beta diversity

After filtering, all metagenomes combined included 19243883 reads assigned to taxa (mean per sample = 641463, range = 237573–1220646) and 10156357 total identified orthologs (mean = 338545, range = 126380–645082) (Table S1). Microbial taxonomic richness differed among site types, with remnant soils significantly lower in richness than restored soils and agricultural soils intermediate (Table 2, Fig. 1). The same general pattern was present for taxonomic Shannon diversity and evenness, which was highest in summer and lowest in autumn. Functional alpha diversity followed similar patterns, with all three measures significantly affected by site type, and values lowest in remnant soils and highest in restored soils. Both taxonomic and functional beta diversity differed significantly among the three site types, but there were no consistent differences among seasons when considering all sites (Table 3, Fig. 2A and C). That is, there were distinctive compositions for agricultural fields, restorations, and remnants. Because of concerns that removing features present in 10% or less of all samples might be more likely to drop taxa or orthologs specialized to lower-replication remnant or agricultural sites, we repeated both taxonomic and functional alpha diversity analyses without dropping the <10% samples, but all of the diversity patterns described above were qualitatively the same (results not shown).

Indicator species analysis identified both taxa and orthologs that distinguished site types (Figs. 3A, Tables S2–S4). A higher number of taxa distinguished remnant sites from both restorations (469 taxa) and agricultural fields (407 taxa) than taxa differing between restorations and agriculture (86 taxa), which corresponds to the distinct remnant compositions shown in Fig. 2A. Several bacterial and archaeal lineages tended to distinguish each site types, either having many OTUs identified or particularly large differences in abundance of particular OTUs between the site types under comparison (Table 4). Few fungi were identified as indicator taxa, except families within the Ascomycota (classes Dothideomycetes and Leotiomycetes) that were more abundant in restorations. Similarly, there were more functional orthologs distinguishing remnant sites from restorations (160) and from agricultural fields (204), while only 46 orthologs differed between restorations and agricultural fields (Fig. 3B).

Fire effects and restoration characteristics

Within restorations, prescribed fire reduced taxonomic richness but increased all three functional measures (Fig. 4). Functional Shannon diversity and evenness both declined with restoration age, in contrast to taxonomic measures that were unaffected by age or, in the case of evenness, marginally increased with age. Taxonomic and functional composition also differed significantly with prescribed fire and restoration age, and there were marginally significant differences associated with season (Fig. 2B, D).

Indicator species analysis revealed 9 taxa that had significantly higher abundance in burned sites, and 40 taxa that were more abundant in unburned sites (Fig. 3A). Of these 40 taxa, 45% were Firmicutes, particularly members of Bacillales and Clostridiales. There were 8 orthologs more abundant in burned restorations, and 9 more abundant in unburned restorations (Fig. 3B).

Carbohydrate degradation and nitrogen cycling

Cellulose degrading enzyme genes were significantly more abundant in restorations compared to remnants, with agricultural sites intermediate ($\chi^2 = 16.50$, df = 2, $P < 0.001$) (Fig. 5A). These genes were also significantly more abundant in burned sites than unburned sites ($\chi^2 = 15.74$, df = 1, $P < 0.001$) (Fig. 5B) but did not differ with site age or season. Nitrification and nitrogen fixing genes were significantly more abundant in agricultural sites than in either other site type ($\chi^2 = 26.08$, df = 2, $P < 0.001$), and they declined in abundance with restoration age ($\chi^2 = 9.53$, df = 1, $P = 0.002$) but did not differ with prescribed burning or season.

Discussion

We used shotgun metagenomic sequencing to examine soil microbial communities in agricultural fields, remnant tallgrass prairies, and former agricultural fields restored to prairie ecosystems. We found distinct differences in both taxonomic and functional diversity and composition among the three site types. Remnant soil tended to have low levels of richness and diversity, from both taxonomic and functional perspectives, particularly compared to highly diverse restored soils. Prescribed fire also influenced communities, especially functional richness, diversity, and evenness, which were significantly increased by fire in prairie restorations but tended to decline as restorations became older. These distinct communities in remnants and post-fire soils were characterized by a variety of taxonomic groups. Finally, restored and post-fire soils also included high abundances of genes encoding cellulose-degrading enzymes.

