

Ecosystem recovery from chronic fertilization: Biotic mechanisms underpinning soil nitrogen legacies in burned and unburned grasslands

by

Matthew Aaron Nieland

B.S., Morningside College, 2018

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Abstract

Nitrogen (N) is an essential element for life. Though most forms of N cannot be used by biota, humans have transformed the global N cycle and enriched terrestrial ecosystems with bioavailable N. Since soil microorganisms, plants, and soil organic matter all have incredible capacity to retain N from exogenous inputs, N accrued from the past can persist as a N source to support the functions of future biota once exogenous input ceases. This N legacy may cause ecosystem recovery from higher N conditions to lag, or it may lead to asynchronous recovery of ecosystem N pools and fluxes. Moreover, fire is a key driver of N dynamics in mesic grasslands, and it may shape responses to past and current input of N by combusting exogenous N in aboveground litter. Therefore, the overarching goal of this dissertation is to assess how fire affects plant and microbial mechanisms of ecosystem N cycling and loss after the cessation of decades of chronic N input. To address this goal, I measured N availability, soil microbial community structure and function, and plant and soil N and carbon (C) stocks in a long-term N fertilization and cessation experiment crossed with different fire treatments at the Konza Prairie Biological Station, KS, USA.

Before cessation of fertilization, N-addition and fire suppression both independently decreased soil microbial enzymatic investment for N relative to C, indicating reduced microbial N-limitation, and this response was connected to substrate availability feedbacks, rather than life history tradeoffs or pH-related suppression of activity. In the first year after ceasing N-fertilization, soil N availability, measured as resin-sorbed N, dropped by 86%, nitrification potential recovered more than denitrification potential, and key microbial N-cycling populations were differentially sensitive to fertilization, together suggesting that microbial N removal and fire could be equally important mechanisms of ecosystem recovery in unburned and burned

prairies, respectively. Three years after cessation of N-fertilization however, nitrification potential recovered in burned prairies only, while denitrification potential recovered by the fourth year in both fire treatments. Five years after ceasing N-fertilization, while soil N stocks in previously fertilized prairies were recovering in both fire treatments, soil microbial community structure did not recover in either fire treatment, and aboveground net primary productivity remained elevated in burned prairies. Plant shoot C:N ratios recovered in both fire treatments, and root C:N ratios recovered in burned prairies, but soil C:N ratios did not recover, suggesting different mechanisms support plant, soil, and soil microbial recovery.

Collectively, while ecosystem attributes in burned and unburned prairies are not recovering similarly because of contrasting plant and soil mechanisms, loss of the soil N accumulated over decades of fertilization is happening at similar rates in both fire treatments, with fire and nitrate leaching the likely respective loss pathways in burned and unburned prairies. Overall, this dissertation shows that ecosystem N pool and flux responses to cessation of N eutrophication are caused by different feedbacks driven by fire history, which have large-scale consequences to N loss during ecosystem recovery; and that N-fertilization legacies in plant and microbial community structure can persist even as ecosystem function recovers.

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Approved by:

Major Professor
Dr. Lydia Zeglin

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Table of Contents

List of Figures	x
List of Tables	xiv
Acknowledgements	xv
Chapter 1 - Introduction	1
1.1 References	9
Chapter 2 - Product-inhibition feedbacks, not microbial population growth tradeoffs or soil pH, regulate decomposition potential under nutrient eutrophication	14
2.1 Abstract	14
2.2 Introduction	15
2.3 Methods	19
2.4 Results	23
2.5 Discussion	27
2.6 References	36
Chapter 3 - Differential resilience of soil microbes and ecosystem functions following cessation of long-term fertilization	49
3.1 Abstract	49
3.2 Introduction	50
3.3 Materials and Methods	54
3.4 Results	59
3.5 Discussion	64
3.6 Conclusion	73
3.7 References	75
Chapter 4 - Biotic feedbacks drive soil nitrogen legacies in burned and unburned grasslands	91
4.1 Abstract	91
4.2 Introduction	92
4.3 Methods	96
4.4 Results	100
4.5 Discussion	103
4.6 Conclusion	113

4.7 References	115
Chapter 5 - Limited recovery of soil microbial communities reveals fertilization legacy in a burned and unburned grassland	128
5.1 Abstract	128
5.2 Introduction	129
5.3 Methods.....	133
5.4 Results	139
5.5 Discussion	141
5.6 References	146
Chapter 6 - Conclusions.....	159
6.1 References	165
Appendix A - Supplementary tables and figures for Chapter 2	167
Appendix B - Detailed methods for Chapter 3	176
Appendix C - Supplementary tables and figures for Chapter 3	187
Appendix D - Supplementary tables and figures for Chapter 4.....	193
Appendix E - Supplementary tables and figures for Chapter 5	201

List of Figures

- Figure 2-1. Log response ratios (LRRs) of extracellular enzyme activities from fertilization separated by fertilization (+N, N-alone; +P, P-alone; +N+P, N- and P-fertilized) and fire treatments. Each point is a LRR for the fertilization treatment within each experimental block at one sample month. Asterisks above boxplots indicate statistical difference from zero at the $*P \leq 0.05$, $**P < 0.01$, and $***P < 0.001$ thresholds..... 46
- Figure 2-2. Relative soil microbial demand for C:N, C:P, and N:P during the study time course summarized by boxplots. Each point is the ratio between C-, N-, and P-acquiring enzymes for each plot corresponding to the fertilization treatment (+N, N-alone; +P, P-alone; +N+P, N- and P-fertilized), represented by color. Capital letters within boxplots or near mean values for fire treatments indicate group differences at $P \leq 0.05$ 47
- Figure 2-3. Log response ratios (LRRs) of microbial phyla and classes predicted to be copiotrophs or oligotrophs, separated by fertilization (+N, N-alone; +P, P-alone; +N+P, N- and P-fertilized) and fire treatments. Each point is a LRR for the fertilization treatment within each experimental block at one sample month. Asterisks above boxplots indicate statistical difference from zero at $*P \leq 0.05$, $**P < 0.01$, and $***P < 0.001$ thresholds. Labels on x-axis are shortened for clarity [Actino, Actinomycetota (formerly Actinobacteria); Alpha, Alphaproteobacteria; Gamma, Gammaproteobacteria; Nitroso, Nitrososphaera (formerly Thaumarchaeota); Acido, Acidobacteriota (formerly Acidobacteria); Delta, Deltaproteobacteria; Planco, Planctomycetota (formerly Planctomycetes); Verruco, Verrucomicrobiota (formerly Verrucomicrobia)]. 48
- Figure 3-1. Our conceptual model of tallgrass prairie ecosystem sensitivity and resilience to long-term nitrogen (N) fertilization under contrasting fire treatments. Ecological states may change proportionally with increasing N availability. Therefore, the sensitivity of ecological states to annual fertilization (height of the shaded box) can increase independently in unburned prairies because of the higher baseline level of ecosystem N. Following the cessation of fertilization, the magnitude of recovery (difference between fertilized and recovering prairies) is hypothesized to depend on the prairie's fire treatment. We predict that ecological states of burned prairies will recover quicker than unburned prairies..... 86

Figure 3-2. Estimated concentrations of (a) $\text{NH}_4^+\text{-N}$ and (b) $\text{NO}_3^-\text{-N}$ sorbed to resin bags buried in soils under contrasting fertilizer treatments and fire regimes. The height of the bars (mean $\pm$ standard error [SE]) correspond to the plot and subplot means for each treatment combination and each point representing the individual plot and subplot concentration means during the course of the growing season. Tukey's honest significant difference (HSD) post-hoc analysis results are shown with different letters indicating field treatment groups that differed at the $P < 0.05$ level.	87
Figure 3-3. Measured (a) nitrification potential (NP) and (b) denitrification potential (DNP) rates of the soils from contrasting fertilizer and fire treatments. Bar heights (mean $\pm$ SE) are the means of the NP and DNP rates from each plot and subplot at each month with the points showing the monthly average of each (sub)plot. Tukey's HSD post-hoc analysis results are shown with dissimilar letters indicating fertilization treatment groups that differed at the $P < 0.05$ level. Both NP and DNP rates were affected by the burning treatment, but no interaction between fire and fertilization.	88
Figure 3-4. Gene abundance of (a) archaeal and (b) bacterial <i>amoA</i> , (c) clade I and (d) clade II (<i>A. dehalogenans</i>) of <i>nosZ</i> , and (e) bacterial 16S rRNA in soils with contrasting treatments of burning and fertilization. The bar height (mean $\pm$ SE) represents the mean gene abundance at each plot and subplot for each given treatment combination during the span of the growing season. Points are the monthly average for each (sub)plot. Tukey's HSD post-hoc analysis results are shown with dissimilar letters indicating fertilization treatment groups that differed at the $P < 0.05$ level.	89
Figure 3-5. Yearly variation of aboveground net primary productivity (ANPP), estimated from end-of-growing season biomass, between 2013-2017 of (a) grasses, (b) forbs, and (c) woody plants. Data are shown as boxplots with the central line representing the median, and the upper and lower quartiles being the lines above and below the median, respectively. The lines extending from the quartiles end at the upper and lower fences of the data distribution. Yearly plot means are shown as points; each color represents a different year. Tukey's HSD post-hoc analysis results are shown with dissimilar letters indicating Fertilizer $\times$ Burn treatment groups for grasses and forbs, and burning treatment groups for woody plants, that differed at the $P < 0.05$ level.	90

Figure 4-1. Total available inorganic N extracted from surviving resin bags during the growing season in 2017 and 2019-2021 (a, b), and nitrification potential (c, d) and denitrification potential (e, f) rates estimated twice during the growing season in 2017-2021, for both burned and unburned prairie treatments. Each point is the average annual value for the fertilization treatment, and the error bars are ± 1 standard error (SE). Letters denote significant differences between fertilization treatments within the year and fire treatment at $P \leq 0.05$	124
Figure 4-2. Total (a, b) and herbaceous (c, d) ANPP in 2017-2021 for both burned and unburned prairie treatments. Each point is the average rate for the fertilization treatment, and the error bars are ± 1 SE. Letters denote significant differences among years at $P \leq 0.05$	125
Figure 4-3. Plant (a, b) and soil (c, d) N stock in 2021 for both burned and unburned prairie treatments. Each bar is the average stock for the fertilization treatment, and the error bars are ± 1 SE. Capital letters denote significant differences among fertilization treatments at $P \leq 0.05$	126
Figure 4-4. C:N ratio of shoots (a), roots (b), and soils (c) in burned and unburned prairies at the end of the 2021 growing season. Each point is the average C:N ratio for each individual plot corresponding to the fertilization treatment, with the different fertilization treatments denoted by color. Capital letters for root C:N denote significant differences between combined field treatments, while lowercase letters in soil C:N indicate significant differences between fertilization treatments, at $P \leq 0.05$	127
Figure 5-1. NMDS of soil microbial communities along field treatments at the BGP experiment. Each ordination point represents a single time point, and lines connect the samples across time. The gradient of color intensity reflects sample collection date, with the lightest points reflecting the 2017 soil microbial communities. The ordination is split by fire management treatment for clarity purposes.	155
Figure 5-2. Observed ASV richness, Shannon diversity, and evenness of soil microbial communities during the growing season from 2017-2021. Each point is the average alpha diversity metric for the fertilization treatment, split by the burned and unburned prairies, and the error bars are ± 1 standard error (SE). Letters near points denote significant differences between years within the fertilization treatment at $P \leq 0.05$	156

Figure 5-3. Average ASV log-fold changes from previous and continuous N-fertilization, relative to ASV abundance in the control treatment, binned by phyla and classes defined as putative copiotrophs (top facet) and oligotrophs (bottom facet). Asterisks near points indicate phylum or class is statistically higher than zero at $**P < 0.01$, and $***P < 0.001$ thresholds.

..... 157

Figure 5-4. Microbial enzymatic investment for C relative to N (C:N), C relative to P (C:P), and N relative to P (N:P) summarized by boxplots. Each point is the enzymatic ratio for each plot corresponding to the fertilization treatment. Letters next to the mean $\pm$ standard deviation in C:N plots denote significant difference between fire treatments at $P \leq 0.05$. 158

List of Tables

Table 2-1. Soil chemistry in soils with different fire and fertilization treatments.	44
Table 2-2. Permutational analysis of variance (PERMANOVA) results for soil microbial communities under different fixed effects.	45
Table 3-1. Statistical results for two-way ANOVA on the direct and interactive effects of fertilization (“Fertilizer”) and burning (“Burn”) treatments on soil chemistry response variables.	83
Table 3-2. Statistical results for three-way ANOVA on the direct and interactive effects of fertilization (“Fertilizer”), burning (“Burn”), and time (“Month” or “Year”) on soil microbial and plant response variables.	84
Table 3-3. PERMANOVA results on the direct and interactive effects of fertilization (“Fertilizer”), burning (“Burn”), and sampling month (“Month”) for the 16S rRNA gene composition of microbial communities based on the Bray-Curtis dissimilarity.....	85
Table 5-1. Permutational analysis of variance (PERMANOVA) results for soil microbial community composition different treatments and time.	154

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Chapter 1 - Introduction

Nitrogen (N) is the seventh element on the periodic table and is one of the most abundant elements by mass on our planet (Canfield et al. 2010). At normal atmospheric pressures and temperatures on Earth, most N exists as a practically inert diatomic gas (N_2) with strong triple covalent bonds, a form that cannot be used by most living organisms. However, N is a fundamental element for all life on Earth: Nitrogenous bases are components of nucleotides that make up the DNA and RNA, amino acids polymerize into long chains that shape into proteins, and peptidoglycans form long polymers that mesh into an important cell wall layer in most bacteria. At higher orders of biological organization, N content in plants is strongly correlated to their photosynthetic rates (Evans 1989), N in forage affects grazing behavior of large ungulates (Raynor et al. 2015), and assimilable N quantity regulates the growth of microorganisms (Schimel and Weintraub 2003). Therefore, structure and function of biological organizations across all levels are affected by N.

In terrestrial ecosystems, N exists as inorganic and organic pools that are transformed within and between these pools by organisms (Galloway et al. 1995, Schlesinger and Bernhardt 2020). Plants and soil microbes in most temperate ecosystems take up inorganic ammonium (NH_4^+) or nitrate (NO_3^-) ions and incorporate them into their biomass through assimilation and immobilization (Schimel and Bennett 2004). When these organisms die, the N in this non-living organic material is retained in plant litter or microbial necromass, or converted to N in soil organic matter (SOM) that cannot be reassimilated by plants or soil microbes in its polymeric form. Instead, soil microbes must synthesize extracellular enzymes to catalyze the depolymerization of the non-living organic material to labile forms so N can be used again (Burns 1982, Sinsabaugh 1994). This depolymerization process is the rate-limiting step for the

entire N cycle of an ecosystem (Schimel and Bennett 2004), explaining why 96-97% of total organic N is stored in soil globally (Post et al. 1985, Batjes 1996, Schlesinger and Bernhardt 2020). Once N in the non-living organic pool re-enters the inorganic pool by N mineralization, soil microbes that utilize NH_4^+ as a source of energy or NO_3^- as an electron acceptor compete against plants and microbial heterotrophs for the inorganic N that are subject to loss from the ecosystem (Robertson and Groffman 2015). For example, nitrification is the oxidation of NH_4^+ to nitrite by chemolithoautotrophic nitrifiers known as ammonia oxidizers, after which nitrite is transformed rapidly to NO_3^- by a separate group of nitrifiers (Prosser 2011). Though the fate of NO_3^- varies in different ecosystems, soils in most temperate climates will leach NO_3^- from the soil under wet conditions. On the other hand, NO_3^- can serve as an alternative electron acceptor and be reduced by heterotrophic denitrifiers during anaerobic respiration when oxygen is in short supply (Wallenstein et al. 2006). The end-product of denitrification is a gaseous form of N, either the potent greenhouse gas nitrous oxide (N_2O), or N_2 when N_2O is further reduced by denitrifiers that can synthesize nitrous oxide reductase (Hallin et al. 2018). From N_2 , this element returns to the biosphere via N fixation. Collectively, assimilatory (plant assimilation and microbial immobilization) and dissimilatory (nitrification and denitrification) processes, along with N fixation, regulate the flux and pools of ecosystem N.

Prior to modern industrialization, many terrestrial ecosystems were limited by N availability. Here, limitation is defined as in ‘Liebig’s Law of the Minimum’, in which biomass production or other ecosystem function rates are fixed by the resource in lowest supply relative to biotic demand (von Liebig 1840). With the exception of late successional ecosystems, the limited availability of N meant that ecosystem N loss was minimal as strong competition between plants and soil microbes retained this nutrient from exiting an ecosystem limited by N

(Vitousek and Reiners 1975, Lovett et al. 2018). However, global industrialization has made biologically usable, ‘reactive’ N more available globally due to the Haber-Bosch process to produce N fertilizer to improve cereal crop yield (Fixen and West 2002) and fossil fuel combustion through atmospheric deposition (Vitousek et al. 1997, Galloway et al. 2008, Schlesinger 2009). As a result, reactive N from human systems has augmented N cycling in terrestrial ecosystems.

The question among ecologists is now less about how N availability impacts terrestrial ecosystems, but rather about what the magnitude and duration of *previous* higher N input from humans will be on *current* and *future* ecosystem structure and function (Mason et al. 2022). Higher N availability often increases net primary production in historically N-limited ecosystems (Gough et al. 2000, LeBauer and Treseder 2008), accelerates N-cycling processes (Lu et al. 2011, Ouyang et al. 2018), decreases local plant diversity (Stevens et al. 2004, Suding et al. 2005), and acidifies soils (Aber et al. 1998, Lovett and Goodale 2011). Terrestrial ecosystems can also leach excess N into aquatic ecosystems that may cause harmful effects downstream such as triggering harmful algal blooms (Schlesinger 2009). Yet, the N that slowly accrued in plant and soil pools from past chronic inputs may also serve as N sources for current and future ecosystem processes. Thus, this emerging phenomenon that previous increases in available N may continue to alter contemporary ecosystem processes is important, since global models project that atmospheric N deposition will decline across large sections of the planet’s land surface during the 21st century (Lamarque et al. 2013, Kanakidou et al. 2016). In addition, the stored reservoir of exogenous N could cause a significant lag in ecosystem recovery (i.e., the return of ecosystem properties of processes to pre-fertilized or reference conditions) from higher reactive N input (Van Meter et al. 2016). However, N pools and fluxes are not expected to

recover equally under this proposed model (Gilliam et al. 2019): Some aspects of N-cycling, such as the inorganic N pool, could recover within a few years (Bredemeier et al. 1995, Koopmans et al. 1995, O’Sullivan et al. 2011, Stevens et al. 2012), while others (e.g., soil organic N pool) may occur slowly, i.e., a decade or longer (Stevens 2016), suggesting that these higher-than-expected N pools or flux rates can create a positive legacy effect even when the driver is removed (Sala et al. 2012). Therefore, differential recovery of ecosystem structures and functions is expected (Nieland et al. 2021), with potential coupling of the recovery of certain ecosystem properties (e.g., inorganic N availability and aboveground net primary productivity (ANPP)) if they are reliant on or correlated to each other.

Grasslands cover over a third of Earth’s ice-free land surface (White et al. 2000), and in temperate regions this biome was historically maintained by regular fire disturbances that play an important role for the N and carbon (C) cycles (Bond et al. 2005, Ahmed et al. 2017, Pellegrini et al. 2020). At shorter time scales, fire removes aboveground litter and combusts the organic N stored in this pool (Ojima et al. 1994, Blair 1997) but can char a small percentage of the organic C to fire-derived organic matter that is hypothesized to be more resistant to microbial breakdown (Preston and Schmidt 2006). Consequently, grasslands managed with short fire return intervals (e.g., annually burned) are characterized as being limited in available N due to N loss from the volatilization of N in aboveground litter (Connell et al. 2020). On the other hand, fire suppression enables litter to decompose and return organic N and C back belowground; thus, long fire return intervals, or grasslands left unburned, increase N availability (Connell et al. 2020) and the proportion of soil organic matter that is derived from undecomposed fragments of plant debris (i.e., particulate organic matter) that is believed to be more vulnerable to microbial degradation (Lavallee et al. 2020, Pellegrini et al. 2022). As a result, N cycles faster and plants

grow less roots that are also lower in C:N ratio (more N-enriched) in fire-suppressed grasslands relative to annually burned grasslands (Johnson and Matchett 2001, Kitchen et al. 2009).

Ecosystems that experience frequent fire like mesic grasslands may benefit from prescribed fires if the disturbance is a mechanism that can release past and current inputs of exogenous N stored in plant and soil organic N pools. Dissimilatory N cycle processes will also serve as ecosystem N loss mechanisms via increased NO_3^- leaching or gaseous N emission, but in the absence of prescribed fires, the microbially-mediated processes will be the exclusive pathway for exogenous N loss. However, the potential N loss from both fire and soil microbes may diminish over time after higher N input ceases. Frequent fires support a grassier ecosystem (Bragg and Hulbert 1976, Bond et al. 2005), and perennial C_4 grasses can assimilate and retain large pools of N to decrease NO_3^- leaching (Tilman and Wedin 1991); in addition to the immobilization potential of soil microbial biomass, plants and microbes may then strongly compete for the shrinking inorganic N supply (Dell and Rice 2005) and slow ecosystem recovery if the N cycle becomes more closed to conserve this nutrient. Additionally, microbial dissimilatory processes may recover more quickly than plant and microbial assimilatory processes, which could further extend the time the ecosystem recovers as the exogenous N continues to support altered ecosystem structure and/or function. Discerning persistent or decreased responses of plants and soil microbes to a decline in N input would require careful examination of pertinent ecosystem processes across time, in a manner to understand if, and how, fire causes differential recovery of ecosystem functions, communities, and of the total ecosystem.