Soil microbial communities in restorations generally had high diversity, especially compared to low-diversity remnant prairies. These results correspond to the hump-shaped pattern of taxonomic bacterial diversity following restoration in a larger set of prairie restorations at the same site (Barber et al. 2023), as well as studies in other systems that have found increased soil microbial diversity compared to unrestored or remnant soils (Turley et al. 2020, but see Hui et al. 2018). Although agricultural soils are moderately diverse, the process of restoration supports highly rich and even communities that later decline in diversity. High microbial diversity in young restorations could be a result of both the remaining elevated nutrient levels caused by fertilization in the former agricultural fields and the high plant diversity of these sites, which provides a variety of organic litter inputs. The lower soil nitrogen (Baer et al. 2002) and decline in plant richness over time that are consistently seen in tallgrass prairie restorations (Klopf et al. 2017, Bach and Kleiman 2021) may limit community membership to oligotrophic microbes that associate with slow-degrading carbon substrates. Our analyses support this, with remnant soils that are not just lower in diversity but distinctly different in taxonomic composition from both pre-restoration agricultural soils and those of the restorations themselves. Plant species richness in the remnants is lower than all but the oldest restorations.

Unlike previous analyses of these soils that used targeted amplicon sequencing to focus on bacterial taxonomic diversity and composition (Barber et al. 2017a, 2023), whole genome shotgun metagenomic sequencing employed in this study allows us to examine differences in microbial functional potential. We find that functional characterizations of restored soil communities largely reflect the taxonomic characterizations: restored soils are functionally rich, even, and diverse compared to both agricultural and remnant soils, and composition among the three site types differ. That is, rather than just providing functional redundancy by additional lineages, restored soils had a larger number and diversity of identified functional orthologs, which again may reflect the range of organic inputs provided by high plant diversity. Some of this added functional capacity may also be due to higher abundances of fungi, including some Ascomycota that were more abundant in restorations (Treseder and Lennon 2015, Frąc et al. 2018). The low functional richness of agricultural soils is not unexpected, given the homogeneity (through both space and time) of farm fields that are highly cultivated and planted with a rotation of just two crops (Verbruggen and Kiers 2010).

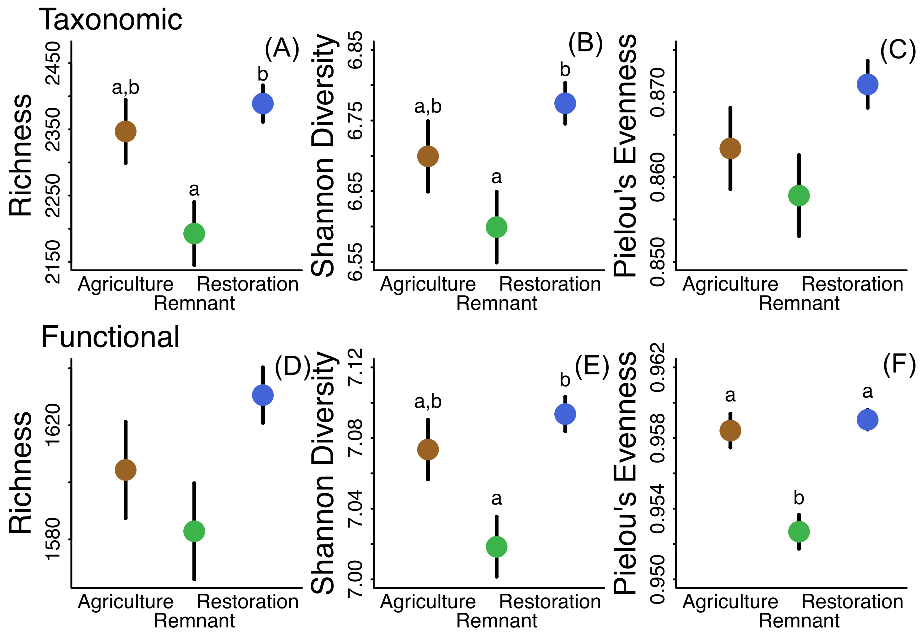

Figure 1. Taxonomic (A–C) and functional (D–F) alpha diversity measures by site type. Values are estimated marginal means ± 1 s.e. Letters indicate significant differences from Tukey post-hoc tests at $P < 0.05$.