In this dissertation, I asked: 1) Which feedbacks underpin soil microbial enzymatic responses to chronic nutrient fertilization? 2) Do ecosystem processes share similar sensitivities

and recoveries from chronic N-fertilization? 3) Do fire-adapted ecosystems, with their communities and functions, recover faster from chronically elevated N inputs under annual burning? And 4) What are the mechanisms that may control current ecosystem responses to past N-fertilization? To answer these questions, I used a fertilization and cessation experiment in a tallgrass prairie managed by different fire treatments to assess ecosystem recovery.

In Chapter 2, I analyzed soil extracellular enzyme activities and microbial community composition from samples collected monthly in 2016 from a 30-year field experiment at Konza Prairie Biological Station near Manhattan, KS, USA (Seastedt et al. 1991). In this fertilization experiment, N ($10 \text{ g N m}^{-2} \text{ yr}^{-1}$ as NH_4NO_3) and phosphorus (P) ($1 \text{ g P m}^{-2} \text{ yr}^{-1}$ as $\text{Ca}(\text{H}_2\text{PO}_4)_2$) were added alone or together (N and P) every spring and fertilization was crossed with annually burned or unburned treatments. P is also a limiting nutrient in this ecosystem, and was included in this chapter to assess whether feedbacks were similar following N and P fertilization, and if microbial responses were stronger under dual fertilization. I found that chronic N-fertilization and fire suppression independently increased microbial enzymatic investment for C relative to N, indicating lower microbial N-limitation, but that P-fertilization did not induce the same response. Also, I found that fertilization with both N+P led to stronger shifts in soil microbial taxa than N and P alone, but there was no evidence for a tradeoff between microbial population growth and resource acquisition via enzyme production, and soil pH did not strongly affect enzyme activities. Collectively, I found the strongest support for a product-inhibition feedback, in which higher availability of a nutrient lowers the investment to synthesize extracellular enzymes that depolymerize the nutrient from SOM, but only under higher N conditions.

In Chapter 3, I measured N pools, N fluxes and their related microbial populations, microbial community composition, and ANPP in the first growing season (2017) after chronic N-

fertilization ceased. I used the long-term N-fertilization treatments from the same experiment described in Chapter 2, except that N fertilization was ceased at the plot scale and subplots fertilized at the same rate were established to assess belowground sensitivity and recovery. I found that N availability dropped after ceasing N-fertilization, but ANPP and soil %N remained high. Moreover, nitrification potential declined more than denitrification potential, but N-cycling microbial populations had different sensitivities and recoveries from N-fertilization, despite overall microbial community composition not shifting. Additionally, N pools and fluxes were always higher in unburned prairies. Altogether, these results suggest that ecosystem functions are differentially sensitive to chronic N-fertilization and are likely not to recover at similar rates, and show that there may be different mechanisms at play in the burned and unburned prairies.

In Chapter 4, I continued measuring N pools, N fluxes, and ANPP the next four growing seasons (2018-2021) to assess how prescribed fires shaped the trajectory of tallgrass prairie recovery. Using the same fertilization and cessation experiment as in Chapter 3, I found that N availability did not subsequently decrease further the first growing season, that nitrification potential recovered faster in burned prairies than denitrification potentials, regardless of fire treatment, and that ANPP never returned to control levels. As in Chapter 2, available N pools and fluxes remained higher in unburned prairies. I also measured the combustible N pool in burned prairies after the 2020 growing season and found that this pool was only marginally higher in previously fertilized plots. Finally, I measured plant and soil N and C stocks in 2021 and found that soil N stock in previously fertilized prairies was intermediate between control and continuously fertilized prairies in both fire treatments; and that shoot and root C:N ratio recovered in burned prairies, but soil C:N ratio did not recover in either fire treatment. This chapter suggests that effective plant assimilation and/or retranslocation of N is a possible

mechanism for maintaining high ANPP and limiting N loss via combustion, and substrate limitation for nitrification (NH_4^+) and denitrification (NO_3^-) may be a similar mechanism for the recovery of dissimilatory processes. In addition, NO_3^- leaching from topsoil might be an overlooked mechanism of N loss from unburned tallgrass prairie.

In Chapter 5, I surveyed soil microbial community structure (composition, relative abundance, and diversity) from 2018-2021 to assess this assemblage's recovery and evaluate evidence for the copiotrophic-oligotrophic framework as an underlying recovery mechanism. In the same fertilization and cessation experiment as in previous chapters, I found that microbial community structure was more sensitive to fertilization in the unburned prairie, and did not recover from chronic N-fertilization in either fire treatment over five years following fertilizer cessation. Yet, there was no support for the copiotrophic hypothesis, in which the relative abundance of putative fast-growing (sub)phyla increased in relative abundance while putative slow-growing (sub)phyla decreased. In 2021, I also measured soil extracellular enzymes to assess recovery of soil microbial community function and found that microbial enzymatic investment for C relative to N was lower, albeit insignificantly, in recovering burned prairies but not for unburned prairies. Collectively, these results suggest that soil microbial community structures have potentially shifted to alternative states, although not necessarily predictable at high taxonomic ranks, but fire suppression in combination with N-fertilization may have pushed the assemblages past an environmental threshold that may further constrain future soil microbial community recovery.

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Chapter 2 - Product-inhibition feedbacks, not microbial population growth tradeoffs or soil pH, regulate decomposition potential under nutrient eutrophication

2.1 Abstract

Global eutrophication of growth-limiting soil nutrients, including nitrogen (N) and phosphorus (P), can affect enzymatic feedbacks at different levels of biological organization that drives soil organic matter (SOM) decomposition. Because land management practices, such as fire management in mesic grasslands, also impact nutrient availability, it is important to understand how soil enzymatic feedbacks may change or mask each other when a management layer is considered. Here, using a thirty-year field experiment that crosses N and P fertilization (alone and dual) with contrasting fire treatments (annual burning and fire suppression) in a tallgrass prairie ecosystem, we evaluated evidence for three enzymatic feedbacks (product-inhibition, copiotrophic, and pH shift hypotheses) by measuring soil extracellular enzyme activities (EEAs) and microbial community composition (16S rRNA gene sequencing) monthly for a year. Consistent with predictions of the product-inhibition hypothesis, N-fertilization and fire suppression, which increases N availability, decreased N-acquiring EEAs and microbial enzymatic investment for C relative to N (ratio of β -glucosidase and N-acquiring enzyme activities). Although N- and P-fertilization alone and together shifted microbial population growth from oligotrophic to copiotrophic groups, a concomitant decrease in all soil EEAs that is expected as a tradeoff for growth under the copiotrophic hypothesis was not observed, except for some EEAs in unburned prairie soils fertilized with N alone. Interestingly, P-fertilization did not lower soil phosphatase activity, where other biological and chemical properties at this site might

be affecting the response. Finally, there was no strong evidence for soil pH influencing soil EEAs, though this has been observed at other fertilized grassland sites. Overall, these results suggest higher N conditions affect enzymatic feedbacks primarily through product-inhibition, but a threshold-like response is possible under certain management practices like fire suppression in combination of N-fertilization. Further, site-specific responses to fertilization are to be expected as evident by a lack of soil EEA responses to P-fertilization. Finally, soil enzymatic feedbacks could be independent of taxonomic turnover of soil microbial communities from fertilization.

2.2 Introduction

Soil decomposer communities redistribute energy and nutrients from the aboveground ‘green’ world to form the base level of belowground ‘brown’ foodwebs (Hairston *et al.* 1960; Allison 2006). However, modern global industrialization risks disrupting microbial feedbacks that shape carbon (C) and nutrient cycling through soil organic matter (SOM) decomposition. Specifically, inputs of bioavailable nitrogen (N) and phosphorus (P) to ecosystems have soared (Cordell *et al.* 2009, Kanakidou *et al.* 2016), due largely to the rise in atmospheric deposition from fossil fuel combustion and fertilizer application (Vitousek *et al.* 1997, Fixen and West 2002), and, as a result, elevated soil N and P availability has increased net primary production as plants fix more C (Elser *et al.* 2007, Harpole *et al.* 2011). Prior to contemporary eutrophication, plants relied on soil microbes to release nutrients from SOM, and over decades to centuries, positive feedbacks between litter input and decomposition would enhance soil fertility, leading to higher net primary production (Hobbie 2015). Following anthropogenic augmentation of nutrient availability, microbial feedbacks that maintain soil fertility may be altered.

Soil microorganisms (including bacteria, archaea, and fungi) produce an arsenal of extracellular enzymes that attack unprotected SOM to release labile N and P compounds needed by microbes and plants (Burns 1982, Sinsabaugh 1994). The synthesis and activity of extracellular enzymes reflects microbial demand for particular nutrients, in that soil microbes reallocate intracellular resources to increase the production and extracellular release of enzymes to target a limiting nutrient (Chróst 1991, Sinsabaugh et al. 2008). In turn, as extracellular nutrient concentrations increase, the synthesis of enzymes responsible for that nutrient's release decreases (Olander and Vitousek 2000, Schimel and Weintraub 2003, Sinsabaugh and Follstad Shah 2012). Thus, this cellular level product-inhibition feedback of extracellular enzymes responsible for SOM decomposition explains observations of lower soil N- or P-acquiring enzymatic activity following the addition of N or P, respectively (Olander and Vitousek 2000, Schimel and Weintraub 2003, Allison and Vitousek 2005). In addition, the copiotrophic hypothesis suggests that microbial populations with faster growth rates and lower investment in synthesizing all extracellular enzymes (i.e., copiotrophs) outcompete populations with slower growth rates (i.e., oligotrophs) in soil environments with higher nutrient availability. This population-level tradeoff between growth and resource acquisition predicts that fertilization will shift the relative abundance of functionally important taxa within soil microbial communities, with the consequence of lower SOM decomposition (Fierer et al. 2007, Ramirez et al. 2012).

However, while these mechanisms have been well supported in lab experiments, results from field fertilization experiments show weaker support for both cellular- and population-level feedbacks (Riggs and Hobbie 2016, Keller et al. 2022). Also, other studies have found mixed responses of soil extracellular enzyme activities to exogenous nutrient additions, and while the copiotrophic hypothesis is based on studies of N-fertilization, it is not clear whether P-

fertilization invokes similar mechanisms and feedbacks (Allison and Vitousek 2005, Hernández and Hobbie 2010, Jian et al. 2016, Ochoa-Hueso et al. 2020). Lower SOM decomposition potential following fertilization can be mediated by other ecosystem-scale feedbacks as well, such as inhibition of total microbial activity due to decreased soil pH (Treseder 2008, Riggs and Hobbie 2016). Furthermore, the magnitude of enzymatic responses to fertilization can be constrained by ambient soil properties (Zeglin et al. 2007), such that even if microbial enzymatic feedbacks are universal, the impact of exogenous nutrient input on enzyme activity and decomposition may vary among ecosystems with different biotic and abiotic baseline conditions.

In grassy ecosystems, which cover over a third of the planet's land surface (White et al. 2000, Bai and Cotrufo 2022), local land management practices such as fire suppression have altered grassland nutrient dynamics concurrently with the global enrichment of bioavailable N and P. Historically, the frequent occurrence of fire in mesic grasslands combusted N stored in senesced plant litter and limited its return belowground, supporting an N-limited ecosystem (Ojima et al. 1994, Blair 1997). In contrast, fire suppression allows litter-N accumulation and decomposition, and positive feedbacks develop, as lower litter C:N promotes faster litter breakdown, and higher plant N availability (Parton et al. 2007). In addition, fire suppression limits P availability as P is immobilized in biomass and detritus and not subsequently released through pyromineralization (Seastedt 1988, Butler et al. 2018). Also, long-term fire suppression shifts plant community composition to reduced dominance of C₄ grasses and can increase the cover and abundance of woody plants (i.e., woody encroachment; Briggs et al., 2002b, 2002a; Ratajczak et al., 2011). Woody encroachment is a widespread and growing concern in the tallgrass prairie ecosystem of North America (Beilmann and Brenner 1951, Bragg and Hulbert 1976, Ratajczak et al. 2014), and impacts local C and nutrient cycles through changes to litter

quality and soil microclimate (Acharya et al. 2017, Connell et al. 2020). Fire suppression also shifts soil bacterial, archaeal, and fungal community composition (Carson and Zeglin 2018, Carson et al. 2019), but it is not clear whether these community shifts are linked to differences in soil microbial enzyme activity. Thus, due to multiple feedbacks operating at different scales, SOM decomposition responses to concurrent anthropogenic eutrophication and land management change are difficult to predict. To begin disentangling these mechanisms, we tested hypotheses related to soil enzymatic feedbacks using a long-term field experiment that crosses fertilization treatments with contrasting fire management practices in a tallgrass prairie ecosystem.

Specifically, we evaluated the product-inhibition, copiotrophic, and soil pH shift hypotheses, collecting evidence to discern which of the soil enzymatic feedbacks that affect SOM decomposition are most prevalent in soils that have been fertilized with N and P (alone and in combination) under contrasting fire management treatments (annual burning and fire suppression) for thirty years. Recent work at this field experiment supported the copiotrophic hypothesis, in that soils chronically fertilized with N had a higher relative abundance of microorganisms putatively classified as copiotrophs, with greater shifts associated with the highest N availability in unburned prairie soils (Carson and Zeglin 2018). However, we do not know whether soil enzymatic activity has been simultaneously affected, or whether chronic P-fertilization in this P-limited system (Wilson et al. 2009, Johnson et al. 2015) has similar effects. We predicted that the product-inhibition feedback would be supported if just N- and P- acquiring enzyme activities decrease following N- and P-fertilization, respectively; and that the copiotrophic feedback would be supported if all soil enzyme activities decrease concomitant with microbial community turnover towards higher copiotroph abundance under eutrophic conditions

(Ramirez et al. 2012). Further, we predicted that the soil pH feedback would be supported if decreases in soil enzyme activity strongly correlates with decreases in soil pH. Using this framework to understand how cellular, population, and ecosystem level feedbacks to SOM decomposition may respond to terrestrial eutrophication, we tested all three hypotheses by collecting soils from the field experiment monthly for a year and measuring soil extracellular enzyme activities (EEAs), bacterial and archaeal community composition, and soil chemistry.

2.3 Methods

Field experiment description and sample collection

The Belowground Plot (BGP) experiment was established in 1986 at an ungrazed area of the Konza Prairie Biological Station (KPBS) near Manhattan, KS, USA (39° 05' N, 96° 35' W) (Seastedt et al. 1991). Mean annual precipitation at KPBS is 835 mm, but total precipitation in the study year (2015) was considerably wetter at 1002.5 mm (Carson and Zeglin 2018). As a strip-plot design, the BGP experiment crosses fertilization across four blocks that are burned annually in early spring (burned prairie) and another four that are not burned (unburned prairie). Strips within blocks are either not fertilized (control) or fertilized with N (10 g N m⁻² yr⁻¹ as NH₄NO₃), P (1 g P m⁻² yr⁻¹ as Ca(H₂PO₄)₂), or N and P (dual nutrient), totaling eight fire × fertilization field treatments, each with four 12.5 m × 12.5 m replicate plots ($n = 4$). At the BGP experiment, soils are deep and silty-clayey loams belonging to the Irwin series (fine, mixed, mesic, pachic Arguistolls) with no organic horizon.

Plot-scale samples were aseptically collected as the composite of four 2 cm diameter, 15 cm deep mineral soil cores within each plot in control and N-only treatments in January 2015 and in all fertilization treatments from March to December 2015. Samples were kept on ice until

stored at -20°C in the laboratory for further processing. Soils were collected one week after the prescribed fire in April 2015 and one week after fertilizer application in June 2015.

Soil chemistry

Soil %C and %N were quantified using a FlashEA 1112 NC Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Soil %C, %N, and pH (pH in 1:1 ratio of soil and deionized water) were measured only in soils collected in July because we did not expect these parameters to change monthly. Soil %C and %N were converted to molar amounts to calculate soil C:N ratios. Gravimetric water content (GWC) was measured in all samples by drying soils overnight at 105°C.

Extracellular enzyme activities

Soil extracellular enzyme activity potentials were measured using short-term, room temperature assays with fluorometric organic substrates (methylumbelliferone (MUB) or methylcoumarin (MC)) for hydrolytic enzymes and a colorimetric substrate (L-3,4,-dihydroxyphenylalanine (L-DOPA)) for the oxidative enzyme (Saiya-Cork et al. 2002 p., Zeglin et al. 2013). Activities of β -glucosidase (BG; EC 3.2.1.21), cellobiohydrolase (CBH; EC 3.2.1.91), *N*-acetylglucosaminidase (NAG; EC 3.2.1.14), leucyl aminopeptidase (LAP; EC 3.4.11.1), acid phosphatase (PHOS; EC 3.1.3.1), and phenol oxidase (POX; EC 1.10.3.2) were measured in 96-well plates with 6-8 technical replicates per sample and enzyme combination. All assays were run in 50 mM sodium acetate buffer (pH 5) with a final substrate concentration at either 40 μ M (hydrolytic enzymes) or 5 mM (POX) for 2 (BG and PHOS), 4 (NAG and CBH), or 18 h (LAP and POX) before raising the pH > 8 with 0.5 M NaOH to measure MUB and MC fluorescence at 360/450 nm (excitation/emission) and absorbance of L-DOPA at 450 nm using a FilterMax F5 Multimode Microplate Reader (Molecular Devices, San Jose, CA, USA). Blanks

and quench controls were included in each assay plate. Hydrolytic EEAs below detection were reported as 0 nmol g⁻¹ dry soil h⁻¹, and POX activities were reported as 60 nmol g⁻¹ dry soil h⁻¹, if below detection. To assess relative microbial investment towards acquiring certain nutrients, we calculated ratios between ln(BG) and the sum of N-acquiring enzyme activities ln(NAG+LAP) for C relative to N, ln(BG) and ln(PHOS) activities for C relative to P, and ln(NAG+LAP) and ln(PHOS) activities for N relative to P (Sinsabaugh and Follstad Shah 2012).

Microbial community composition

Soil genomic DNA was extracted using a modified CTAB method and allowed to precipitate overnight in PEG 6000, followed by an ethanol wash the next day (DeAngelis et al. 2010). Polymerase chain reaction (PCR) was performed to target the 16S rRNA gene V4 region using 515F/806R primers with existing protocols, except 25 cycles were run instead of 35 to minimize PCR bias (Carson and Zeglin 2018). Libraries were gel-purified, and three separate libraries were sequenced with 2 × 150 paired-end Illumina MiSeq runs (Illumina, San Diego, CA, USA).

Sequence data were processed using QIIME2 (Bolyen et al. 2019). Sequence reads from demultiplexed data were trimmed to 150 bp based on the lowest library quality, denoised using DADA2 (Callahan et al. 2016), and then classified taxonomically as amplicon sequence variants (ASVs) using the Ribosomal Database Project classifier V2.13 (Wang et al. 2007, Callahan et al. 2017). Afterwards, we merged the libraries. ASVs were removed if they had less than three reads (Bokulich et al. 2013) and if they were classified as mitochondria or chloroplasts. This quality control ended in a dataset with 19,251,474 sequence reads and 21,816 ASVs. Because of the uneven distribution of reads among samples, we first removed samples that had less than 15,000 reads, losing 30 of the 340 samples, and then normalized the data by proportionally transforming

each ASV read within a sample using *phyloseq* (McMurdie and Holmes 2013). Raw sequence data used in this study can be found in the NCBI BioProject database under the accession PRJNA398249 (<https://www.ncbi.nlm.gov/bioproject/>).

Statistical analyses

We assessed whether fire management and fertilization affected soil chemistry and soil EEAs by using a linear modeling approach in R V4.2.1 (R Core Team 2022) in the RStudio environment V2022.07.0 (RStudio Team 2022). Fire, N-fertilization, and P-fertilization treatments were set as interacting fixed effects in all models. For GWC, soil EEAs (natural log-transformed +1), and EEA ratios, we used linear mixed effect models that included month as an interacting fixed effect and block as a random intercept to account for repeated sampling with the *lmerTest* package (Kuznetsova et al. 2017). For soil %C, %N, molar C:N ratio, pH, we used linear models. Also, correlations between soil chemistry and EEAs were tested using linear models. We checked for homoscedasticity and residual normality for every model; in cases where model assumptions were not satisfied, outlier data were removed if they fell outside the 1.5× interquartile range from the 25 and 75% quartiles and new models were applied, though inference was similar between models that included outliers and those without them. All models except for soil chemistry and soil EEA correlations were assessed using analysis of variance (ANOVA) in the *stats* package (R Core Team 2020), and pairwise comparisons between months or for significant interactions were further evaluated using Tukey's post-hoc analyses with the *emmeans* package (Lenth 2016). Significance was set at $\alpha \leq 0.05$.