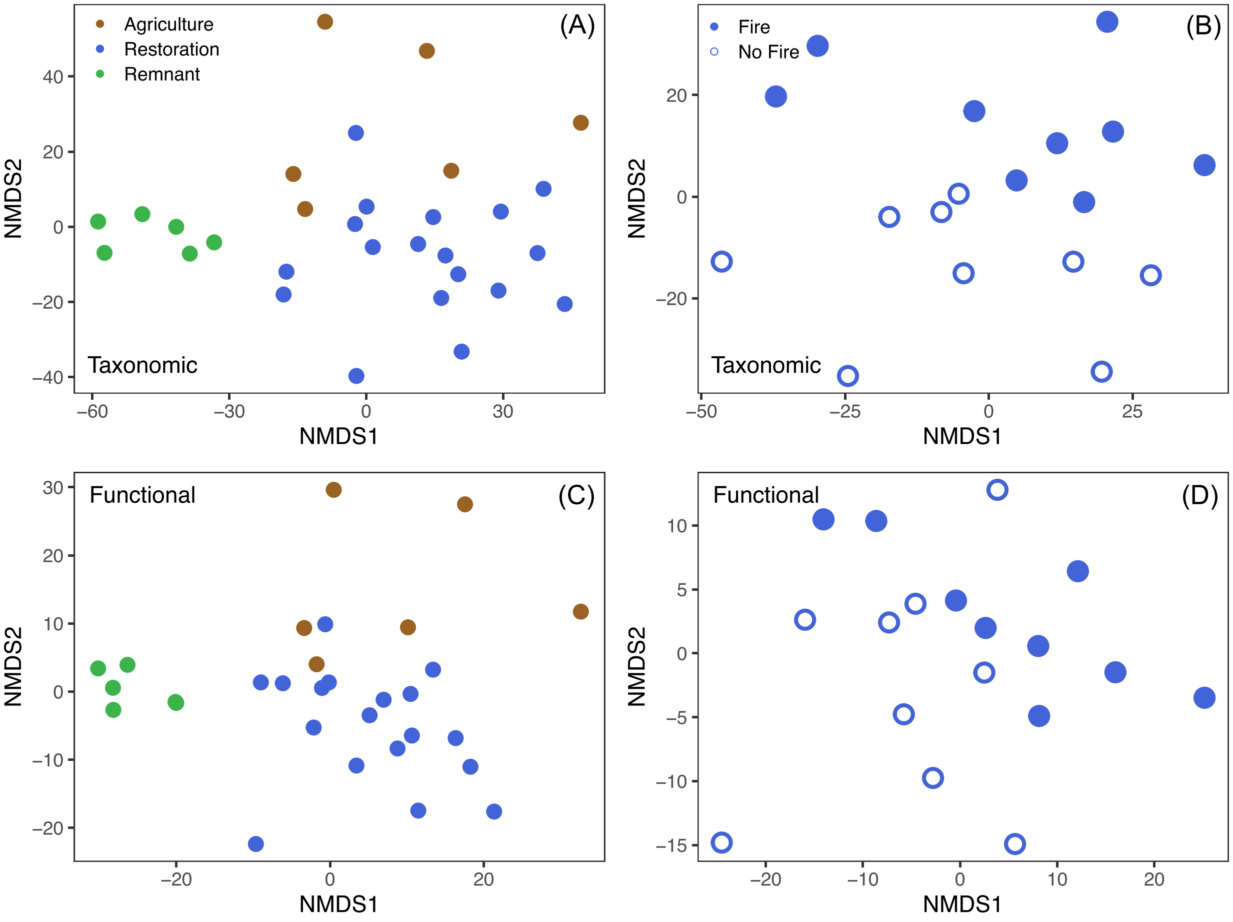

Figure 2. NMDS ordinations illustrating taxonomic (A–B) and functional (C–D) composition by site type or fire status. Fire indicates sites that received prescribed fire in the previous dormant season. For all ordinations, stress < 0.18.

Table 2. Results of linear mixed models analyzing taxonomic and functional alpha diversity measures.