We assessed whether fertilization differentially affected soil EEAs and putative copiotroph and oligotroph relative abundances between fire management treatments by testing

their log response ratios (LRRs) against zero using LMMs that included blocks as random intercepts. LRRs were calculated at each month using the equation

$$\text{LRR} = \frac{\ln(R_F)}{\ln(R_C)}$$

where R_F is the soil EEA or normalized abundance of the microbial group in the N- and P-fertilized (alone and dual) plot, and R_C is the soil EEA or normalized abundance of the microbial group in the control plot within the same block. Putative copiotrophic or oligotrophic phyla and classes were defined based on nutrient responses identified in previous studies (Ramirez et al. 2012, Leff et al. 2015). We assessed whether microbial community composition shifted in response to fire management, N-fertilization, or P-fertilization treatments, and within the year, by using a permutational analysis of variance (PERMANOVA) that included field treatments and month as interacting fixed effects based on the Bray-Curtis dissimilarity in the normalized dataset. Based on the PERMANOVA, we compared microbial community composition between combined field treatments using the *pairwiseAdonis* package (Martinez Arbizu 2017) and visualized microbial community composition using non-metric multidimensional scaling (NMDS) ordination with the lowest stress value from twenty iterations.

2.4 Results

Soil chemistry

Chronic fertilization had minimal effects on most soil chemistry parameters (Table 2-1, Appendix A Table A-1). Soil pH was not different among fertilization or fire management treatments, and neither %C nor %N increased following chronic fertilization or fire suppression. Yet, N-fertilization in unburned prairie soils lowered the mean soil C:N ratio from 13.6 to 12.7 ($P = 0.029$), and burned prairie soils fertilized with P had a higher mean soil C:N ratio than the

N- or dual-nutrient (N and P) fertilized burned prairie soils (post-hoc: all $P < 0.05$). GWC differed monthly (Appendix A Figure A-1), ranging from 0.192 g water g⁻¹ dry soil in October to 0.322 g water g⁻¹ dry soil in December, with a time by fire interaction ($F = 6.50$, $P < 0.001$) reflecting the lower water content in unburned prairie soils relative to burned prairie soils in August, September, and November (post-hoc: all $P \leq 0.01$).

Extracellular enzyme activities

Annual burning decreased soil C-acquiring BG and CBH activities, but not POX activity, with no interactive effects (Appendix A Table A-1, Appendix A Figure A-2). Supporting the product-inhibition hypothesis, chronic N-fertilization consistently reduced N-acquiring NAG and LAP activities by 11 and 14%, respectively (NAG: $F = 7.84$, $P = 0.012$; LAP: $F = 19.9$, $P < 0.001$); but, there was no analogous PHOS decrease under P-fertilized conditions, and no N- by P-fertilization interaction. Contrary to predictions supporting the copiotrophic hypothesis, neither N- nor P-fertilization lowered all soil EEAs (Figure 2-1, Appendix A Figure A-2). Instead, fertilization effects were consistently stronger in unburned prairie soils, in that all EEAs except for POX tended to decrease (i.e., all LRR means were negative) under nutrient fertilization, and the only LRRs that differed from zero were in unburned prairie soils (Appendix A Table A-2, Figure 2-1). Interestingly, P-fertilization effects on soil EEAs always emerged with a temporal interaction (Appendix A Table A-1, Appendix A Figure A-2) that was not consistent with any of our predictions. Post-hoc tests showed that P-fertilization increased soil CBH activities in July and December, and decreased them in November; and decreased soil NAG activities in August (Appendix A Figure A-2).

Microbial enzymatic investment for C relative to N, an index of microbial N limitation, was lower (i.e., more N-limited) in burned prairie soils, and higher (i.e., less N-limited) under N-

fertilization with no interactive effects (Appendix A Table A-1, Figure 2-2), both consistent with the product-inhibition hypothesis. However, microbial enzymatic investment for C relative to P and N relative to P, indices of microbial P limitation, were not higher (i.e., less P-limited) in P-fertilized soils (Appendix A Table A-1, Figure 2-2), inconsistent with the product-inhibition hypothesis. Instead, P-fertilization had a significant interaction with time; post-hoc tests showed that microbial enzymatic investment for C relative to N was higher, and enzymatic investment for N relative to P was lower, in soils not fertilized with P during June and July, but relative enzymatic investment for nutrients did not change in the P-fertilized soils (Appendix A Figure A-3).

Plot-scale soil pH and EEAs of soils sampled in July were not correlated, though P-acquiring PHOS activities tended to be higher at lower soil pH ($R^2 = 0.13$, $P = 0.05$). Soil BG activities and soil %N were weakly positively correlated ($R^2 = 0.18$, $P = 0.03$), but no other enzyme activities were correlated to %C or %N. Across the time series, BG, NAG, and PHOS activities were weakly positively correlated to GWC, reflecting a trend of higher values in August and September with subsequent decreases in October, particularly in burned prairie soils (Appendix A Table A-3, Appendix A Figure A-1, Appendix A Figure A-2).

Microbial community composition

Data on the relative abundance of bacterial and archaeal soil populations provided moderate support for the copiotrophic hypothesis. Of the 48 total comparisons of putative copiotroph and oligotroph relative abundance, 16 groups showed significant responses, 15 of which changed as expected due to chronic fertilization (i.e., increase in copiotrophs, decrease in oligotrophs); the exception was Acidobacteria, a putative oligotrophic group which increased in burned P-fertilized prairie soils (Appendix A Table A-2, Figure 2-3). Relative abundance within

all putative copiotrophic groups across the N-, P-, and dual nutrient- fertilized treatments increased by an average 29% ($t = 4.49$, $P = 0.003$), opposed to relative abundance within putative oligotrophic groups which declined by 11% ($t = -5.01$, $P = 0.002$). Interestingly, P-fertilization had a greater impact in burned prairies, where relative abundances of four groups changed significantly in burned P-alone prairie soils in contrast to one in unburned P-alone prairie soils, and five groups in burned dual nutrient fertilized prairie soils compared to three in unburned dual nutrient prairie soils. Also, dual nutrient fertilization resulted in higher-magnitude LRRs and eight significantly responding groups, in contrast to the three or five significantly responding groups in the N- or P-alone treatments, respectively (Appendix A Table A-2, Figure 2-3).

Soil bacterial and archaeal community composition also changed in response to chronic N- and P-fertilization. Independently, N- and P-fertilization explained 2.8 and 3.5% of the variation in the PERMANOVA, respectively, while their interactive effect was weaker (0.7% of variation; Table 2-2). Fire management treatment also explained 2.3% of the variation, and interactions among the three field treatments together explained 4.3%. Pairwise PERMANOVAs showed that, except for control soils between fire management treatments, community composition was different among all combined field treatments (Appendix A Table A-4), and the NMDS supported these findings in community structure change (Appendix A Figure A-4). Also, a relatively large amount of variation in community composition was attributed to monthly variability (4.5%), and this further depended on whether or not prairies were fertilized with P (3.5% of variation; Table 2-2). From January through May, microbial community composition in soils not chronically fertilized with P was distinct, then converged towards higher similarity with

P-fertilized soils as the growing season progressed, and remained similar through fall and winter (Appendix A Figure A-5).

2.5 Discussion

By measuring soil chemistry, extracellular enzyme activities, and microbial community composition monthly at a thirty year field experiment that crossed N- and P-fertilization with different fire management treatments in a tallgrass prairie ecosystem, we found strongest support for the product-inhibition hypothesis as a result of higher N conditions, conditional support for the copiotrophic hypothesis to N-fertilization, weaker support for both hypotheses to P-fertilization, and weakest support for the soil pH shift hypothesis. The cellular level product-inhibition feedback, in which higher bioavailability of a nutrient lowers the synthesis of enzymes that catalytically release the nutrient from SOM, was broadly supported under higher N conditions from N-fertilization or fire suppression, but not under P-fertilized conditions. The population level copiotrophic feedback, in which fertilization increases relative abundance of microbial populations that tradeoff growth over extracellular enzyme synthesis, had moderate support in population relative abundance responses under both N- and P-fertilization, but evidence for resource acquisition tradeoffs was only seen in the enzyme activities measured under N-fertilized conditions in unburned prairie soils. Soil pH response to fertilization was weak, and there were no strong correlations between enzyme activity and soil pH to implicate in mediating the biological responses. Consequently, we suggest that SOM decomposing enzyme responses to eutrophication are more consistently regulated by the nutrient and energy demand of microbial cells under local environmental conditions, not by a universal tradeoff in resource

acquisition for faster growth of copiotrophic populations, and that soil pH mediated feedbacks are more likely to occur in different soil types than the one found at this site.

Strongest support for the product-inhibition hypothesis, only under higher N conditions

The cellular level product-inhibition feedback mechanism was supported under higher N conditions, where N-acquiring enzyme activities, but not other functional enzyme groups, declined as bioavailable N increased from N-fertilization or fire suppression. A recent meta-analysis of experiments in a variety of biome types also supports the generality of this phenomenon by finding N-fertilization rates at or above $10 \text{ g N m}^{-2} \text{ yr}^{-1}$ often result in lower soil NAG activities, and that soil BG and PHOS activities tend to increase at the same N-fertilization rates (Jia et al. 2020), suggesting soil microbes become less N-limited, relative to other elements, with N enrichment. This mechanism has also been broadly supported across varying soil types, and a variety of aquatic and terrestrial habitats (Zeglin et al. 2007, Sinsabaugh et al. 2009). Importantly, our study shows that this product-inhibition response to N is not exclusive to the context of mineral N-fertilization, since microbial investment to acquire N was also lower (i.e., C relative to N increased) in unburned prairie soils. In tallgrass prairie, fire suppression is known to increase bioavailable N (Blair 1997, Connell et al. 2020), including at this experiment where soil N availability is 3 to 5 times higher under fire suppression (Nieland et al. 2021); thus, product-inhibition feedback on N-acquiring soil microbial enzyme activities was apparent regardless of the driver of increased soil N availability.

In contrast, chronic P-fertilization did not decrease relative PHOS activities. This was somewhat surprising since soils at this site have low levels of bioavailable P relative to N (Johnson et al. 2003), and P-fertilization at other sites, including grasslands, can decrease microbial enzymatic investment for P (Allison and Vitousek 2005, Riggs and Hobbie 2016).

However, there are possible soil chemical and biological reasons for this result. First, the P-fertilization rate at this experiment is an order of magnitude lower than that of N, and soil P availability is low in these soils due in part to low inorganic P solubility and rapid sorption to calcium or iron minerals (Ippolito et al. 2010, Pierzynski 2016); thus it is possible that chronic P-fertilization did not relieve limitation of microbial P requirements. Second, increased P availability has minimal effects on aboveground net primary productivity at this tallgrass prairie site (Avolio et al. 2014) since the dominant grasses here rely heavily on arbuscular mycorrhizal (AM) fungi to uptake P from the soil solution, such that AM colonization may be more important for P-acquisition than phosphatases for plant growth (Wilson and Hartnett 1997, Johnson et al. 2015). Reduction of root-AM relationships also decreases soil aggregation and SOM accumulation (Wilson et al. 2009). Therefore, soil microbial responses to P-fertilization in this experiment are likely restricted by soil mineralogy, and feedbacks on SOM dynamics are more likely related to loss of plant-fungal symbioses following P-fertilization than to enzyme product inhibition in the bulk soils.

Weaker, conditional support for the copiotrophic hypothesis

The copiotrophic feedback mechanism was supported most strongly under dual nutrient fertilization, which produced more significant and stronger changes in the relative abundance of putative copiotrophic and oligotrophic groups than did N- or P-fertilization alone. Similar soil microbial groups across different ecosystems have positively responded to N-fertilization (Ramirez et al. 2012, Leff et al. 2015), so greater N availability appears to support the growth of certain taxa with a competitive advantage under non-limiting growth conditions (Fierer et al. 2007). But, in contrast to this hypothesis, there was no evidence for a tradeoff between microbial population growth and resource acquisition, since dual nutrient fertilization did not decrease soil

EEAs. Thus, in contrast to results of laboratory experiments in which N addition decreased soil EEAs concurrent with shifts in microbial community composition (Ramirez et al. 2012), “copiotrophic” turnover in bacterial and archaeal community composition should not necessarily be expected to affect ecosystem scale SOM decomposition dynamics, because of limited effects to soil enzymatic feedbacks. Only under fire suppression combined with N-alone fertilization did all cellulolytic (BG and CBH) and NAG activities decrease significantly, despite weaker shifts in copiotroph and oligotroph relative abundance. Soil N availability was approximately 40 times higher in this treatment than in the burned, unfertilized treatment (Nieland et al. 2021), suggesting that a threshold of high N load must be crossed before any growth-enzyme production tradeoff occurs, and that functional changes can be independent of microbial community turnover. This may help explain why chronic N-fertilization does not change soil C concentrations across many grasslands (Keller et al. 2022) despite some consistency in microbial taxonomic responses among the same distributed experiments (Leff et al. 2015).

Weakest support for the soil pH shift hypothesis

Soil enzyme activity was not well correlated with soil pH, suggesting that the small increase in soil acidity from chronic N-fertilization is not a primary driver of SOM decomposition feedbacks at this experiment. Recent meta-analyses compile evidence that long-term N-fertilization often lowers soil pH (Ouyang et al. 2018, Jia et al. 2020), which can in turn decrease the activity of soil enzymes by moving away from their pH optima for maximum catalytic activity or by decreasing soil microbial biomass and its activity (Treseder 2008, Turner 2010, Sinsabaugh and Follstad Shah 2012). For example, 13 years of N-fertilization in hardwood and pine forests lowered soil pH in the O-horizon by 0.2-0.4 units and led to a decrease in soil microbial biomass and soil microbial respiration (Bowden et al. 2004, Compton et al. 2004).

However, this study site might be different because of soil characteristics. Limestone parent material and base cation levels near saturation in these soils (Ransom et al. 1998) help to buffer soil pH for longer periods of time. Therefore, soil pH change seems to be happening much slower at this experiment relative to other locations, and thus becomes a weaker factor in regulating ecosystem functional shifts measured after 30 years of fertilization.

Temporal dynamics of soil microbial structure and function

There was no consistent monthly shift in soil microbial community structure or extracellular enzyme activity over the sampling time course, despite the strong seasonality of temperature and plant growth in this temperate ecosystem (Knapp et al. 1998). This observation contrasts with Mediterranean or snowpack-dominated ecosystems, where strong links between soil microbial taxonomic turnover and C and N cycling can occur (Schmidt et al. 2007, Cruz-Martínez et al. 2009). During the same time course of sampling at this experiment, we observed a large increase in bacterial gene copy number during June, July, and August, across all control and N-fertilized field treatments (Carson and Zeglin 2018), which we attributed to higher labile C availability from root exudation during the plant growing season. The lack of seasonality in C cycling enzyme activities suggests that root exudate C may be rapidly assimilated and sequestered into enzyme-protected pools following bacterial death (Bai and Cotrufo 2022), or that a large proportion of active soil enzymes are stabilized and not rapidly responsive to changes in substrate availability (Burns 1982). In support of the latter possibility, there was no transient decrease in N-acquiring enzyme activity following fertilizer application in June, despite a pulse in nitrification potential at that time (Nielsen et al. 2021), and there was a consistent increase in enzyme activity across all field treatments through the sampling time course, perhaps in response to increased root tissue input through what was a relatively wet year (Carson and Zeglin 2018).

Interestingly, however, both soil microbial communities and enzyme activities varied through time in a manner dependent on whether or not the soils were fertilized with P. While most interactions between P-fertilization and time on soil enzyme activities were idiosyncratic, soil microbial communities not fertilized with P clearly shifted across the year, converging after the fertilization application in June (Appendix A Figure A-5). There are likely to be factors beyond fertilization and fire management playing out at different time scales that affect soil microbial community composition and soil enzymatic feedbacks, not necessarily in a synchronous manner.

In addition to elevated N availability and lower N-acquiring enzyme activities, chronic fire suppression also caused higher cellulolytic enzyme activities, likely driven by the multiannual accumulation of aboveground litter that is not consumed by fire (Ajwa et al. 1999, Pellegrini et al. 2020). However, POX activity did not respond to fire suppression, despite this increase in litter input and a concurrent shift toward greater woody plant cover, which adds more litter with higher lignin content (Norris et al. 2001, Carson et al. 2019). One reason for this may be related to the limited change in the soil fungal community following fire suppression: Fungal communities in unburned prairie soils are not dominated by the lignin-decomposing basidiomycetes typical of forest soils, despite concurrent woody encroachment (Morrison et al. 2016, Carson et al. 2019). This stability in the fungal community has implications for decomposition responses to N fertilization: In forests, higher N availability slows decomposition because of the decline in basidiomycete relative abundance and soil oxidative enzyme activity (Frey et al. 2004, Edwards et al. 2011 p. 20). In other grassland soils, N fertilization tends to either increase or not change oxidative enzyme activity (Keeler et al. 2009, Riggs and Hobbie 2016). In our study, N-fertilization also did not affect POX activity even under fire suppression and woody encroachment, showing an apparent “grassland-like” legacy to soil microbial

community functioning under prairies that have transitioned from a grassland state to a woodland state (Ratajczak et al. 2014, Hawkes and Keitt 2015).

Summary and conclusions

The product-inhibition feedback (cellular level), copiotrophic feedback (population level), and soil pH shift feedback (ecosystem level) mechanisms we evaluated are likely active over different time scales in response to N eutrophication, so there is a possibility that soil microbial community function and composition responses emerged at different points in time during the three decades of treatment. On the cellular level, increased N availability from fertilizer application in soils with no fertilization history may quickly repress N-acquiring enzyme synthesis, but soil microbial response to one pulse of N is likely short-lived as immobilization and plant uptake also quickly reduces N availability in the soil solution (Dell and Rice 2005). Any changes to microbial community composition from early fertilization events may have also been ephemeral, as there was no strong shift in response to a one-time fertilization event of N in a switchgrass field with no recent fertilization history (Chen et al. 2019). But, as soil N pools begin to increase due to accumulation of higher quality plant litter following multiple growing seasons of N fertilization, or from total accumulated aboveground litter resulting from fire suppression (Johnson and Matchett 2001, Connell et al. 2020), the alleviation of microbial N-limitation could transition from being transient to persistent, which might explain the lack of fertilizer event responses to soil enzyme activities in this experiment after thirty years of fertilization. Moreover, elevated N availability from internal mineralization rather than supplemental addition could also support the growth of copiotrophs over time (Hart et al. 1994, Blaško et al. 2013), and their associated smaller biomass pool could explain the small decrease in soil microbial biomass in N-fertilized and unburned prairie soils at this experiment and others

(Nieland et al. 2021). Although soil pH is not a leading mechanism for altered SOM decomposition potential at our experiment, this mechanism can shape soil microbial responses at other sites on a faster trajectory; for example, soil pH declined within a decade of annual N-fertilization at a different tallgrass prairie with sandy soil that resulted in significant shifts in the soil bacterial and archaeal community composition (Widdig et al. 2020). Given the potential for strong changes to abiotic (i.e., SOM pools and pH) and biotic (i.e., microbial populations) soil properties from fertilization, soil microbial responses may reverse in a similar manner if chronic fertilization is ceased (e.g., Clark *et al.* 2009; Bowman *et al.* 2018; Nieland *et al.* 2021), but with significant, and potentially asynchronous, time lags for recovery of both soil microbial community function and composition (Gilliam et al. 2019). In addition, fire suppression may continue to constrain these microbial responses and recovery.

Our repeated measurements of the soil microbial community composition and enzyme activities found cellular level product-inhibition feedbacks tied to microbial nutrient demand can vary between nutrients, with their sensitivities to fertilization depending on baseline nutrient levels. Specifically, N-fertilization reduced microbial investment to acquire N because it alleviated N-limitation and the need to produce N-acquiring enzymes, but this phenomenon did not occur under P-fertilization. Additionally, there was limited evidence for a tradeoff between microbial population growth and nutrient acquisition; therefore, mild shifts to the soil microbial community may not result in drastic changes to enzymatic feedbacks tied to SOM decomposition, instead a threshold-response dynamic may be more widespread. Soil properties at this site prevented soil pH to decrease, and thus had minimal effect on soil enzyme activities. In conclusion, changes to nutrient availability induced by global change drivers and land management practices at the local level will affect the N and P cycles in different ways since

microbial nutrient demand is constrained by local environmental conditions (Burns et al. 2013), but soil microbial community change may not always portend a slower breakdown of organic matter supplied by the aboveground world.

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Table 2-1. Soil chemistry in soils with different fire and fertilization treatments.