	Richness		Shannon diversity		Pielou's evenness	
	χ^2	P	χ^2	P	χ^2	P
Taxonomic						
Site type	**18.09**	**<0.001**	**13.41**	**0.001**	**9.06**	**0.010**
Season	2.23	0.329	**6.69**	**0.035**	**10.52**	**0.005**
Age	1.30	0.255	2.55	0.110	2.95	0.086
Fire	**5.54**	**0.019**	0.26	0.612	0.00	0.972
Season	2.23	0.328	4.01	0.135	3.94	0.139
Functional						
Site type	**9.42**	**0.009**	**21.13**	**<0.001**	**47.98**	**<0.001**
Season	1.77	0.413	0.34	0.843	0.25	0.883
Age	3.04	0.081	**4.20**	**0.040**	**5.16**	**0.023**
Fire	**10.72**	**0.001**	**9.92**	**0.002**	**6.76**	**0.009**
Season	0.66	0.721	0.282	0.868	2.41	0.300

Note: df for Site type & Season = 2; df for Fire & Age = 1.

Table 3. Results of PERMANOVAs examining taxonomic and functional beta diversity.

	Taxonomic			Functional		
	F	P	R^2	F	P	R^2
Site type	**4.70**	**<0.001**	**0.26**	**5.87**	**<0.001**	**0.30**
Season	1.08	0.279	0.06	1.06	0.325	0.05
Fire	**1.78**	**0.004**	**0.09**	**2.12**	**0.005**	**0.10**
Age	**2.04**	**<0.001**	**0.11**	**2.56**	**0.001**	**0.13**
Season	1.25	0.051	0.13	1.33	0.062	0.13

Note: df for Site type & Season = 2,25; df for Fire & Age= 1,13; df for Season = 2,13.

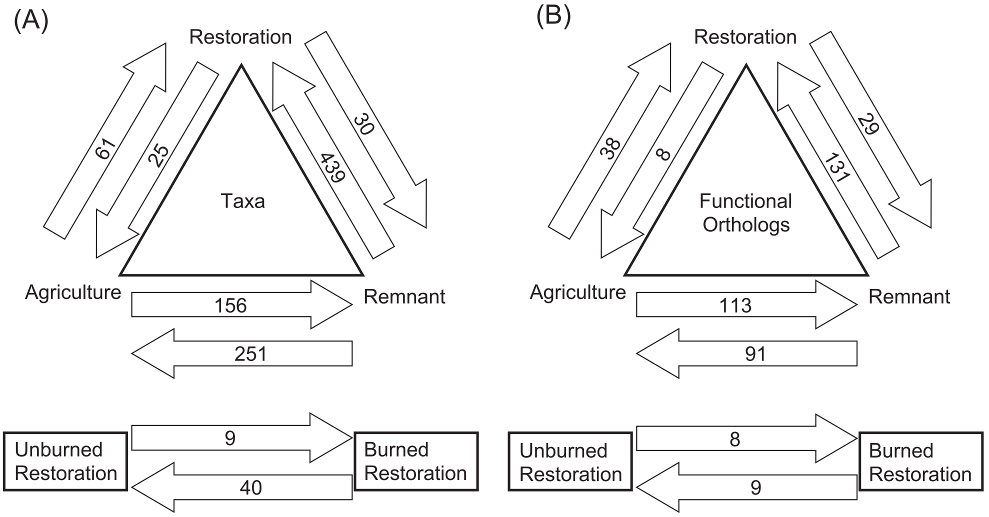

Figure 3. Number of indicator (A) taxa and (B) functional orthologs per site type comparison, identified by indicator species analysis. Direction of arrow gives the site type with higher abundance, and the number in each arrow is the number of taxa or functional orthologs that were more abundant in that site type.

Management effects and outcomes

Restored and remnant tallgrass prairies are commonly managed using prescribed fire—indeed, in the absence of fire, these habitats can transition to ecosystems dominated by woody plants and cease to be prairies (Bond and Keeley 2005, Nippert et al. 2021). At Nachusa Grasslands, all prairies receive prescribed fire during the dormant season approximately every two years, although the exact schedule varies depending on annual weather conditions. Taxonomic richness was slightly lower in sites that received fire in the previous winter or spring, compared to restorations that had not received fire in at least a year, but the composition of these soils was distinct. In contrast, functional alpha diversity was consistently higher in burned soils, but functional composition was less distinct. Together, these results signify a community shift, in which fire shapes microbial communities where a smaller number of distinct taxa remain but possess a higher diversity of func-

Table 4. Bacterial and archaeal lineages distinguishing between site types based on indicator species analysis. “Indicator” identifies the site type(s) distinguished by those lineages.