Fire	Fertilization	<u>g water g⁻¹ dry soil</u>	Soil %C	Soil %N	Molar soil C:N	Soil pH
		GWC				
Burned	Control	0.259 (± 0.057) ^a	2.45 (± 0.05) [°]	0.21 (± 0.00) [°]	13.6 (± 0.3) ^{°a}	5.50 (± 0.23)
	+N	0.258 (± 0.060) ^a	2.44 (± 0.45)	0.22 (± 0.04)	13.1 (± 0.4) ^b	5.05 (± 0.12)
	+P	0.272 (± 0.080) ^a	2.81 (± 0.29) [°]	0.23 (± 0.02) [°]	14.1 (± 0.4) ^{°a}	5.34 (± 0.06) [°]
	+N+P	0.269 (± 0.061) ^a	2.52 (± 0.16) [°]	0.22 (± 0.02) [°]	13.4 (± 0.3) ^{°b}	5.24 (± 0.25)
Unburned	Control	0.237 (± 0.066) ^b	2.66 (± 0.33)	0.23 (± 0.03)	13.5 (± 0.5) ^a	5.23 (± 0.19) [°]
	+N	0.224 (± 0.042) ^b	2.93 (± 0.77)	0.27 (± 0.07)	12.7 (± 0.2) ^b	5.36 (± 0.67)
	+P	0.233 (± 0.056) ^b	2.34 (± 0.24)	0.20 (± 0.03)	13.5 (± 0.3) ^a	5.40 (± 0.12)
	+N+P	0.236 (± 0.065) ^b	3.00 (± 0.44)	0.27 (± 0.04)	13.2 (± 0.1) ^b	5.40 (± 0.66)

Values are given as means (± standard deviation)

+N, nitrogen-alone; +P, phosphorus-alone; +N+P, nitrogen- and phosphorus-fertilized

° denotes only three plot samples instead of four

Letters within the same column indicate differences between fire treatments (GWC) and N-fertilization (molar soil C:N) with different letters indicating significant differences based on Tukey post-hoc tests.

Table 2-2. Permutational analysis of variance (PERMANOVA) results for soil microbial communities under different fixed effects.

Fixed effects	Degrees of freedom	Pseudo- <i>F</i>	<i>R</i> ²	<i>P</i> -value
Fire (F)	1	7.98	0.023	0.001
Nitrogen (N)	1	9.80	0.028	0.001
Phosphorus (P)	1	12.39	0.035	0.001
Month (M)	10	1.56	0.045	0.001
F × N	1	4.64	0.013	0.001
F × P	1	4.32	0.012	0.001
N × P	1	2.32	0.007	0.008
F × M	10	0.95	0.027	0.660
N × M	10	0.97	0.028	0.575
P × M	9	1.35	0.035	0.006
F × N × P	1	3.97	0.011	0.001
F × N × M	10	0.82	0.024	0.979
F × P × M	9	0.85	0.022	0.937
N × P × M	9	0.89	0.023	0.869
F × N × P × M	9	0.65	0.017	1.000
Residuals	227		0.650	

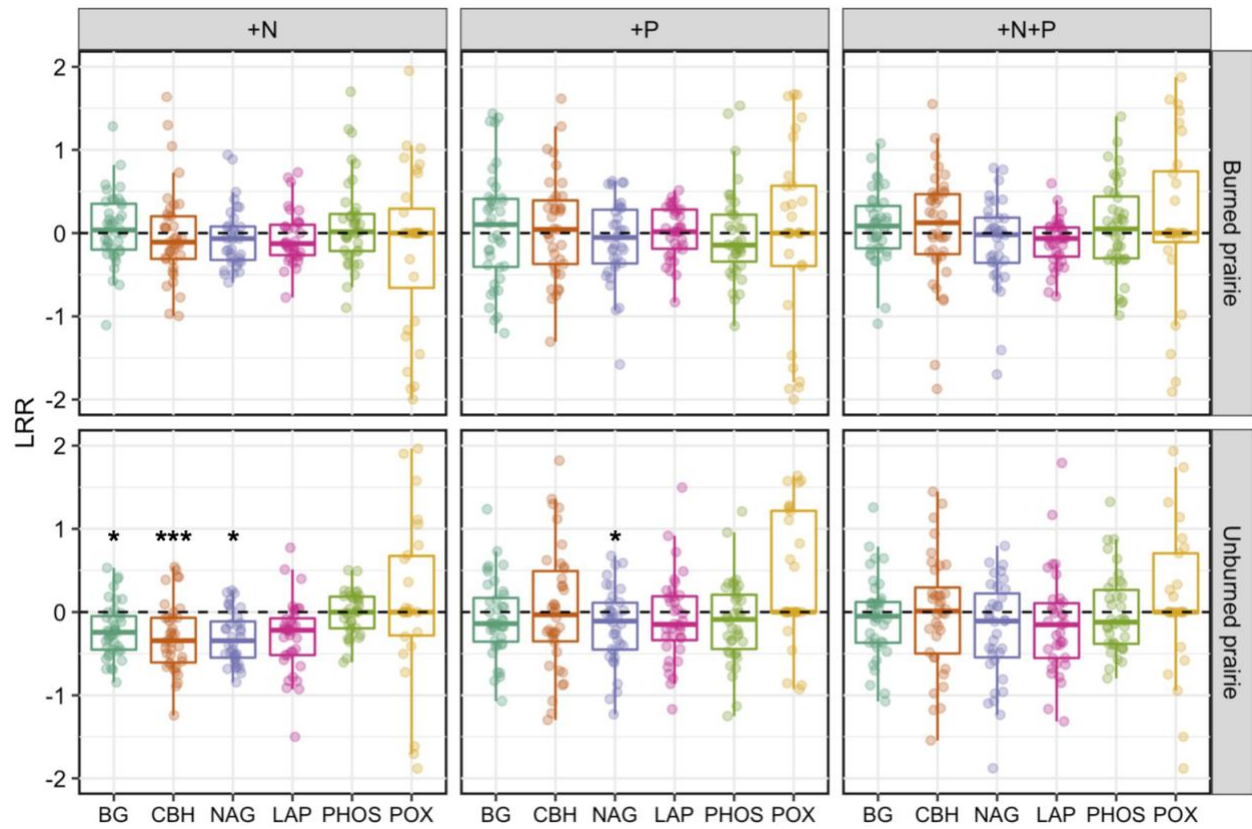


Figure 2-1. Log response ratios (LRRs) of extracellular enzyme activities from fertilization separated by fertilization (+N, N-alone; +P, P-alone; +N+P, N- and P-fertilized) and fire treatments. Each point is a LRR for the fertilization treatment within each experimental block at one sample month. Asterisks above boxplots indicate statistical difference from zero at the $*P \leq 0.05$, $**P < 0.01$, and $***P < 0.001$ thresholds.

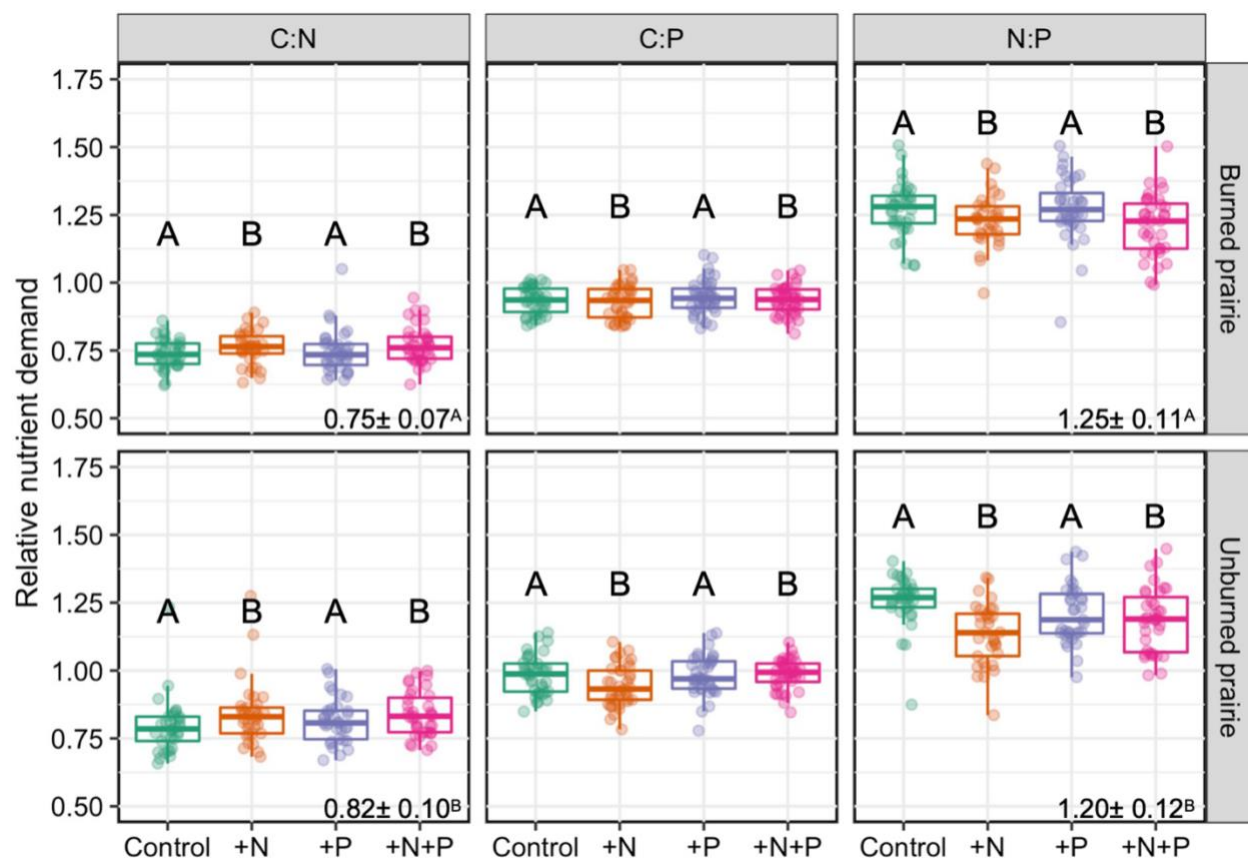


Figure 2-2. Relative soil microbial demand for C:N, C:P, and N:P during the study time course summarized by boxplots. Each point is the ratio between C-, N-, and P-acquiring enzymes for each plot corresponding to the fertilization treatment (+N, N-alone; +P, P-alone; +N+P, N- and P-fertilized), represented by color. Capital letters within boxplots or near mean values for fire treatments indicate group differences at $P \leq 0.05$.

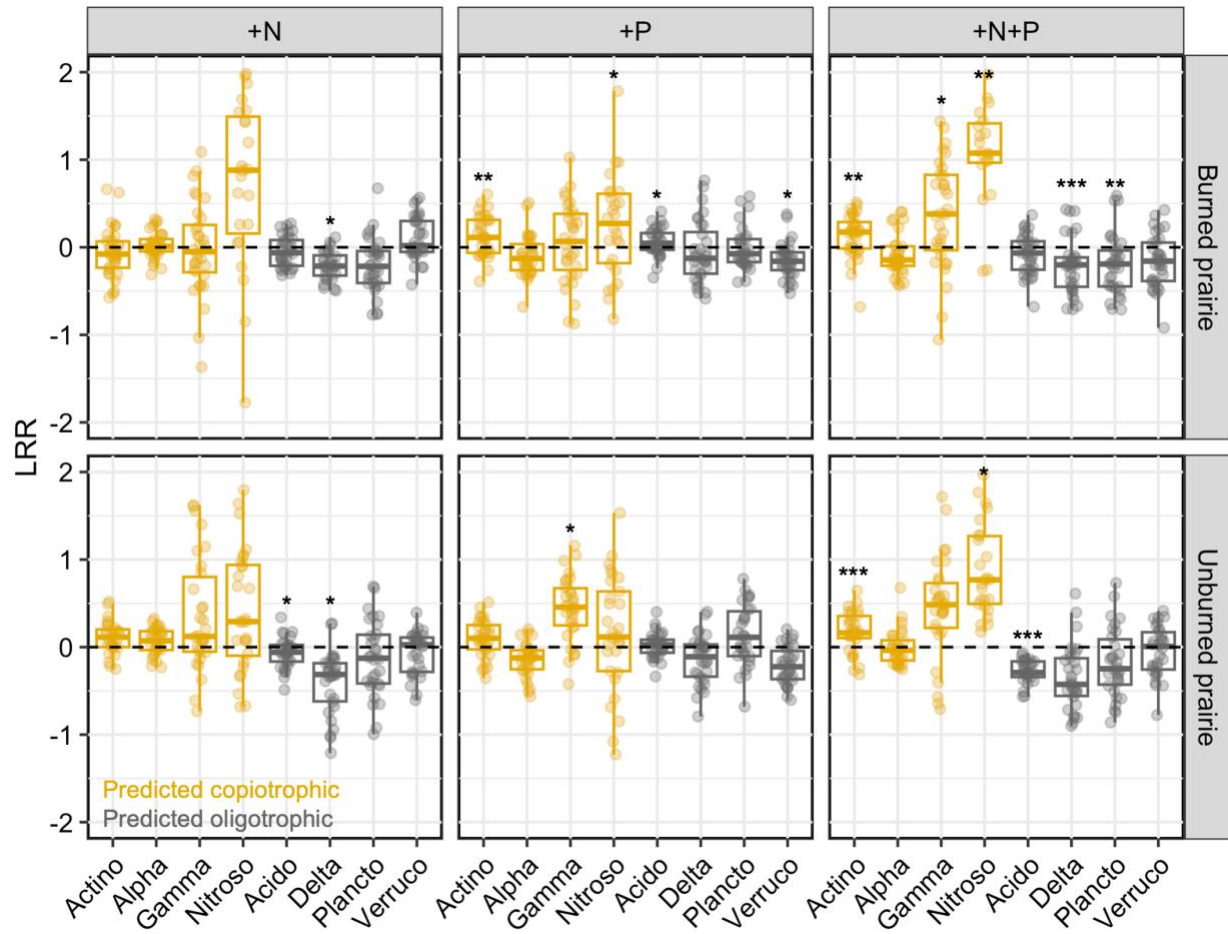


Figure 2-3. Log response ratios (LRRs) of microbial phyla and classes predicted to be copiotrophs or oligotrophs, separated by fertilization (+N, N-alone; +P, P-alone; +N+P, N- and P-fertilized) and fire treatments. Each point is a LRR for the fertilization treatment within each experimental block at one sample month. Asterisks above boxplots indicate statistical difference from zero at * $P \leq 0.05$, ** $P < 0.01$, and *** $P < 0.001$ thresholds. Labels on x-axis are shortened for clarity [Actino, Actinomycetota (formerly Actinobacteria); Alpha, Alphaproteobacteria; Gamma, Gammaproteobacteria; Nitroso, Nitrososphaera (formerly Thaumarchaeota); Acido, Acidobacteriota (formerly Acidobacteria); Delta, Deltaproteobacteria; Plancto, Planctomycetota (formerly Planctomycetes); Verruco, Verrucomicrobiota (formerly Verrucomicrobia)].

Chapter 3 - Differential resilience of soil microbes and ecosystem functions following cessation of long-term fertilization

Nieland, M. A., P. Moley, J. Hanschu, and L. H. Zeglin. 2021. Differential Resilience of Soil Microbes and Ecosystem Functions Following Cessation of Long-Term Fertilization. *Ecosystems* 24:2042-2060. Reproduced with permission from Springer Nature.

3.1 Abstract

Nitrogen (N) from anthropogenic sources has dramatically increased in terrestrial ecosystems globally. Although belowground microbial processes and events that release N into the atmosphere, such as fire, could support ecosystem resilience to eutrophication, little is known about how these factors might affect N loss following chronic fertilization, thus promoting ecosystem recovery. We studied how N pools, N-cycling potential rates and their affiliated microbial populations, and microbial community composition responded to the factorial effects of cessation of 30 years of chronic N fertilization and annual burning at a tallgrass prairie. Soil N availability in previously fertilized plots recovered by 86% (using never-fertilized control plots as a 100% recovery reference), while plant and microbial biomass did not change. Nitrification potential recovered (80%), and denitrification potential partially recovered (50%), in previously fertilized plots. There were differential responses among key groups of nitrifiers (archaea vs. bacteria) and denitrifiers (*nosZ* clade I vs. clade II from *Anaeromyxobacter dehalogenans*) to long-term N fertilization and cessation despite the whole microbial community composition not shifting significantly after fertilization ceased. Also, N-cycling potentials were consistently higher in unburned prairie. Together, results suggest that fire is a prominent mechanism for ecosystem N removal in annually burned prairie, while N-cycling microbes will have an important role in the absence of fire; however, the recovery to pre-fertilized conditions, with or

without fire, will take longer than 1 year. Overall, differential resilience of biotic populations and processes can potentially shape different outcomes of soil N loss and tallgrass prairie ecosystem recovery from long-term N fertilization.

3.2 Introduction

Across the globe, nitrogen (N) availability has increased substantially in terrestrial ecosystems that were historically limited in N (Galloway et al. 2008, Schlesinger 2009). Elevated atmospheric deposition of N from fossil fuel emissions (Vitousek et al. 1997) and increasing rates of N fertilizer application to croplands (Fixen and West 2002) have augmented N availability for plants and soil microbes. This terrestrial “eutrophication” tends to increase aboveground net primary productivity (Gough et al. 2000, LeBauer and Treseder 2008) but can also lower the richness of plant species (Stevens et al. 2004, Clark and Tilman 2008), and cause turnover of plant functional groups (Suding et al. 2005) particularly the loss of plants that are more competitive in nutrient-poor soils (Tilman and Wedin 1991). Belowground, the linked composition and function of soil microbial communities is also responsive to N fertilization (Ramirez et al. 2012), with a consistent taxonomic response across many grassland soils (Leff et al. 2015). Microbial biomass often decreases with N addition in non-agricultural soils (Treseder 2008, Zhang et al. 2018), while nitrification and denitrification activities, and the populations that carry out these processes, tend to increase (Lu et al. 2011, Ouyang et al. 2018). Together, both plant and microbial dynamics underlying ecosystem N-cycling may shift substantially with chronic N addition.

In some terrestrial biomes, like the tallgrass prairie, suppressing natural disturbances such as fire can also increase total soil N by preventing the combustion of organic N from plant litter

(Raison et al. 1985, Ojima et al. 1994). Shifts from high to low fire frequency can concurrently promote net soil N accumulation (Turner et al. 1997) and increase N loss over time (Dell et al. 2005) from leaching and denitrification, pointing to higher N-cycling rates and changes in the microbiota driving these processes. While microbes are consistently responsive to N addition in prairie soils with contrasting fire regimes, the magnitude of response is substantially higher in soils with elevated baseline N availability due to fire suppression (Carson and Zeglin 2018). The consequences of interactions between these two common global change factors, fire suppression and increased N availability, may allow the establishment of woody plant species (Silva et al. 2013) and further increase total soil N. Furthermore, while both fire and microbial activity have substantial roles in ecosystem N loss following chronic fertilization, they have rarely been compared as mechanisms for ecosystem recovery from chronic fertilization (Stevens 2016).

Ecosystem resilience to eutrophication can be assessed by manipulating nutrient availability. Ecological states, measured as key population sizes, community metrics, and ecosystem process rates, often respond to altered environmental drivers, including exogenous N addition. The sensitivity of a state to an altered driver reflects the magnitude of responsiveness (Smith et al. 2009, Ratajczak et al. 2018). The resilience of the state to a driver is assessed by measuring the recovery, defined as the time until, or magnitude of, the sensitive state's return to a prior or present reference control upon ceasing the driver (Grimm and Wissel 1997, Scheffer et al. 2015, Hodgson et al. 2015, Angeler and Allen 2016, Ratajczak et al. 2018). Thus, a more resilient state recovers more quickly, or rebounds by a greater magnitude in the same period of time. Resistance, the inverse of sensitivity, is the state's lack of response to a driver ((Grimm and Wissel 1997, Hodgson et al. 2015, Angeler and Allen 2016). Tracking multiple biotic and abiotic ecological states under current or ceased drivers allows for an understanding of how interactions

of drivers, such as fire and fertilization, affect a state's resilience and an assessment of potential legacy effects (Gilliam et al. 2019). Assessing the sensitivity and recovery of prairie ecosystems with different fire regimes to long-term N fertilization will aid our understanding of the mechanisms underlying ecosystem responses to anthropogenic change, both now and into the future.

Microbial diversity can modulate ecosystem responses to environmental change (Folke et al. 1996, Loreau 2001). A recent meta-analysis showed that the compositional or functional properties of soil microbes to abiotic change, while driver- or site-dependent, are often sensitive to both short- and long-term alterations, with a somewhat surprisingly low prevalence of recovery (Shade et al. 2012). Thus, differential and asynchronous responses of soil microbes to chronic fertilization can contribute to the sensitivity and recovery of ecosystem processes, including the N cycle (Bardgett and Caruso 2020). Ammonia oxidizing archaea (AOA) and bacteria (AOB), for example, complete the first step in nitrification as a requirement of their chemolithoautotrophic lifestyle, but differences in AOA and AOB sensitivity to added N substrate can moderate rates of nitrate (NO_3^-) production (Verhamme et al. 2011, Prosser and Nicol 2012, Carey et al. 2016). Because of their differences in growth rate and activity, shifts in AOA and AOB abundances, quantified as the AOA:AOB ratio, can indicate changes to the nitrification potential and NO_3^- availability in soil (Prosser 1990, Jung et al. 2011, Prosser and Nicol 2012), with nitrification potentials correlating more positively with AOB abundance (Di et al. 2010, Carey et al. 2016). Denitrifier responses to N fertilization, in contrast, may be weaker and less coherent due to the facultative nature of denitrification, as well as the reliance of some N_2 producers on other soil microbes for N_2O production (Sanford et al. 2012, Hallin et al. 2018). Yet, denitrifiers together regulate the loss of soil N as leached NO_3^- , inert atmospheric gas, or

heat-trapping atmospheric gas. Therefore, in order to understand ecosystem resilience, the sensitivity and potential recovery of soil microbes and their functional traits need to be addressed, especially following chronic changes to N availability.

Nitrogen loss from a tallgrass prairie ecosystem following long-term fertilization could be underpinned by either fire or microbes. Dominant grasses in tallgrass prairie (e.g., *Andropogon gerardii*) can assimilate and retain substantial amounts of N (Tilman and Wedin 1991). Higher N mineralization along with greater plant uptake can increase the aboveground biomass and N content in plant tissue (Tilman and Wedin 1991). Fire, therefore, could ameliorate nutrient eutrophication by combusting plant litter N and preventing its remineralization. However, soil N mineralization in the absence of fire can remain elevated up to 12 years following fertilizer cessation (Clark et al. 2009, O'Sullivan et al. 2011), during which time subsequent belowground N loss mechanisms depend heavily on soil microbes. Nitrification and denitrification, together with mineralization, would support ecosystem N loss through increased leaching and gaseous N emission. After decades of N addition, a lack of recovery in soil microbial populations and their functions may result in high potential for N loss even in the absence of fertilizer, independent of fire.

We assessed the strength of mechanisms underlying ecosystem resilience to chronic N enrichment by ceasing long-term N fertilization at a 30-year grassland field experiment with contrasting fire treatments (annual fire versus no fire since 1986). During the first growing season without N application (2017), we measured the sensitivity and recovery of plants and soil microbes in burned and unburned prairie using long-term plant data collected from unfertilized and fertilized soils and by continuing to fertilize subplots of soil to collect microbial data not obtained in previous years. We hypothesized the magnitude of recovery towards an unfertilized

state relative to long-term fertilized conditions would depend on how directly each functional group's metabolism and growth relied on the supplemental inorganic N. Thus, we predicted microbial nitrifiers would have the greatest recovery from fertilizer cessation, denitrifiers and plants would recover to some extent, and the total heterotrophic microbial community, reliant primarily on soil organic nitrogen, would recover the least. We also expected greater recovery in the annually burned prairie, due to fire's role in reducing N availability in this ecosystem, and higher reliance of all biota on supplemental N in that more N-limited system (Figure 3-1).

3.3 Materials and Methods

Site description and experimental design

This work was performed at Konza Prairie Biological Station (KPBS), a 3,487-hectare tallgrass prairie reserve in the Flint Hills region of Northeastern Kansas near Manhattan, KS, USA (39°05'N, 96°35'W). The Belowground Plots (BGP) experiment, located at KPBS, was established in 1986 by the Konza Prairie Long-Term Ecological Research (LTER) program to evaluate linked plant-soil responses to nutrient amendments and fire. This experiment is situated on Irwin series (fine, mixed, mesic, pachic Arguistolls) silty clay loam soil, has a mean annual precipitation of 835 mm and mean annual temperature of 26.6 °C, and is dominated by perennial C₄ grasses (*A. gerardii*, *Panicum virgatum*) in burned treatments and encroaching woody plants (Carson et al. 2019) in unburned treatments (Carson et al. 2019).

The BGP experiment includes eight 50 m × 25 m field blocks with split-strip plots. Blocks have either burned annually each spring (March-April), or remain unburned since the experiment's inception. A mowing treatment to mimic grazing originally split all the blocks, and N and phosphorus fertilizers were applied at the beginning of each growing season (June) as

strips. For this project, we focused on responses in the unmowed, annually N fertilized ($10 \text{ g N m}^{-2} \text{ y}^{-1}$ as NH_4NO_3) versus unfertilized control plots ($12.5 \text{ m} \times 12.5 \text{ m}$). In 2017, fertilizer application at the BGP ceased, while long-term plant biomass and community data collections continued, allowing for a comparison of plant recovery. To enable recovery assessment of the soil microbial parameters not monitored prior to 2017, we continued to fertilize four $1 \text{ m} \times 1 \text{ m}$ subplots randomly within each historically fertilized plot. Thus, with each field treatment having four separate replicate plots ($n = 4$), we compared three fertilization treatments (control, recovering, and fertilized), crossed with burned and unburned treatments, for soil chemistry and microbial responses.

Soil collection

Soils were collected every five weeks between April and September 2017. In June, samples were collected one week after fertilizer application on the subplots. Four 2 cm diameter, 20 cm depth mineral soil cores were randomly sampled from each plot and combined for one composite sample per plot and treatment. Fertilized treatment soils were collected similarly by extracting one core from each fertilized subplot and combining them into one composite plot-scale sample. Soils were aseptically sieved to remove particles larger than 4 mm and as many roots as possible, then a ~15 g subsample of each composite sample was stored at -80°C for molecular work, a ~50 g subsample was stored at -20°C for soil chemistry and microbial biomass, and remaining soil was kept at 5°C before N-cycling activity potential assays. All response variable measurements are described below, with more methodological detail available in the Supplemental Text (Appendix B).

Soil chemistry and microbial N-cycling

Soil inorganic N available for plant and microbial uptake was estimated using resin bags buried from June to September (Baer and Blair 2008). The integrated amount of resin-sorbed ammonium ($\text{NH}_4^+\text{-N}$) and $\text{NO}_3^-\text{-N}$ was quantified using a modified indophenol method and VCl_3 /Griess reagent method (Hood-Nowotny et al. 2010) and measured spectrophotometrically with a FilterMax F5 Multimode Microplate Reader (Molecular Devices, San Jose, CA, USA). Soil %C and %N were measured by the Kansas State University Soil Testing Laboratory in Manhattan, KS, USA using a LECO TruSpec CN Combustion Analyzer (LECO Corporation, St. Joseph, MI, USA), and soil pH was measured in a 1:1 slurry of field-moist soil and DI H_2O , on soils collected in September 2017.

Gravimetric water content (GWC) of fresh soil was estimated by drying ~5 g of soil at 105 °C overnight. Microbial biomass C (MBC) and N (MBN) were estimated using the chloroform fumigation-extraction method (Brookes et al. 1985, Vance et al. 1987, Zeglin and Myrold 2013). For fumigated and unfumigated samples, dissolved organic C (DOC) was quantified via combustion using a Shimadzu TOC analyzer (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA), and organic N was converted to $\text{NO}_3^-\text{-N}$ using a persulfate digestion and quantified as noted above. Difference in DOC and extractable total inorganic N between fumigated and unfumigated samples was used to estimate microbial biomass C and N.

Nitrogen cycling activity potential assays were run on fresh soil samples within 48 hours of collection. Nitrification potential (NP) was estimated as the rate at which a saturating concentration of NH_4^+ was oxidized to NO_3^- in 24 hours in an aerobic soil slurry (Taylor et al. 2010). Denitrification potential (DNP) and denitrification enzyme activity (DEA) were measured together. DEA assays were supplemented with glucose and KNO_3 (Groffman et al. 1999) to estimate the maximum enzymatic potential for NO_3^- reduction in each soil sample, while DNP

assays were not amended with nutrients, to estimate the NO_3^- reduction potential attainable under substrate-limited field conditions. Activities were estimated as the rate at which NO_3^- was reduced to nitrous oxide (N_2O) over 1 (DEA) or 4 (DNP) hours in an anaerobic soil slurry, in the presence of acetylene to prevent N_2O reduction to N_2 . N_2O -N was measured using a Shimadzu 2014 GC analyzer (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA).

Molecular microbial analysis

Genomic DNA (gDNA) was extracted from 0.5 g soil using a modified CTAB approach (DeAngelis et al. 2010), and yields were estimated using Quant-iT PicoGreen assay kits (Life Technologies, Grand Island, NY, USA). The abundance of archaeal and bacterial *amoA* genes (Rotthauwe et al. 1997, Mosier and Francis 2008), the bacterial *nosZ* clade I (Henry et al. 2006) and clade II genes (Sanford et al. 2012), and bacterial 16S rRNA genes (Fierer et al. 2005) were estimated using quantitative polymerase chain reactions (qPCR), with non-template controls, melting curves and standard curve efficiencies to assure target specificity (detail in Appendix 1). Gene abundance was calculated from standard curves and normalized against the gDNA yield g^{-1} dry soil. The bacterial and archaeal V4 region of the 16S rRNA gene was PCR amplified using an established one-step protocol (Caporaso et al. 2012), with the exception that only 25 cycles were used instead of 35 (Carson and Zeglin 2018). Sequencing was performed with Illumina MiSeq Technology (Illumina, San Diego, CA, USA) using 2×150 paired end cycles at the Kansas State University Integrated Genomics Facility. QIIME1 bioinformatics software was used to process the raw data collected from the Illumina sequencing run (Caporaso et al. 2010). All raw sequence data are deposited with links in the NCBI BioProject database under accession PRJNA577961 (<https://www.ncbi.nlm.nih.gov/bioproject/>).

Aboveground plant responses

Aboveground biomass of grasses, forbs, and woody plants was collected annually from 1986-2017 at the end of the growing season (September-October) for all plots. Plant mass was clipped in the field at two 0.1 m² quadrats per plot, dead litter removed, live tissue sorted into the functional groups, oven-dried, and weighed. Aboveground net primary production (ANPP) was estimated for each functional group by converting the dry weight accumulated in one year to g m⁻². To evaluate whether plant biomass changed following fertilizer cessation, the 2013-2017 data (four years before and one year after fertilizer cessation) were compared. All data are available at the Konza LTER Data Portal in dataset PBB01 (Blair and Zeglin 2020).

Statistical analysis

Mixed-effect models were used to test the null hypotheses that soil chemistry, microbial, and plant variables had no responses to N fertilization or contrasting fire treatments, or to the cessation of chronic fertilizer application. The mixed-effect models, with a plot within block random effect, were performed for variables with repeated sampling to test the fire, fertilization, and date-of-sampling (i.e., Month or Year) fixed effects by using the lme4 package in RStudio version 1.2.1335 (Bates et al. 2015). Shapiro-Wilk tests and QQ-plots were used to assess the distribution of the response variables, and data were log₁₀ transformed (except for plant biomass which was square root transformed) to meet normality assumptions when needed. The assumption of homoskedasticity for the mixed-effect models was tested by plotting the residual versus fitted values prior to analysis of variance (ANOVA). Tukey's honest significant difference (HSD) post-hoc tests were used to distinguish differences between the levels in the fixed effects or their interactions using the lsmeans package (Lenth 2016). The contribution of the contrasting treatments to variation in soil bacterial and archaeal 16S rRNA gene community composition was assessed using indirect ordination and permutational analysis of variance

(PERMANOVA). Ten iterations of multivariate non-metric multidimensional scaling (NMDS) visualized the Bray-Curtis dissimilarity of microbial communities using the vegan package (Oksanen et al. 2019); the model of best fit (stress = 0.1409) was accepted. PERMANOVA was performed with the Bray-Curtis dissimilarity matrix and comparing the sampled communities to 999 null communities drafted from the composition data. Null hypotheses were rejected when $P \leq 0.05$. More detail for the statistical analysis is found in the Supplemental Text (Appendix 1).

We considered *sensitivity* to chronic fertilization as the change of measured variables in the long-term fertilized treatment relative to the unfertilized control. To quantify the relative level of *recovery* from chronic fertilization, we calculated the percent recovery of the variable of interest using this equation,

$$\left[1 - \left(\frac{\bar{X}_R - 1}{\bar{X}_C - 1} \right) \right] \times 100$$

where $\bar{X}$ is the mean value of the variable in control (C), recovering (R), or fertilized (F) soils.

3.4 Results

Soil available nitrogen

Total available soil inorganic N was always higher in unburned than annually burned treatments and dropped substantially after fertilization ceased in both fire treatments (Table 3-1, Figure 3-2). Average NH_4^+ -N fell from 4.6 and 20.2 mg NH_4^+ -N per kg resin in the chronically fertilized burned and unburned soils, respectively, to 1.4 and 0.6 mg NH_4^+ -N per kg resin in the recovering soils, which was comparable to control soils ($P < 0.001$; Figure 3-2a). Between the fertilized and recovering soils, average NO_3^- -N dropped from 19.8 to 3.5 mg NO_3^- -N per kg resin

in annually burned prairie and from 59.4 to 10.2 mg NO₃⁻-N per kg resin in the unburned prairie, but these levels were still higher than control soils (0.7 and 2.0 mg NO₃⁻-N per kg resin in annually burned and unburned treatments, respectively; $P < 0.001$). The control, annually burned treatment soils had the lowest available NO₃⁻-N and total inorganic nitrogen (Figure 3-2b). The unburned treatment soils had higher NO₃⁻-N availability than the annually burned treatment soils ($P = 0.001$), and the ratio of NO₃⁻-N:NH₄⁺-N was highest in the recovering soils of the unburned treatment ($P = 0.044$; Table 3-1, Figure 3-2b). Total inorganic N availability showed no interaction between fire and fertilization treatment effects, recovered significantly by 86.0% across all soils, yet remained 5 times higher in the recovering versus control treatment soils ($P < 0.001$; Table 3-1).

Soil nitrification potential rates and functional composition

Nitrification potential (NP) rates were higher in both unburned and fertilized soils, and rates significantly declined in the first year of ceasing chronic fertilization to a level comparable to the control soils (Table 3-2, Figure 3-3). Comparing the fertilized and recovering soils, seasonal average NP rates dropped from 1.30 to 0.59 mg N kg⁻¹ dry soil h⁻¹ and from 3.07 to 1.25 mg N kg⁻¹ dry soil h⁻¹ in burned and unburned prairie soils, respectively ($P < 0.001$; Figure 3-3a), corresponding to a 79.9% recovery in both fire treatments. A significant three-way interaction between fire, fertilization, and month reflects temporal variation in NP ($P = 0.038$): in fertilized, unburned treatment soils, rates were significantly higher than all other treatments in June after fertilizer was added, and remained high; in contrast, NP was higher in September than June in all other treatments (Table 3-2, Appendix C Figure C-1a,b).

Soil archaeal *amoA* gene abundance (AOA) and bacterial *amoA* gene abundance (AOB) differentially responded to fire regime and the cessation of long-term N fertilization (Table 3-2,

Figure 3-4a,b). AOB was consistently higher in unburned than annually burned treatment soils ($P = 0.042$), and their abundance in recovering soils was significantly similar to control soils, but lower than chronically fertilized soils, for a 68.3% recovery magnitude ($P < 0.001$; Figure 3-4b). In contrast, AOA did not change with fire regime, was higher in fertilized soils, and showed no significant recovery from chronic fertilization ($P < 0.001$; Figure 3-4a). AOA was higher than AOB by about an order of magnitude, and the AOA:AOB ratio was higher in the annually burned than unburned prairie soils ($17.4 \pm 14.0 > 12.4 \pm 9.3$; $P = 0.015$; Table 3-2). The AOA:AOB ratio did not differ between fertilization treatments. Like NP in control and recovering soils, both AOA and AOB abundance increased as the growing season progressed ($P < 0.001$; Table 3-2, Appendix C Figure C-2a-d).

Soil denitrification potential rates and functional composition

Maximum denitrification enzyme activity potential (DEA) rates were low in April and peaked in July and August, but were not affected by fire or chronic fertilization (Table 3-2, Appendix C Figure C-1e,f). However, with only field-available levels of soluble C and NO_3^- , substrate-limited denitrification potential (DNP) rates were higher in all unburned treatment soils ($P = 0.007$), and rates were similar in the recovering and fertilized soils ($P < 0.001$), with no fire by fertilizer interaction (Table 3-2, Figure 3-3b). Average DNP rates for both burned and unburned fire treatments, respectively, were 35.8 and $77.4 \mu\text{g N}_2\text{O-N kg}^{-1} \text{ dry soil h}^{-1}$ in fertilized soils, 15.2 and $48.9 \mu\text{g N}_2\text{O-N kg}^{-1} \text{ dry soil h}^{-1}$ in recovering soils, and 0.8 and $16.0 \mu\text{g N}_2\text{O-N kg}^{-1} \text{ dry soil h}^{-1}$ in control soils.

Bacterial *nosZ* gene abundances were consistently higher in annually burned than unburned treatment soils (clade I: $P = 0.036$; clade II: $P = 0.016$), but clade I and clade II responses differed in fertilizer response and temporal change (Table 3-2, Figure 3-4c,d,

Appendix C Figure C-2). After post-hoc comparison, *nosZ* clade I gene abundance did not shift in response to long-term fertilization or its cessation, but did increase between April and September like NP, AOA, and AOB. In contrast, after long-term addition of N ceased, the abundance of *nosZ* clade II in *A. dehalogenans* in recovering soils dropped below that in control soils ($P < 0.001$). *NosZ* clade II abundance was an order of magnitude greater than clade I and showed a distinct peak in July in all treatments; thus, the clade I:clade II ratio was most strongly influenced by sampling month ($P < 0.001$; Table 3-2, Appendix C Figure C-2e-h).

Soil microbial biomass and 16S rRNA gene abundance and composition

Microbial biomass C (MBC) and N (MBN) were lower in fertilized and recovering soils relative to control soils ($P = 0.02$; Table 3-2, Appendix C Figure C-3a-d). Both MBC and MBN peaked in August for all fire and fertilization treatments ($P < 0.001$). The MBC:MBN ratio was variable and high, but it matches other MBC:MBN ratios reported from Konza Prairie (Baer and Blair 2008, Zeglin et al. 2013). Also, the MBC and MBN extraction efficiencies were likely different and could explain the variability (Vance et al. 1987). Bacterial 16S rRNA gene abundance was also highest in the control soils and lowest in recovering soils, like the *nosZ* clade II gene ($P = 0.035$; Table 3-2, Figure 3-4e). Soil 16S rRNA abundance was not affected by fire regime and increased through the growing season in all field treatments (Table 3-2, Appendix C Figure C-2i,j).

Soil bacterial and archaeal 16S rRNA gene composition responded to chronic fertilization, and more strongly so in the unburned treatment (Table 3-3, Appendix C Figure C-4), but the soil microbial community composition did not recover following fertilizer cessation. PERMANOVA indicated that more (11.5%) of the variation in community composition was attributed to the fertilizer treatments alone than the fire treatments (5.9%), their

interaction (3.4%), or sampling month (6.8%; Table 3-3). The relative abundance of many common ($\geq 0.10\%$ relative abundance) phyla or classes changed in response to long-term fertilization, fire treatment, or their interaction; however, no group's relative abundance shifted towards recovery in the first year following cessation of chronic fertilization (Appendix C Table C-1).

Plant aboveground biomass

Aboveground net primary productivity (ANPP) changed substantially in response to long-term fire and fertilization treatments, but did not respond to the cessation of fertilization in 2017 (Table 3-2, Figure 3-5). Grass ANPP in annually burned prairie increased by an average of 156% in response to chronic fertilization ($P = 0.020$), and while post-hoc comparisons showed no significance, forb ANPP tended to be highest in the unburned fertilized prairie, but neither changed in the year fertilization was ceased (Figure 3-5a,b). Annual fire suppressed woody plant growth ($P = 0.010$), but neither fertilization nor its cessation affected woody plant ANPP (Figure 3-5c).

Soil chemistry

Soil %C, %N, and pH responded to chronic fertilization, and soil %C and %N also responded to the fire treatments, but no recovery in soil pH, %C, or %N was detected in the first year of fertilizer cessation (Table 3-1, Appendix C Figure C-5a,b,d). After 30 years of fertilization, soil pH decreased from an average of 6.9 in control soils to 6.3 in recovering and fertilized soils ($P < 0.001$; Table 3-1, Appendix C Figure C-5d). Soil %C was highest in unburned and fertilized soils ($P = 0.21$), and soil %N increased in response to both long-term fertilization ($P < 0.001$) and fire suppression ($P < 0.001$; Table 3-1). C:N was variable, but tended to be highest in the annually burned and unfertilized prairie soils. Variation among field

replicates also reflects sub-plot scale heterogeneity that is typical of this and other grassland soils.

3.5 Discussion

We predicted that ecosystem N-cycling processes, and the organisms supporting these functions, would show levels of recovery from chronic (30 years) fertilization that reflect how directly that process or population was supported by supplemental inorganic N. Further, we expected that recovery would be greater in the annually burned, more N-depleted, tallgrass prairie relative to the unburned prairie treatment. Nitrogen availability for both plants and microbes was indeed independently higher in both unburned (3 times greater) and fertilized (30 times greater) treatments than the burned and control treatments, respectively, and most response variables were also sensitive to long-term fertilization (i.e., differences between control and fertilized treatments were statistically significant). During the first growing season in thirty years with no supplemental N addition, N availability in recovering soils recovered by 86%, still 5 times greater than that of the controls, nitrification activity potential recovered by 80%, to levels statistically indistinguishable from that of controls, and while denitrification activity potential decreased 51%, it did not show statistically significant recovery. N-cycling microbial functional populations also showed differential recovery, in that nitrifying bacterial populations recovered by 68%, but nitrifying archaeal populations did not, and both nitrous oxide reducing populations and total bacterial populations were insensitive to the chronic fertilization. Soil microbial biomass, which decreased in response to long-term fertilization, did not recover, and neither did the 16S rRNA gene community composition. Finally, grass biomass was also sensitive to chronic fertilization, but did not recover in the first year of its cessation. The unburned prairie treatment

had elevated soil available N, NP, and DNP relative to the burned treatment, but a comparatively weak plant biomass response to chronic fertilization. This suggests that microbially-mediated N loss could constrain ecosystem recovery in the absence of fire, while plant litter combustion may remain a primary driver for recovery after chronic fertilization in annually burned prairie.