Archaea **Phylum**	**Family**	**Genus**	**Indicator**
Thaumarchaeota	Nitrososphaeraceae	Nitrosocosmicus	agriculture
Bacteria **Phylum/Class**	**Family**	**Genus**	**Indicator**
Actinomycetota	Mycobacterieaceae	Mycobacterium	restoration & remnant
	Streptomycetaceae	Streptomyces	restoration & remnant
Bacteroidota	Chitinophagaceae	Chitinophaga	restoration & agriculture
	Sphingobacteriaceae	Pedobacter	restoration & agriculture
	Flavobacteriaceae	Flavobacterium	restoration
Alphaproteobacteria	Bradyrhizobiaceae	Bradyrhizobia	remnant
	Rhizobiaceae	Rhizobia	remnant
	Erythrobacteraceae	Erythrobacter, Altererythrobacter	agriculture
	Sphingomonadaceae	Sphingomonas, Sphingopyxis	agriculture
Betaproteobacteria	Comamonadaceae	Acidovorax, Diaphorobacter	restoration & agriculture
	Methylophilaceae	Methylopumilus, Methylobacillus, Methylophilus, Methylotenera, Methylovorus	restoration & agriculture
	Nitrosomonadaceae	Nitrospira	restoration & agriculture
Gammaproteobacteria	Xanthomonadaceae	Arenimonas, Luteimonas, Lysobacter, Pseudoxanthomonas, Stenotrophomonas, Xanthomonas	restoration & agriculture
	Pseudomonadaceae	Pseudomonas	restoration & agriculture
Verrucomicrobiota	(multiple families)	–	remnant
Fungi **Phylum**	**Family**	**Genus**	**Indicator**
Ascomycota	(multiple families)	–	restoration

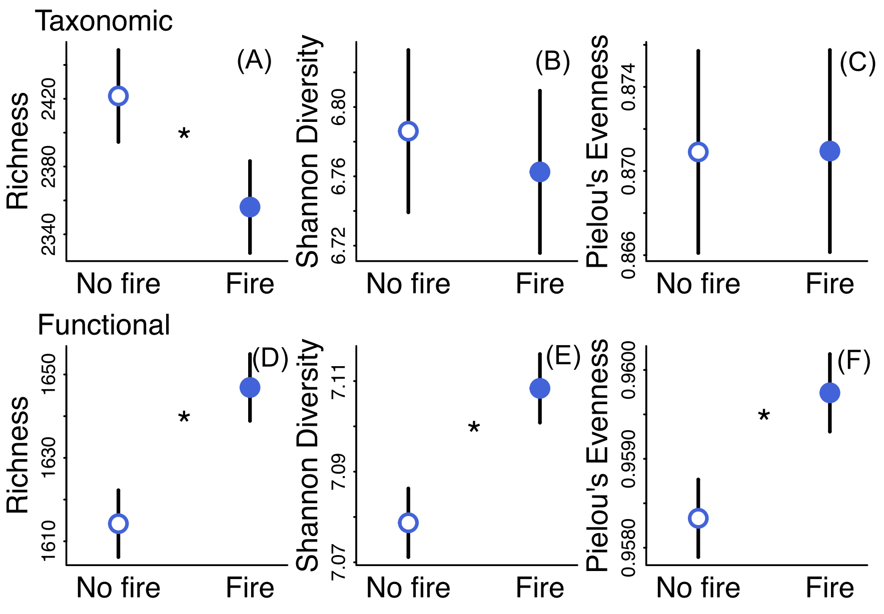

Figure 4. Taxonomic (A–C) and functional (D–F) alpha diversity measures by fire status. Fire indicates sites that received prescribed fire in the previous dormant season. Values are estimated marginal means ± 1 s.e. Asterisks indicate significant differences at $P < 0.05$.

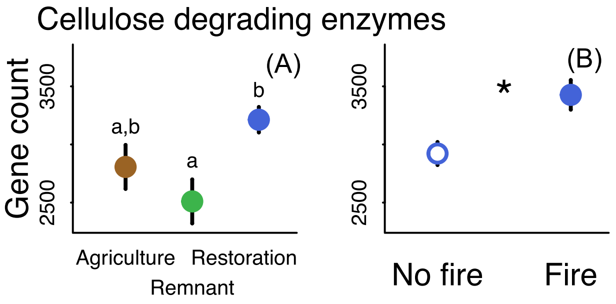

Figure 5. Cellulose degrading enzyme gene counts by (A) site type and (B) fire status. Gene counts are expressed as counts per 10^6 sequences. Values are estimated marginal means ± 1 s.e. Letters in (A) and asterisk in (B) indicate significant differences at $P < 0.05$.

tional capacities, including more genes for enzymes associated with carbohydrate-degradation (see below). In some ways, this result is surprising because unburned sites have large amounts of litter from the previous growing season that is mostly eliminated by fire. Eliminating these diverse organic inputs might be expected to reduce functional diversity, but their combustion may also increase accessible organic materials for microbes through inputs of dissolved organic C into the soil. In forest soils with longer fire return intervals, Dove et al. (2022) found that the relative abundance of genes that encode for carbohydrate-active enzymes, particularly cellulolytic genes, were highest shortly after fires and decreased over following decades. Although fire cycles are much more rapid in grasslands, a similar process may be selecting for these carbon acquisition strategies that are beneficial in soils that tend to be low in C after fires.

If a goal of ecosystem restoration is to re-create the conditions in reference remnant sites (McDonald et al. 2016), then our results indicate restorations are only partially successful: over time, soil microbial diversity declines, and composition becomes more similar to remnants (from both taxonomic and functional perspectives), but remnants remain distinct from even the oldest restorations, which were removed from agricultural cultivation and planted with a diverse native plant community 30 years before. This is also apparent in the high number of taxa and functional orthologs identified by indicator species analysis as being significantly more or less abundant in remnants compared to both restorations and agriculture. Nonetheless, remnant soils may not always be appropriate references for ecosystem restoration. Areas remaining as remnant habitats were often avoided for cultivation specifically because they were different; in tallgrass prairies, including at Nachusa, remnant sites are generally rocky “knobs” where the underlying sandstone is near the surface and soils would be challenging to plow (Taft et al. 2006). These edaphic differences likely contribute to the distinct microbial communities in remnants (Barber et al. 2017a, 2023), making direct assessments of restoration progress difficult without comparable pre-agricultural conversion soils. Unfortunately, the nearly complete conversion of tallgrass prairie in northern Illinois to rowcrop agriculture means there may not be any appropriate remnants with which to compare restorations. Restoration goals may need to be defined relative to starting conditions at the intended restoration site rather than as a comparison to existing references (Higgs et al. 2014).

Characteristic taxa

The lineages identified as indicator taxa may reveal some of the causes of diversity and composition differences among site types and following prescribed fire. Several families and genera had either many identified OTUs or showed particularly strong differences in the abundances of specific OTUs. For example, members of the archaeal family Nitrosphaeraceae, particularly in the genus *Nitrocosmicus*, were more abundant in agriculture, while nitrogen-fixing Alphaproteobacteria, *Rhizobia* and *Bradyrhizobia*, were common in prairie remnants. These are likely due to important differences in N cycling between agricultural sites, which receive regular inputs of high-N fertilizer for corn production, and remnant sites where both native perennial legumes in the family Fabaceae, as well as leguminous weeds like clovers (*Trifolium* spp.) and sweet-clovers (*Melilotus* spp.), are common (Mackelprang et al. 2018). *Nitrosocosmicus* and other Thaumarchaeota are widespread ammonia-oxidizing archaea and important contributors to nitrification (Alves et al. 2018) and have been found in other agricultural soils and where ammonia fertilizer is applied (Kim et al. 2012, Carey et al. 2016). Their lower abundance in restored and remnant soils probably reflects the rapidity with which accessible nitrogen leaves soils following the cessation of fertilization, as well as the lower pH of prairie soils compared to these agricultural soils (Barber et al. 2023), as high pH has increased relative abundance and diversity of some ammonia-oxidizing archaea groups (Gubry-Rangin et al. 2011). In support of this, we also found higher abundance of genes for enzymes involved in nitrification in agricultural sites than in restored or remnant prairie soils, and a general decline in the abundance of these genes as time since restoration increases. These same patterns may also explain the high abundances of multiple genera of ammonia-oxidizing Nitrosomonadaceae in our, and others’, agricultural samples (Mackelprang et al. 2018), although many were also relatively abundant in restored soils as well.