Soil available N sensitivity and recovery

The whole growing season's accumulation of resin-sorbed N dropped significantly in the first year of ceasing N fertilizer application, such that N availability in the recovering soils, relative to the control and fertilized soils, recovered more than 80% in both the annually burned and unburned treatments (Table 3-1, Figure 3-2). This suggests that after 30 years of chronic fertilization, the large majority of available N for plant and microbial uptake, nitrification, and denitrification was derived directly from the annual fertilizer application. In addition, available NO_3^- -N remained five times higher in the recovering versus control soils, reflecting elevated N mineralization and nitrification associated with the legacy of fertilization. Nutrient addition experiments at a Minnesota grassland showed evidence for elevated net N mineralization rates up to 12 years after ceasing long-term N application (Clark et al. 2009). Similarly, net N mineralization in a Netherlands grassland took 20-45 years to decline following fertilizer cessation (Olff et al. 1994). Although we did not directly measure N mineralization rates, a legacy of higher net N mineralization might be supported by the larger total soil N pool in chronically fertilized soils. Notably, the resin-sorbed NO_3^- -N: NH_4^+ -N ratio was highest in the unburned recovering treatment, despite substantially recovered nitrification potential rates in the first year of ceasing N fertilization. This is related to the larger excess of NH_4^+ -N in the unburned, chronically fertilized soils, and points to possible saturation of plant uptake or denitrification activity in the unburned treatment, as we will address later in the discussion. With

all of this in mind, plant and microbe available N in recovering tallgrass prairie soils could remain elevated for an extended period and subsequently feed ecosystem N loss by promoting NO_3^- leaching, supporting gaseous emission from denitrification, and supplying N to plant biomass that is combusted eventually by fire.

Sensitivity and recovery of soil nitrification potential rates and ammonia oxidizers

Nitrification potential (NP) rates also significantly declined in the first year of ceasing chronic fertilization, recovering approximately 80%, and reaching a statistically similar level to the control soils (Table 3-2, Figure 3-3a). At other N cessation experiments, nitrification activity uniformly decreased as NH_4^+ availability declined over time (Stienstra et al. 1994, Olf et al. 1994), and the clear drop of NP in our experiment is also likely explained by the reduced input of soil NH_4^+ . Interestingly, the significant three-way interaction highlights the increase in NP rates later in the growing season in all but one combinatory fertilization and fire treatment (Table 3-2, Appendix C Figure C-1a,b). Plant uptake of N early in the growing season, when shoot and root production is highest in annually in grasses (Knapp 1984, 1985) and new biomass production in woody plants also peaks, can limit available NH_4^+ for nitrifiers and prevent nitrification (Schimel and Bennett 2004). This pattern, indicating plant success over soil nitrifiers in the competition for limiting levels of ammonium, was apparent in all treatments except the unburned, chronically fertilized treatment, suggesting that N may no longer be limiting plant uptake under those conditions. However, because our data do not directly target the interaction, it is not clear whether plant competition for N is related to the recovery of NP rates following cessation of long-term fertilization.

Ammonia oxidizing archaea (AOA) and bacteria (AOB) differentially recovered in the first year of N fertilization cessation. While both groups were sensitive to long-term N

fertilization, AOA showed only a 38% recovery, in contrast with the significant 68% recovery of the AOB (Table 3-2, Figure 3-4a,b), under both burned and unburned conditions. Although AOA and AOB are both considered poor competitors against plants and soil microbial heterotrophs for NH_4^+ (Verhagen et al. 1995, Kaye and Hart 1997, Schimel and Bennett 2004), previous work suggests AOA and AOB differ in response to supplemental N. Increased NH_4^+ availability from N addition can support greater AOB abundance than AOA (Di et al. 2010, Wertz et al. 2012). Other studies detect an increase in AOA abundance when organic N, but not inorganic N, is added (Levičnik-Höfferle et al. 2012, Ouyang et al. 2018). In this study, AOB could have been more directly reliant on fertilizer for substrate, thus recovered to a greater extent when long-term fertilization ceased, or the lack of AOA recovery could be linked to the incomplete recovery of NO_3^- availability or the relatively small but significant changes in pH in the historically fertilized soils (Table 3-1, Appendix C Figure C-5d). While some AOA isolates have higher affinity for NH_4^+ than AOB and are thus better competitors at lower N availability (Martens-Habbena et al. 2009), this cannot be extrapolated to all AOA taxa; similarly, we cannot assume that all AOA prefer low soil pH, though some do (Prosser and Nicol 2012). Finally, though we did not measure the contribution of AOA versus AOB to NP rates, group-specific contributions to nitrification activity are possible (Taylor et al. 2010). Thus, if AOA abundance remains elevated after fertilizer cessation, the complete recovery of nitrification could be prolonged until AOA abundance declines to levels similar to control soils.

Partial recovery of denitrification potential and resistance of denitrifiers

Denitrification potential (DNP) rates, representing activity under ambient (possibly limiting) levels of N and C, was higher in both the recovering and fertilized soils, and were therefore sensitive but less resilient than nitrification to long-term N fertilization (Table 3-2,

Figure 3-3b). Substrate-saturated denitrification enzyme activity (DEA) did not change following, and therefore was resistant to, long-term N fertilization. Together, this suggests that soil NO_3^- or DOC availability, not enzyme abundance, controls denitrification rates in these soils (Firestone et al. 1980, Wallenstein et al. 2006). A lower NO_3^- supply due to recovery of NP could have reduced DNP; however, ample amounts of NO_3^- must have been present to maintain the still-elevated DNP rates in recovering soils. In fact, NP rates remained substantially higher than DEA and DNP rate through the study season, suggesting that DNP was close to its maximum and that N loss through nitrate leaching continued, despite cessation of fertilization. This phenomenon was more likely in the unburned treatment, where the resin-sorbed NO_3^- -N: NH_4^+ -N was highest. The resistance of DEA to chronic fertilization means ecosystem N loss through denitrification might be limited by enzyme abundance when N availability remains elevated.

As the DEA data suggest, bacterial populations with the *nosZ* gene were largely resistant to 30 years of fertilization (Figure 3-4c,d). Under the rationale that these organisms are predominantly facultative, not obligate, denitrifiers, this fits our predictions; yet, the resistance to long-term fertilization was a bit of a surprise given the significant shift in the total bacterial community. Further, patterns of sensitivity to N fertilization and intra-seasonal dynamics were very different between taxa with the *nosZ* clade I and clade II genes (Table 3-2, Appendix C Figure C-2e-h). While the *nosZ* clade I abundance did not respond significantly to long-term N addition, but peaked in September concurrent with NP, the abundance of *nosZ* clade II from *A. dehalogenans* tended to be lower in fertilized and recovering soils and peaked in July. Considering that at least 50% of taxa with the *nosZ* clade II gene lack the genes for the full denitrification pathway (Sanford et al. 2012, Hallin et al. 2018), and because this variant of *nosZ*

appears dominant in the study soils (mean ratio clade II:clade I = 14.5), we might predict a lower N₂O sink and higher losses of N as a potent greenhouse gas in soils recovering from chronic fertilization (Jones et al. 2014), especially late in the growing season. However, although *nosZ* gene abundance often responds positively to N fertilizer addition, it is not a reliable predictor of denitrification rates (Rocca et al. 2015, Ouyang et al. 2018), so we can only speculate on N₂O sink or efflux potentials. Measurement of *in situ* N₂O emission may be a priority for a follow-up study.

Sensitivity and lack of recovery in total microbial biomass and community composition

Total microbial biomass carbon (MBC) and nitrogen (MBN) varied more by month than between the fertilization treatments, but tended to be lower in the recovering and fertilized soils than the control soils, which agrees with observations from others (Treseder 2008, Zhang et al. 2018). The peak in MBC and MBN in August is likely related to microbes growing more on labile C root exudates and experiencing less competition with plants for inorganic N as the growing season progresses, as also observed by others (Garcia and Rice 1994, Ajwa et al. 1999, Kuzyakov and Xu 2013). While bacterial abundance at this site is higher through the plant growing season (Appendix C Figure C-2i,j; Carson and Zeglin 2018), this peak was asynchronous with the MBC peak, and the tendency towards lower bacterial 16S rRNA gene abundance in the recovering and fertilized soils was weak (Table 3-2, Figure 3-4e), in contrast with the MBC pattern. Gene abundance cannot be equated directly to cell abundance due to variation in 16S rRNA gene copies per genome of different taxa, and cell abundance does not equate to biomass due to the orders of magnitude of range in cell size among diverse soil microorganisms. In fact, total soil microbial biomass is likely dominated by larger but less abundant fungi, not smaller but more numerous bacteria, as also suggested by the high

MBC:MBN ratios for all field treatments (Rice et al. 1998, Strickland and Rousk 2010). There was no recovery in microbial biomass or 16S rRNA gene abundance from fertilizer cessation; instead, the total 16S rRNA gene abundance declined slightly further in the recovering soils. This could be related to a mild shift in the microbial community composition, since 16S rRNA gene numbers in genomes of different taxa, or even the same bacterial population, can vary (Klappenbach et al. 2000, Větrovský and Baldrian 2013). In the context of ecosystem resilience from chronic fertilization, a legacy of lower microbial biomass in chronically fertilized soils indicates that the potential for microbial N immobilization remains lower after long-term N fertilization ceases, making plant assimilation and coupled nitrification-denitrification more important fates of N as whole-ecosystem recovery progresses.

The soil bacterial and archaeal 16S rRNA gene community composition was also sensitive to N fertilization, but did not change significantly in the first year of cessation (Table 3-3, Appendix C Figure C-4). The compositional shift, including directionality of change in certain high-level taxonomic groups (Appendix C Table C-1), is consistent with many other inorganic fertilization studies (Leff et al. 2015, Carson and Zeglin 2018), and thus seems to support the “copiotrophic hypothesis.” This hypothesis predicts that under high-N conditions, fast-growing microbial taxa dominate in relative abundance over slower-growing taxa that are more competitive in nutrient-poor conditions (Ramirez et al. 2012). If this is true, the lack of recovery is interesting, given that N availability dropped so dramatically with cessation of fertilizer addition. A legacy effect from long-term N fertilization could result from the increase in soil total N pools (Table 3-1, Appendix C Figure C-5) and associated higher N mineralization, or slower growth of putative oligotrophic populations could create a lag in turnover towards their recovered dominance. Alternatively, the pH drop, while less extreme than measured in other

experiments, might also constrain microbial recovery (Liu et al. 2020). Finally, the potential for relic DNA to obscure temporal change is a concern; however, relic DNA proportion was very low in samples collected from this study site, in association with the relatively high base saturation in local soils (Carini et al. 2017). While the mechanism is not immediately clear, the legacy of microbial community shift for at least one year after ceasing N application suggests that there is no longer a direct link between supplemental inorganic N and many soil microbial heterotrophs after 30 years of fertilization (Shade et al. 2012, Bardgett and Caruso 2020).

Plant aboveground sensitivity and lack of recovery

Aboveground net primary productivity (ANPP) increased with fertilization by more than double in the annually burned treatment, but did not decline in the first year after 30 years of fertilization was stopped (Table 3-2, Figure 3-5). Nitrogen limits the growth of perennial grasses in the tallgrass prairie (Seastedt et al. 1991, Blair 1997), and to help overcome this limitation, many grasses retranslocate assimilated N belowground during the annual plant senescence period (Ojima et al. 1994, Dell et al. 2005). Thus, perennial grasses may represent an N reservoir that slows ecosystem recovery from chronic fertilization, despite the recovery of soil NH_4^+ -N availability. Although grass and forb biomass responded to long-term fertilization, woody biomass did not; the increase in woody biomass in unburned prairie reflects the well-documented plant community shift toward woody dominance that occurs in prairies following fire suppression (Briggs et al. 2005, Bond 2008). While N remains elevated in plant biomass, fire will be a significant factor for ecosystem N removal in the annually burned prairie. If grass tissue in fertilized treatments was 0.4-0.7% N (Norris et al. 2001, Reed et al. 2005, Roley et al. 2018), and all the N in aboveground biomass was combusted, 2.1-3.7 g N m⁻² y⁻¹ in excess of the unfertilized treatment would be lost to fire. This loss will depend on N concentration in plant

litter, and will be less than the senesced tissue amount, since ~10% of litter N can be incorporated into the soil organic matter in one winter before the typical springtime prescribed fire application (Cotrufo et al. 2015). Still, this substantial N efflux reduces total ecosystem N, and likely underpins differences in total N accumulation due to chronic fertilization (Appendix C Figure C-5), and recovery of the N cycle following its cessation, between burned and unburned prairie.

Fire history effects on microbial N fertilization sensitivity

Many N-cycling rate potentials and microbial populations were higher in unburned treatment soils, independent of fertilization (Table 3-2). While we were somewhat surprised to see no interactions in N-cycling attributes, given the significant interaction detected in the plant and microbial community change (Carson and Zeglin 2018, Carson et al. 2019), this shifted baseline is important. The unburned unfertilized treatment had more than double the available N, almost 4 times the NP and 20 times greater DNP than the burned unfertilized treatment (Figures 3-2 and 3-3). It is also important to note that in addition to increasing N availability, fire suppression contributes to soil C retention through litter accumulation (Appendix C Figure C-5), especially in woody-encroached areas (McKinley and Blair 2008), which could additively support higher denitrification (Firestone et al. 1980, McCulley et al. 2004, Wallenstein et al. 2006). If the DNP rates in unburned recovering soils, integrated across the whole sampling season (late April – late September), were attained in the field, $\sim 22.7 \text{ g N m}^{-2} \text{ y}^{-1}$ could be lost through microbes to the atmosphere. This estimate is an order of magnitude greater than the estimated N loss to fire in annually burned prairie.

3.6 Conclusion

In summary, our observations suggest that competition between plants and microbes for available N, with or without fire in the ecosystem, will constrain the lag time and primary mechanisms of N loss via fire or microbial activity, and thus the resilience of this tallgrass prairie ecosystem to chronic fertilization. Many of the soil, plant, and microbial responses to fertilizer cessation fit our predictions and agree with our hypothesis and conceptual model (Figure 3-1), particularly in that the mediating effect of fire was associated with greater recovery in the first year after ceasing fertilization. Yet, both plant and soil N pools will remain important reservoirs of N as the recovery process proceeds, such that they could represent an “N bank” somewhat analogous to that now hypothesized to underlie lags in N-cycling processes in successional forest ecosystems (Lovett et al. 2018). Suppressing fire, furthermore, can induce similar belowground effects as long-term N fertilization in burned prairies, so the relative importance of these two global change factors might depend on their context across the globe. As a final projection, soil total N would have increased by 0.14% if the 30 years of added N (300 g N m^{-2}) was retained in the top 20 cm of soil, with an estimated bulk density of 1.097 g cm^{-3} of dry soil (Carson 2013). We measured a mean difference of 0.03 and 0.06% in soil total N between the historically fertilized and never-fertilized soils in the burned and unburned treatments, respectively, so up to 21% (with fire) and 44% (without fire) of the total added N has been retained in the soil organic matter. Soil microbes will partially support ecosystem N release in the burned prairie, but will exclusively drive N loss in the unburned prairie. Our rough, yet reasonable, estimates suggest that it could take 17-30 years (with fire alone) or ~6 years (with denitrification in the absence of fire) for the extraneous N in this ecosystem to be depleted. Thus, the long-term rate of ecosystem recovery may not fit our predictions. If the soil microbial biomass or plant tissues return to a

more retentive state (Dell et al. 2005), if nitrification/denitrification potentials continue to drop, or if soil organic N is not biologically available, recovery will take longer or may never be attained (Gilliam et al. 2019). In conclusion, we show that chronic N addition results in different potential legacy mechanisms for soil N loss and ecosystem recovery because of the differential resilience of biotic populations and processes shaped by contrasting fire management regimes. However, because of the shifting dynamics during the rapid period of change post-fertilizer cessation, more study is needed to ascertain the fate of N in this manipulated prairie ecosystem.

3.7 References

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Table 3-1. Statistical results for two-way ANOVA on the direct and interactive effects of fertilization (“Fertilizer”) and burning (“Burn”) treatments on soil chemistry response variables.

Soil chemistry variables	$F_{(\text{numDF}, \text{denDF})}$		
	Fertilizer	Burn	Fertilizer $\times$ Burn
NH ₄ ⁺ -N ^l	74.49 _(2, 13.2)	3.27 _(1, 13.8)	10.00 _(2, 13.2)
NO ₃ ⁻ -N ^l	60.23 _(2, 13.2)	15.87 _(1, 13.8)	0.81 _(2, 13.2)
NH ₄ ⁺ -N+NO ₃ ⁻ -N ^l	72.25 _(2, 13.6)	13.29 _(1, 14.2)	1.50 _(2, 13.6)
NO ₃ ⁻ -N:NH ₄ ⁺ -N ^l	3.56 ⁺ _(2, 12.8)	13.46 _(1, 13.5)	4.04 _(2, 12.8)
%C	4.25 _(2, 14.1)	21.30 _(1, 14.1)	5.14 _(2, 14.1)
%N	11.96 _(2, 14.0)	35.45 _(1, 14.0)	3.18 ⁺ _(2, 14.0)
C:N	5.41 _(2, 17.0)	0.55 _(1, 17.0)	4.41 _(2, 17.0)
pH	14.68 _(2, 15.0)	1.12 _(1, 15.0)	0.31 _(1, 15.0)

Superscript “⁺” denotes variables that were log-transformed transformed to meet assumptions of normality.

Values bolded signify treatments in which $P \leq 0.05$, and superscript “⁺” indicates where $P = 0.10$ - 0.051 .

Table 3-2. Statistical results for three-way ANOVA on the direct and interactive effects of fertilization (“Fertilizer”), burning (“Burn”), and time (“Month” or “Year”) on soil microbial and plant response variables.

<u>Microbial variables</u>	$F_{(\text{numDF}, \text{denDF})}$						
	Fertilizer	Burn	Fertilizer × Burn	Month	Fertilizer × Month	Burn × Month	Fertilizer × Burn × Month
NP ¹	17.34 _(2, 15.2)	20.34 _(1, 14.9)	0.62 _(2, 15.2)	10.27 _(4, 64.7)	3.10 _(7, 64.8)	0.69 _(4, 64.7)	2.28 _(7, 64.8)
DNP (Subs.-lim.) ¹	13.87 _(2, 15.4)	9.90 _(1, 15.1)	0.43 _(2, 15.4)	4.21 _(4, 64.6)	1.43 _(7, 64.6)	2.03 _(4, 64.6)	0.42 _(7, 64.6)
DEA (+CN) ¹	0.07 _(2, 15.4)	0.10 _(1, 15.1)	1.09 _(2, 15.4)	4.18 _(4, 61.4)	1.26 _(7, 61.4)	1.86 _(4, 61.4)	1.36 _(7, 61.4)
Archaeal <i>amoA</i> ¹	15.72 _(2, 15.3)	0.26 _(1, 15.1)	1.34 _(2, 15.3)	12.05 _(4, 55.6)	1.55 _(7, 55.5)	0.67 _(4, 55.6)	1.38 _(7, 55.5)
Bacterial <i>amoA</i> ¹	11.54 _(2, 15.7)	4.90 _(1, 15.5)	0.70 _(2, 15.7)	33.22 _(4, 63.7)	0.83 _(7, 63.6)	0.39 _(4, 63.7)	1.13 _(7, 63.6)
AOA ¹ :AOB ¹	2.85 ⁺ _(2, 15.7)	8.89 _(1, 15.1)	2.34 _(2, 15.7)	15.76 _(4, 57.8)	2.22 _(7, 57.4)	0.66 _(4, 57.8)	1.24 _(7, 57.4)
<i>nosZ</i> clade I ¹	3.34 ⁺ _(2, 15.2)	5.31 _(1, 15.1)	1.15 _(2, 15.2)	104.48 _(4, 63.1)	1.40 _(7, 63.2)	0.14 _(4, 63.2)	1.10 _(7, 63.2)
<i>nosZ</i> clade II (<i>A.deh</i>) ¹	14.71 _(2, 15.8)	7.27 _(1, 15.6)	3.00 ⁺ _(2, 15.8)	181.90 _(4, 63.7)	3.20 _(7, 63.7)	0.80 _(4, 63.7)	1.70 _(7, 63.7)
Clade I ¹ :clade II ¹	1.67 _(2, 15.1)	1.61 _(1, 15.0)	0.21 _(2, 15.1)	370.42 _(4, 63.1)	3.87 _(7, 63.1)	1.21 _(4, 63.1)	0.48 _(7, 63.1)
Bacterial 16S rRNA	4.20 _(2, 15)	0.051 _(1, 15.0)	0.85 _(2, 15.0)	20.83 _(4, 1 595 320)	0.25 _(7, 1 400 142)	0.53 _(4, 1 771 442)	0.36 _(7, 1 735 891)
MBC ¹	4.15 _(2, 15.7)	0.12 _(1, 15.7)	2.74 _(2, 15.7)	37.26 _(3, 51.8)	1.88 _(6, 51.7)	1.00 _(3, 51.8)	0.72 _(6, 51.7)
MBN ¹	5.88 _(2, 16.9)	3.12 _(1, 18.0)	0.88 _(2, 17.2)	19.40 _(3, 47.9)	2.26 _(6, 47.2)	1.99 _(3, 46.8)	0.61 _(5, 46.6)
MBC:MBN ¹	0.85 _(2, 17.8)	1.00 _(1, 18.2)	0.10 _(2, 17.9)	9.27 _(3, 46.2)	2.94 _(6, 45.5)	0.79 _(3, 45.3)	1.16 _(5, 45.0)
<u>Plant variables</u>	Fertilizer	Burn	Fertilizer × Burn	Year	Fertilizer × Year	Burn × Year	Fertilizer × Burn × Year
Grass ANPP ^s	8.81 _(1, 9.0)	81.63 _(1, 9.0)	7.94 _(1, 9.0)	5.05 _(4, 48.0)	1.37 _(4, 48.0)	1.46 _(4, 48.0)	0.78 _(4, 48.0)
Forb ANPP ^s	0.13 _(1, 9.0)	0.0021 _(1, 9.0)	11.01 _(1, 9.0)	1.66 _(4, 48.0)	0.95 _(4, 48.0)	1.48 _(4, 48.0)	0.77 _(4, 48.0)
Woody plant ANPP ^s	0.12 _(1, 9.0)	10.44 _(1, 9.0)	0.12 _(1, 9.0)	3.79 _(4, 48.0)	1.27 _(4, 48.0)	3.79 _(4, 48.0)	1.27 _(4, 48.0)

Superscript “¹” denotes variables that were log-transformed and “^s” that were square root-transformed to meet assumptions of normality.