Restoration soils had a mix of shared taxa with both agricultural and remnant sites, although they were also typified by distinct *Flavobacterium,* Betaproteobacteria in the families Comamonadaceae, and several fungal families in Ascomycota. Interestingly, *Flavobacterium* (phylum Bacteroidota) were particularly enriched in earthworm casts in agricultural and prairie soils (Schlatter et al. 2019), and Wodika et al. (2014) found that earthworms abundance at Nachusa increased after restoration of agricultural soils, but they also found higher earthworm abundances in remnant prairies. Ascomycota have high relative abundance in grassland soils worldwide, and their diversity positively correlates with plant diversity (Prober et al. 2015), so the high plant richness in restorations may support these fungi that rely on diverse organic materials provided by litter and root exudates (Millard and Singh 2010). The higher abundance of cellulose-degrading enzyme gene orthologs in restored prairie soils compared to agricultural and remnant soils, could be due in part to this higher plant diversity in restorations. Although restored prairies often initially support high plant richness and diversity, these values generally decline over time as older restorations become dominated by warm-season C_4 grasses (Sluis 2002, Hansen and Gibson 2014, Barber et al. 2017b). Higher plant diversity (due to both high richness and evenness) may support abundant and diverse microbes through more diverse litter inputs and root exudates.

In addition to high relative abundances of nitrogen-fixing bacteria, remnants were also characterized by several families belonging to the phylum Verrucomicrobiota, which can dominate mature tallgrass prairie soils that are low in nutrients (Bergmann et al. 2011, Fierer et al. 2013). A class-level examination of restored Nachusa soils showed an increase in relative abundance with age in Verrucomicrobiota, particularly Spartobacteria, suggesting that restoration and management practices move post-agricultural soil communities closer to remnant targets (Barber et al. 2017a), but our results indicate that remnant soils remain distinct, and restoration is incomplete. A comparison of pristine calcareous grasslands to arable land and restorations in southern England also identified many of the same genera as indicators of pristine grassland habitats as in our analysis (Armbruster et al. 2021): *Xiphinemobacter* (Verrucomicrobiota); *Bradyrhizobia, Rhodoplanes,* and *Mesorhizobia* (Alphaproteobacteria); and *Mycobacterium* (Actinobacteria). Together, these lineages may help to define "old growth" grassland soil assemblages that could potentially serve as metrics to evaluate long-term restoration success, as suggested by Armbruster et al. (2021).

Conclusions

Overall, we find distinct communities in agricultural, restored, and remnant prairie soils that differ both taxonomically and in functional potential. When prescribed fire is applied to prairie ecosystems, it shows marked effects on taxonomic composition and, especially, functional diversity, producing microbial communities with increased abundance of genes associated with carbohydrate degradation. Nonetheless, these findings underscore the challenges of effective ecosystem restoration, in which efforts to reproduce the composition, structure, and function of less-degraded reference communities and ecosystems are often incomplete. Relevant reference sites may not be available, so restoration practitioners will need to set site-specific benchmarks. Determining if the putative functional differences we show here correspond to actual differences in C and N fluxes would be an important first step, combined with more detailed analysis of which restoration characteristics and management actions could support desirable functions.

Author contributions

Cayla N. Mason (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing), Shayla Shahar (Data curation, Formal analysis, Writing – review & editing), Kendall K. Beals (Data curation, Formal analysis, Writing – original draft, Writing – review & editing), Scott T. Kelley (Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Supervision, Writing – review & editing), David A. Lipson (Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing), Wesley D. Swingley (Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing), and Nicholas A. Barber (Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing)

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Supplementary data

Supplementary data is available at *FEMSEC Journal* online.

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