Values bolded signify comparisons with $P \leq 0.05$, and superscript “⁺” indicates where $P = 0.10$ -0.051.

Table 3-3. PERMANOVA results on the direct and interactive effects of fertilization (“Fertilizer”), burning (“Burn”), and sampling month (“Month”) for the 16S rRNA gene composition of microbial communities based on the Bray-Curtis dissimilarity.

Factor	DF	R^2	Pseudo- F	P value
Burn	1	0.059	7.15	0.001
Fertilizer	2	0.115	7.01	0.001
Month	4	0.068	2.08	0.001
Burn $\times$ Fertilizer	2	0.034	2.08	0.001
Burn $\times$ Month	4	0.022	0.67	0.973
Fertilizer $\times$ Month	7	0.044	0.76	0.862
Burn $\times$ Fertilizer $\times$ Month	7	0.037	0.64	1.000
Residuals	76	0.622	-	-

Observed composition of communities were compared by permutating 999 null communities, resulting in a pseudo- F score and variation explained (R^2) by treatments and their interactions. Bolded rows signify the comparisons in which $P \leq 0.05$.

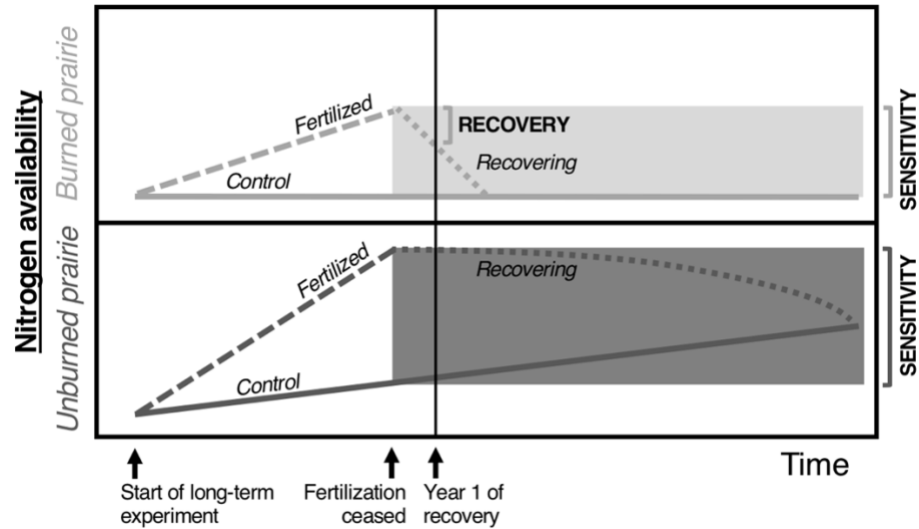


Figure 3-1. Our conceptual model of tallgrass prairie ecosystem sensitivity and resilience to long-term nitrogen (N) fertilization under contrasting fire treatments. Ecological states may change proportionally with increasing N availability. Therefore, the sensitivity of ecological states to annual fertilization (height of the shaded box) can increase independently in unburned prairies because of the higher baseline level of ecosystem N. Following the cessation of fertilization, the magnitude of recovery (difference between fertilized and recovering prairies) is hypothesized to depend on the prairie’s fire treatment. We predict that ecological states of burned prairies will recover quicker than unburned prairies.

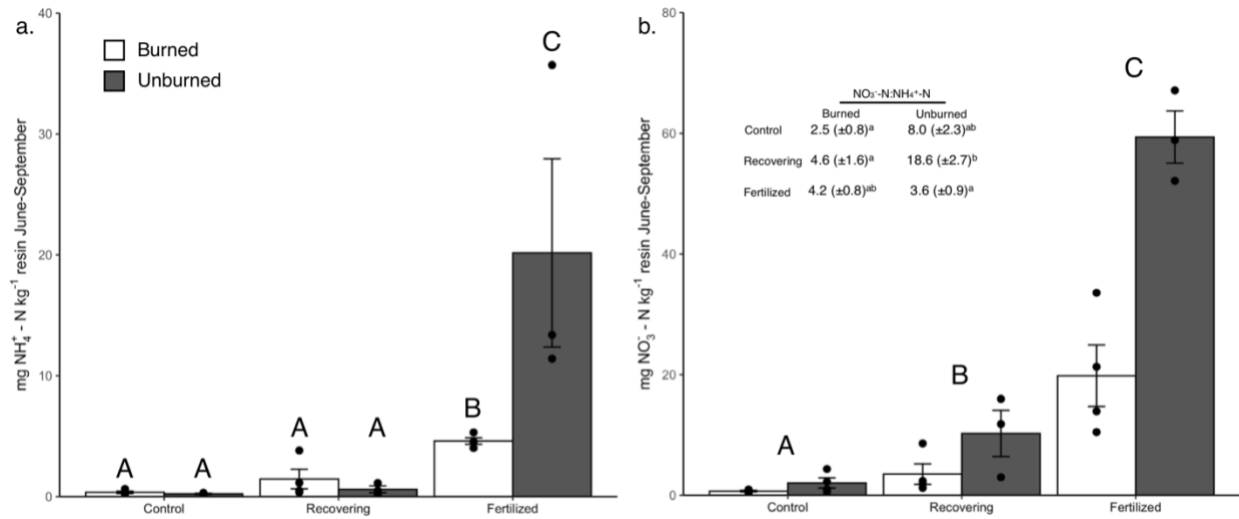


Figure 3-2. Estimated concentrations of (a) $\text{NH}_4^+ - \text{N}$ and (b) $\text{NO}_3^- - \text{N}$ sorbed to resin bags buried in soils under contrasting fertilizer treatments and fire regimes. The height of the bars (mean $\pm$ standard error [SE]) correspond to the plot and subplot means for each treatment combination and each point representing the individual plot and subplot concentration means during the course of the growing season. Tukey's honest significant difference (HSD) post-hoc analysis results are shown with different letters indicating field treatment groups that differed at the $P < 0.05$ level.

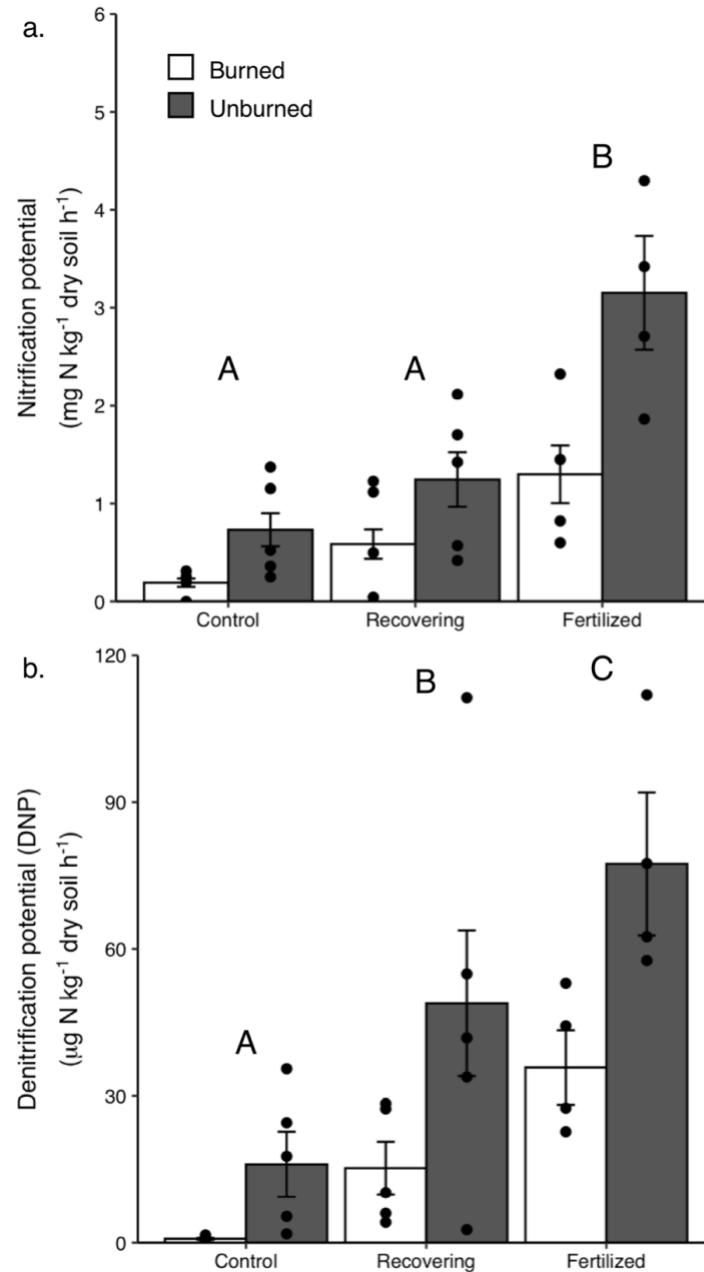


Figure 3-3. Measured (a) nitrification potential (NP) and (b) denitrification potential (DNP) rates of the soils from contrasting fertilizer and fire treatments. Bar heights (mean $\pm$ SE) are the means of the NP and DNP rates from each plot and subplot at each month with the points showing the monthly average of each (sub)plot. Tukey's HSD post-hoc analysis results are shown with dissimilar letters indicating fertilization treatment groups that differed at the $P < 0.05$ level. Both NP and DNP rates were affected by the burning treatment, but no interaction between fire and fertilization.

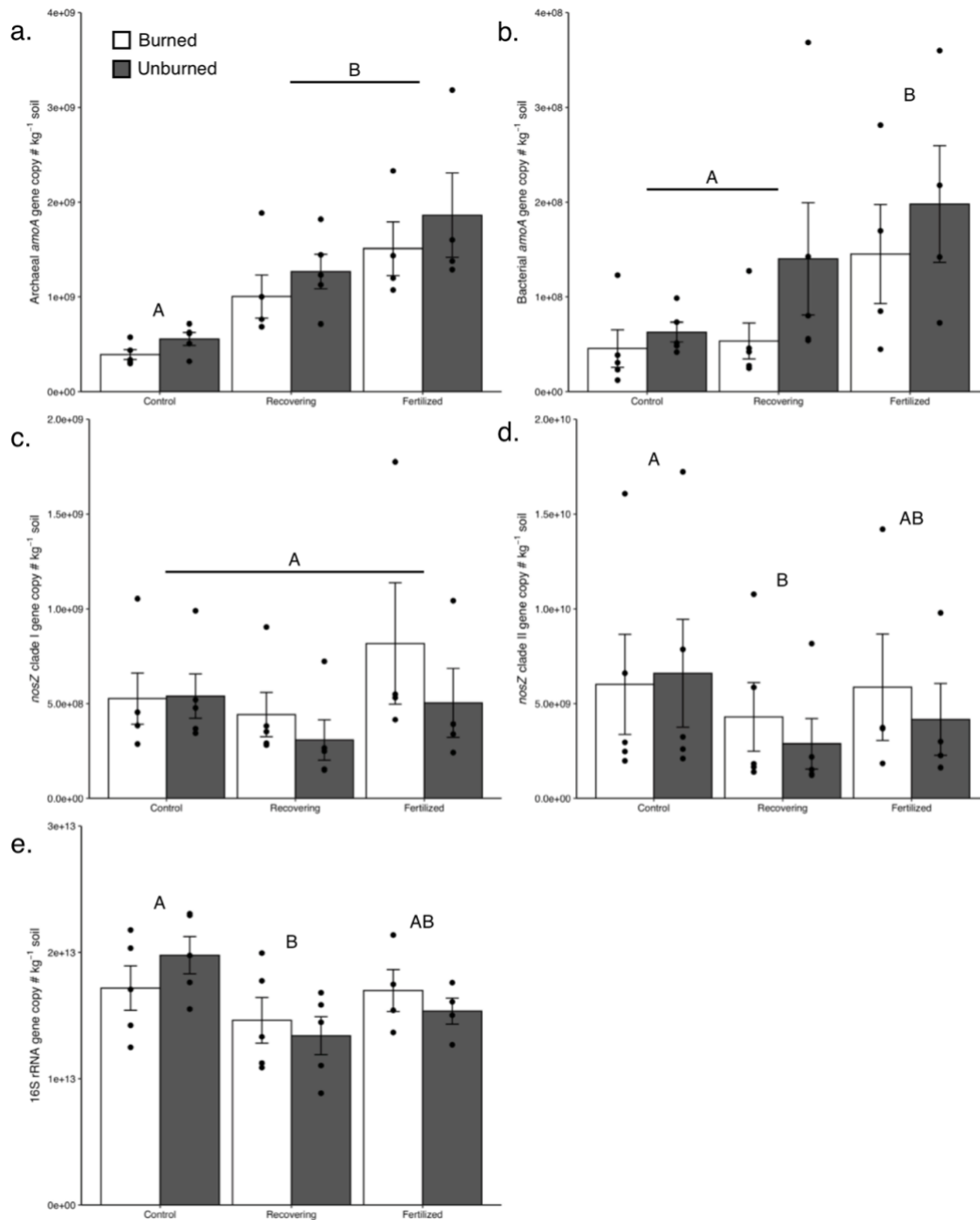


Figure 3-4. Gene abundance of (a) archaeal and (b) bacterial *amoA*, (c) clade I and (d) clade II (*A. dehalogenans*) of *nosZ*, and (e) bacterial 16S rRNA in soils with contrasting treatments of burning and fertilization. The bar height (mean $\pm$ SE) represents the mean gene abundance at each plot and subplot for each given treatment combination during the span of the growing season. Points are the monthly average for each (sub)plot. Tukey's HSD post-hoc analysis results are shown with dissimilar letters indicating fertilization treatment groups that differed at the $P < 0.05$ level.

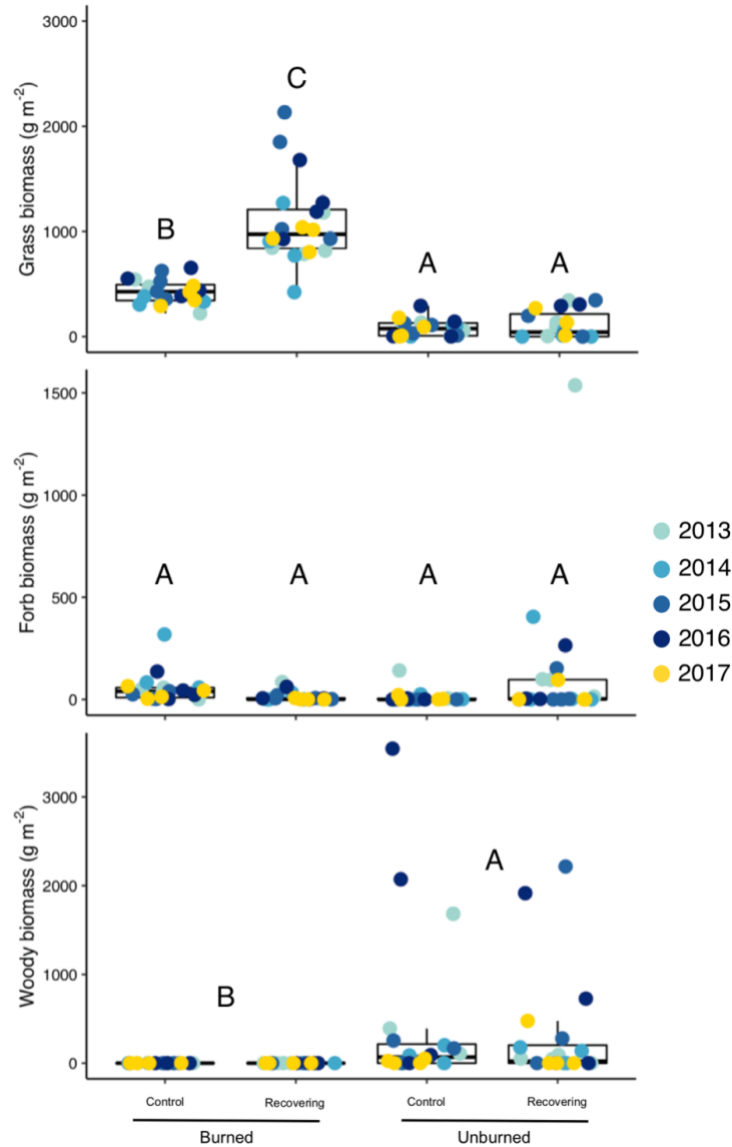


Figure 3-5. Yearly variation of aboveground net primary productivity (ANPP), estimated from end-of-growing season biomass, between 2013-2017 of (a) grasses, (b) forbs, and (c) woody plants. Data are shown as boxplots with the central line representing the median, and the upper and lower quartiles being the lines above and below the median, respectively. The lines extending from the quartiles end at the upper and lower fences of the data distribution. Yearly plot means are shown as points; each color represents a different year. Tukey's HSD post-hoc analysis results are shown with dissimilar letters indicating Fertilizer $\times$ Burn treatment groups for grasses and forbs, and burning treatment groups for woody plants, that differed at the $P < 0.05$ level.

Chapter 4 - Biotic feedbacks drive soil nitrogen legacies in burned and unburned grasslands

4.1 Abstract

Humans have enriched the globe with nitrogen (N), but N availability across many terrestrial ecosystems has been decreasing. Persistent effects by previous long-term N input could arise by exogenous N being released from soil organic matter or by altered plant and soil microbial communities. Management practices that impact the N cycle, such as prescribed fire or fire suppression in mesic grasslands, may mediate this N-fertilization legacy. To assess how previous chronic N-fertilization affects tallgrass prairie ecosystem N-cycling and recovery, we used a long-term fertilization and cessation experiment crossed with contrasting fire treatments to evaluate the strength of legacies between burned and unburned prairies for five years. We found that N availability, measured using resin bags, remained elevated after ceasing N-fertilization, nitrification potential decreased to control levels (i.e., recovered) years 3 and 4 in burned prairies, and denitrification potential recovered by the fourth year in both prairies. While unburned prairies always had higher N availability and flux potentials, herbaceous aboveground net primary productivity (ANPP) never decreased back to control levels. Litter biomass and %N measurements after the fourth growing season revealed only a modest difference in potential combustible N between the control and recovering treatments in burned prairies. When plant and soil (0-15 cm) C and N stocks were quantified in year 5, the root C:N ratio in burned recovering prairies was found to have increased back to levels in burned control prairies, but soil N losses were similar between burned and unburned prairies. Collectively, these findings suggest a comparable soil N legacy between fire treatments, which may have contributed to the relatively

short-lived legacies of N fluxes, resulted respectively by pyrovolatization and, potentially, leaching in burned and unburned prairies, but positive legacies in herbaceous ANPP and N availability may have emerged by the shifts in plant and soil microbial communities.

4.2 Introduction

Nitrogen (N) is an important nutrient that regulates many ecosystem processes, and the past widespread increase in biologically available N has altered global N- and carbon- (C) cycling. Current atmospheric N deposition rates are three-fold higher than prior to modern industrialization (Kanakidou et al. 2016), and fertilizer use augments local N supply in terrestrial ecosystems (Fixen and West 2002). Consequently, primary production in historically N-limited ecosystems has increased (Vitousek et al. 1997, Gough et al. 2000, Galloway et al. 2008) and N slowly has accrued as vegetative and soil pools retain the nutrient (Vitousek and Reiners 1975, Lovett and Goodale 2011), but often at the cost of lower plant diversity (Stevens et al. 2004, Suding et al. 2005) and acidification of soils and associated base cation leaching (Aber et al. 1998, Lovett and Goodale 2011). Though some ecosystems will continue to receive high N inputs, N availability has declined across most terrestrial ecosystems (Mason et al. 2022), and atmospheric N deposition is projected to decrease across large areas of the globe during the 21st century (Lamarque et al. 2013). As a result, the legacy of accumulated N can become a source for current N-cycling processes, with a lag in ecosystem recovery after cessation of elevated exogenous N supply (Van Meter et al. 2016). Therefore, past changes to biologically available N may continue to modify ecosystem processes even after N inputs decline.

Meta-analyses of experiments in agricultural (Ouyang et al. 2018) and nonagricultural (Lu et al. 2011) systems underpin the idea that N-fertilization increases ecosystem N fluxes and

pools. Fertilizer feeds N-cycling microbes with ammonium (NH_4^+) and nitrate (NO_3^-) that fuels nitrification and denitrification (Wallenstein et al. 2006, Verhamme et al. 2011), thus supporting a faster N cycle that increases the likelihood for N to become a pollutant (e.g., leached NO_3^- or gaseous N_2O) (Schlesinger 2009). In addition to increasing aboveground net primary production (ANPP), N-fertilization often lowers the plant C:N ratio to promote faster litter breakdown and slower N immobilization (Parton et al. 2007). In turn, N-addition increases net N mineralization and builds the soil organic N pool as microbes turn over and necromass accumulates, also lowering the soil C:N ratio (Cotrufo et al. 2015, Liang et al. 2017, Lavalley et al. 2020). However, the magnitude of N-cycling and ecosystem function responses to fertilization (i.e., sensitivity) varies among fluxes and pools since inorganic N pools cycle faster than organic N pools, and different microbial (e.g., nitrifiers and denitrifiers) and plant (e.g., grasses, shrubs, and trees) functional groups vary in growth rates and N use physiology (Schimel 1995, Hall et al. 2011, Martiny et al. 2013, Yahdjian et al. 2014, Rocca et al. 2015). Thus, differences in sensitivity among ecosystem pools and functions to N-fertilization together modulate the form and quantity of available N, the net accumulation of fertilizer-N in the ecosystem, and its depletion after cessation of fertilization.

After N-fertilization ceases, ecosystem N pools and fluxes in many ecosystems may recover asynchronously in plants and soils (Stevens 2016, Gilliam et al. 2019, Nieland et al. 2021). Inorganic N pools can return within a decade to levels observed in untreated controls in grasslands (O'Sullivan et al. 2011, Stevens et al. 2012) and forests (Bredemeier et al. 1995, Koopmans et al. 1995). Additionally, leaf N content can decrease in as little as two years (Arróniz-Crespo et al. 2008) and soil nitrification potential within one year (Nieland et al. 2021) after ceasing fertilization, suggesting N availability can decline quickly in some cases. However,

the N reservoir in soil organic matter (SOM) seldom recovers within the same timespan (Stevens 2016), and this pool can persistently support higher net N mineralization rates over unfertilized conditions for over a decade post-fertilization (Clark et al. 2009, O’Sullivan et al. 2011) due to direct (low soil C:N ratio) and indirect (plant C input) effects (Knops et al. 2002). Moreover, ANPP can be higher in previously fertilized than unfertilized ecosystems for at least a year after N-fertilization ceases (Nieland et al. 2021). As evident by these examples, the recovery of plant and soil fluxes and pools can happen quickly (i.e., one or two years) or slowly (i.e., a decade or more); the latter definitive of a positive legacy, i.e., higher-than-expected N-cycling rates or pools relative to those in never-fertilized systems (Sala et al. 2012). While N fluxes controlled by faster N-cycling pools are expected to recover relatively soon once fertilization ceases, the extent and duration of positive legacies are not well understood and limits our ability to predict future ecosystem functions from past N-fertilization.

Grasslands cover approximately 40% of Earth’s land surface (White et al. 2000, Bai and Cotrufo 2022) and were historically characterized by periodic fire, which also plays a critical role in N- and C-cycling (Bond et al. 2005, Ahmed et al. 2017). Frequent fires in mesic grasslands sustain low N availability by combusting organic N stored in aboveground litter (Ojima et al. 1994, Blair 1997), while fire suppression allows aboveground litter to decompose and increase biologically available N, supporting faster soil N-cycling rates (Blair 1997, Fynn et al. 2003, Nieland et al. 2021). In turn, grassland plants allocate less growth belowground and have lower root C:N ratios, further promoting N-cycling (Johnson and Matchett 2001, Kitchen et al. 2009). In the tallgrass prairie of North America, where N deposition has leveled off (National Atmospheric Deposition Program 2023), both prescribed fires and soil microbial processes could drive ecosystem N loss, reducing the legacy effect of elevated N availability, and supporting

faster ecosystem recovery after supplemental N ceases. Indeed, soil in an annually burned prairie retained half as much fertilizer-N as soil in a fire-suppressed, unburned prairie did, likely due to the history of fire (Nieland et al. 2021). However, native perennial prairie grasses and microbial biomass also quickly take up N and effectively retain the nutrient in plant and soil organic pools (Tilman and Wedin 1991, Dell and Rice 2005), making pathways of ecosystem inorganic N loss less likely. Moreover, elevated soil nitrification and denitrification potential rates in unburned prairies previously fertilized with N make leaching and gaseous N losses more likely (Nieland et al. 2021), but they may also represent ephemeral loss pathways if microbial dissimilatory metabolic processes recover more quickly after cessation of exogenous N loss than plant and microbial assimilatory processes. Therefore, disentangling fire- and microbially-mediated mechanisms that facilitate N loss from SOM or other N pools will help resolve the magnitude and trajectory of tallgrass prairie recovery from elevated N conditions.

In this study, we used a long-term fertilization and cessation experiment in a tallgrass prairie to assess how fire shapes the recovery of ecosystem N and C dynamics from previous fertilization. Recent work at this site found that while soil inorganic resin-sorbed N pools recovered to levels comparable to control plots in the first year after ceasing N-fertilization, microbial N-cycling potential rates did not recover equally, and ANPP remained elevated (Nieland et al. 2021). We continued to measure soil and plant N-cycling rates and pools for five years, to evaluate the role of annual prescribed fire on trajectories of ecosystem recovery, and to assess the potential mechanisms underlying the observed positive legacies of past fertilization. We hypothesized that the higher ambient soil total and available N levels from decades of fire suppression would slow recovery of ecosystem N pools and fluxes after fertilization ceased. Moreover, we expected that annual prescribed burning would enhance ecosystem recovery from

N eutrophication by combusting the N assimilated into plant litter during each annual production cycle, thereby removing N from the system faster after ceasing fertilization. To test our hypothesis, we measured resin-sorbed inorganic N, soil microbial N-cycling potential rates, and ANPP for five consecutive growing seasons after N-fertilization ceased, and documented the post-fertilization amounts of combustible litter-N, and plant and soil N and C stocks.

4.3 Methods

Study site

This study was done at the Belowground Plots (BGP) experiment, located in an ungrazed area of the Konza Prairie Biological Station (KPBS) near Manhattan, KS, USA (39° 05' N, 96° 35' W). KPBS is a 3,487-ha experimental research station in the Flint Hills ecoregion, which encompasses the largest remaining tract of continuous tallgrass prairie in North America. Mean annual temperature is 12.8°C, and mean annual precipitation (1986-2021) is 849 mm, most of which falls during the growing season (April-September). During the study period (2017-2021), interannual precipitation varied such that cumulative growing season precipitation was abnormally dry (one standard deviation (SD) under the mean) in 2018, abnormally wet (one SD over the mean) in 2019, and closer to average in other years (Appendix D Figure D-1). The dominant vegetation in frequently burned areas is comprised of perennial C₄ grasses *Andropogon gerardii*, *Sorghastrum nutans*, *Panicum virgatum*, and *Schizachyrium scoparium*, while vegetation in less frequently burned areas is dominated by woody and shrubby plants such as *Juniperus virginiana*, *Cornus drummondii*, and *Rubus occidentalis* (Carson et al. 2019). At the BGP experiment, the soils are deep and silty-clayey loams (Irwin series: fine, mixed, mesic, pachic Argiustolls).

Site and experiment description

The BGP experiment was established in 1986 with a strip-plot experimental design that crosses prescribed fire and fertilization treatments (Seastedt et al. 1991). There are eight main blocks: Four are burned annually in early spring (burned prairie) and the other four are not burned (unburned prairie), so each treatment combination includes four replicate plots ($n = 4$). Within the blocks, we sampled the 12.5×12.5 -m plots that were fertilized for 31 years (1986-2016) with $10 \text{ g N m}^{-2} \text{ yr}^{-1}$ as NH_4NO_3 (recovering treatment), and the plots with no fertilization (control treatment). Also, after the N-fertilization treatment was ceased, we established four 1×1 -m sub-plots within each previously-fertilized plot and continued fertilizing them at the same rate (fertilized treatment), and sampled them to assess belowground recovery (Nieland et al. 2021).

Sample collection and processing

Soil samples were collected twice during the growing season to assess ecosystem recovery from chronic N-fertilization. Four 2 cm diameter, 15 cm deep soil cores were taken from each experimental plot and sub-plot and combined into one composite sample for each treatment replicate. Soils were sieved through a 4 mm mesh to remove rocks and organic debris, while preserving soil aggregates $> 2\text{mm}$, and immediately placed on ice until arriving in the laboratory. Soils were stored at 5°C for no more than one week while N-cycling assays were completed.

Available N and microbial N-cycling

To estimate total available inorganic N, four resin bags were buried 10 cm below the soil surface in each replicate plot during the growing season in 2017 and 2019-2021 (Baer and Blair 2008). At the end of the growing season, surviving bags were removed, and resin-sorbed N was

extracted in 2M KCl. NH_4^+ -N and NO_3^- -N in the extracted solution were quantified using modified indophenol and VCl_3 /Griess reagent methods, respectively (Hood-Nowotny et al. 2010), and measured spectrophotometrically using a FilterMax F5 Multimode Microplate Reader (Molecular Devices, San Jose, CA, USA). Quantities of resin-sorbed N were averaged by plot for subsequent analysis.

Nitrogen flux rates were estimated using N-cycling potential assays. Nitrification potential (NP) was measured by recording the change in NO_3^- -N, quantified similarly as above, 24 h after spiking a slurry of 2.5 g of soil in a 1 mM phosphate buffer solution with a saturating amount of NH_4^+ (Taylor et al. 2010, Petersen et al. 2012). Denitrification potential (DNP) under ambient C and N concentrations was measured by recording the change in N_2O -N over 3 h inside an anoxic vessel containing a slurry of 5 g of soil in deionized water with chloramphenicol, with acetylene added to prevent N_2O reducing to N_2 (Groffman et al. 1999). Similarly, denitrification enzyme activity (DEA) under non-limiting C and N conditions was measured by adding glucose and NO_3^- to a parallel vessel and recording the change in N_2O -N over a 1 h incubation period. N_2O -N was quantified using a Shimadzu GC 2014 fitted with an electron capture detector.

Aboveground plant responses

Aboveground net primary productivity (ANPP) was estimated at the end of each growing season in the control and previously-fertilized plots. Plant biomass was clipped inside two 0.1 m^2 quadrants per plot and dried at 60°C . After the previous year's plant growth was removed, the current year's growth was sorted by plant functional group and weighed (Blair and Zeglin 2023). We report both herbaceous and woody ANPP, but because woody ANPP is underestimated with the quadrat approach as larger shrubs and trees are not sampled, we centered our inferences on herbaceous ANPP. To estimate the amount of N vulnerable to combustion, aboveground plant

biomass was collected four weeks prior to the prescribed fire in spring 2021 in the burned treatment plots. The dead plant biomass was collected similarly as live biomass. After drying and weighing, composite sub-samples were ground using an 8000D mixer/mill and %C and %N was quantified using a FlashEA 1112 NC Analyzer. The combustible N pool was calculated as the sum of aboveground plant N content.

Ecosystem C and N stock

Aboveground and belowground ecosystem C and N stocks were estimated at the end of the growing season in 2021. To estimate aboveground ecosystem C and N stock, plant biomass collected for ANPP in the experimental plots was ground after it was dried and weighed, and %C and %N were quantified using a LECO TruSpec CN Combustion Analyzer at the Kansas State Soil Testing Lab. To estimate belowground C and N stock, four 5 cm diameter, 15 cm depth soil cores each experimental plot or sub-plot were collected and stored at 5°C in the lab during processing. Belowground plant biomass (i.e., roots) was extracted, dried at 60°C, and weighed, and the sieved soil was composited by plot. Both roots and soils were processed as described above to quantify C and N with a FlashEA 1112 NC Analyzer. To calculate soil C and N stock from %C and %N, we used the bulk density of 1.097 g cm⁻³ estimated for surface mineral soils at the BGP experiment by Carson (2013).

Statistical analysis

We evaluated how fire affected the response and recovery of ecosystem N cycle components from chronic N-fertilization across time using linear mixed effect modeling with the *lmerTest* package (Kuznetsova et al. 2017) in R V4.2.1 (R Core Team 2022) in the RStudio environment V2022.07.0 (RStudio Team 2022). Fire, fertilization, and year (as a factor) were included as interacting fixed effects in the mixed effect models. To account for repeated

sampling, we set block as a random intercept, and we also included day of year (scaled to zero) as a random intercept for measurements collected twice a growing season. We evaluated the effects of fertilization and fire using linear modeling for variables measured at one time point only (C and N stocks). As needed, response variables were naturally-log transformed (or square root-transformed, for ANPP) to attain a normal distribution, and homoscedasticity and normality of residuals were checked for each model. Log transforming the DEA data did not satisfy model assumptions of normal distribution because many rates were below detection (zero), so we determined that observing a rate reported as zero was not biased towards any field treatment or time using a generalized linear model with a binomial distribution (Appendix D Figure D-2), and we proceeded with the statistical analysis as described above. We also evaluated the effect of N-fertilization on the C:N ratio of the dead plant biomass using a mixed effect model that included random intercepts for litter type and position within litter type. Analysis of variance (ANOVA) was used to calculate *F*- and *P*-values for each model, and pairwise comparisons for significant effects or their interactions were evaluated using Tukey's post-hoc analyses using the *emmeans* package (Lenth 2016). Data were handled and visualized using *tidyverse* (Wickham et al. 2019).

4.4 Results

Available nitrogen and microbial N-cycling

Both total resin-sorbed inorganic N and the ratio of NO_3^- -N: NH_4^+ -N sorbed were higher in unburned prairie soils (Appendix D Table D-1, Figure 4-1, Appendix D Figure D-3). In both fire treatments, available N decreased in the first year of fertilizer cessation by 86% (Nieland et al. 2021) but did not decrease further in the subsequent three measured years (2019-2021). Independent of its interannual variation, resin-sorbed inorganic N in recovering soils remained

higher than in control soils (post-hoc $P \leq 0.002$), and an order of magnitude lower than continuously fertilized soils (post-hoc $P \leq 0.001$); but there was no statistical support for prescribed fire effects on available N recovery (Figure 4-1, Appendix D Figure D-1). There was an interactive fire and fertilization effect on inorganic N composition, as higher resin-bound NO_3^- -N relative to NH_4^+ -N in the continuously fertilized soils than in both recovering and control soils, only in burned prairies (Appendix D Figure D-3, post-hoc $P \leq 0.024$). Also, the NO_3^- -N: NH_4^+ -N sorbed ratio was ten times higher in the wet 2019 growing season than all other seasons across all field treatments (post-hoc $P < 0.001$).

Soil microbial N-cycling potentials dropped following cessation of chronic N-fertilization, as expected (post-hoc $P < 0.001$), but their five-year trajectories of recovery varied among the different processes measured. Effects of fire treatment on the recovery of N-cycling potentials was contingent on time (Appendix D Figure D-1, Figure 4-1): In the absence of fire, nitrification potential (NP) remained higher in the recovering soils than the control soils (post-hoc $P \leq 0.04$), except in 2020 when NP was similar among all three fertilization treatments. Conversely, NP was similar in recovering and control soils in burned prairies by the third- and fourth-year post-cessation (2019 and 2020), though NP increased in the recovering soils again in 2021. In contrast, by the fourth and fifth year after ceasing fertilization (2020 and 2021), denitrification potential (DNP) under ambient concentrations of C and N were similar in the recovering and control soils, independent of fire treatment (post-hoc $P < 0.001$). Under non-limiting C and N conditions, denitrification enzyme activity (DEA) did not increase from chronic N-fertilization or fire suppression, nor did DEA vary interannually (Appendix D Table D-1, Appendix D Figure D-4). In summary, DNP showed the strongest recovery five years after cessation of fertilization, while NP showed moderate recovery, and DEA was insensitive to the

long-term treatments. After assuming that DNP rates were representative of *in situ* rates, the minimum, maximum, and average cumulative amount of N lost to denitrification in recovering burned prairies each growing season can be roughly estimated as 0.8, 73.3, 39.3 mg N m⁻², respectively, which contrasts with 26.4, 298.2, 113.6 mg N m⁻² for recovering unburned prairies.

Aboveground plant responses

Herbaceous aboveground net primary productivity (ANPP) varied interannually independent of treatment effects, peaking in the wet year of 2019, but did not recover from chronic N-fertilization after five years of treatment cessation (Appendix D Table D-1, Figure 4-2). N-fertilization increased total ANPP only for burned prairies, since higher measurable woody ANPP in the never-fertilized prairies offset the increase from herbaceous ANPP in unburned prairies (Appendix D Table D-1, Figure 4-2). Burned prairies that never were fertilized had higher herbaceous ANPP than either fertilized or unfertilized unburned prairies, and the legacy of historical fertilization on herbaceous ANPP in burned prairies was higher (ANPP_{Recovering} - ANPP_{Control}; 446 ± 247 g m⁻²) than in unburned prairies (80 ± 34 g m⁻²). There was in turn more litter and litter-N in the historically-fertilized burned treatment (Appendix D Figure D-5); however, the amount of litter-N vulnerable to combustion was only 1.1 g N m⁻² greater in the recovering than never-fertilized burned prairie prior to the 2021 spring fire, partially because the C:N ratio of this litter was higher in the recovering prairies (Appendix D Table D-1, Appendix D Figure D-5). Assuming the N concentration measured after the 2020 growing season was representative of all study years and that there is minimal loss of biomass after the growing season, the minimum, maximum, and average amount of N lost to combustion in recovering burned prairies annually can be estimated as 3.1 ± 0.4 (mean $\pm$ SD), 6.0 ± 1.5 , and 4.0 ± 1.2 g N m⁻², respectively.

Ecosystem carbon and nitrogen stock

Plant N stock was not different between control and recovering prairies five years after N-fertilization ceased (Appendix D Table D-2, Figure 4-3). In burned prairies, this apparent recovery in plant N stock was related to a reduction in root %N and an increase in root C:N ratio that returned to control values (Appendix D Table D-2, Figure 4-4), but shoot C:N ratios were higher in recovering than control plots for both fire treatments (Appendix D Table D-2, Figure 4-4). In contrast, soil N stock to a depth of 15 cm in recovering prairies was intermediate between the control and continuous fertilization treatments (Appendix D Table D-2, Figure 4-3), with 46 g N m⁻² difference between the latter treatments (post-hoc $P = 0.029$), after five years post-cessation. Fire suppression increased soil N stock almost identically as chronic N-fertilization (47 g N m⁻²), and both fertilization and fire suppression decreased the soil C:N ratio, which remained low after ceasing long-term fertilization (Appendix D Table D-2, Figure 4-4). Though there was a positive legacy on herbaceous ANPP, plant C stock was not different between control and recovering prairies, and topsoil C stock was not different among fertilization treatments but was higher under fire suppression (Appendix D Table D-2, Appendix D Figure D-6). Collectively, soil C:N ratio did not recover, soil N stock was recovering, and plant N stock and the root C:N ratio in burned prairies recovered five years after cessation of fertilization.

4.5 Discussion

We hypothesized that positive legacy effects of chronic N-fertilization would be enhanced, as evidenced by slower recovery post-fertilization, in prairies with a history of fire suppression. Specifically, we predicted that annual prescribed fire would limit N accrual from

long-term fertilization because of litter removal and associated pyrovolatilization losses, and that upon ceasing N-fertilization, prescribed fire would shorten the recovery time for soil N-cycling rates (i.e., nitrification and denitrification potentials), ANPP, and soil N pools to never-fertilized levels, compared to recovery in unburned, fire-suppressed conditions. By measuring available inorganic N, potential N transformation rates, ANPP, potential combustible N losses, and ecosystem C and N stocks, we found: Ambient soil total and available N levels were consistently higher following 30 years of fire suppression, as predicted; however, N-cycling potential rates and stocks did not always recover faster under annually burned conditions. NP recovered in the third and fourth years post-fertilization in the burned prairies only, while DNP recovered in the fourth and fifth years post-fertilization regardless of fire treatment. Herbaceous ANPP in unburned prairies was insensitive to fertilization, while ANPP in burned prairies showed a strong positive legacy from past fertilization; however, plant N stock and potential combustible N levels showed a minimal legacy effect, because higher shoot and litter C:N ratios following fertilizer cessation relative to never-fertilized conditions. Five years after ceasing N-fertilization, despite insensitivity of root N stocks to chronic fertilization, root C:N ratios had recovered to similarly high values in burned, but not unburned, prairies. Finally, soil N stocks were intermediate between continuously- and never-fertilized treatments in both fire treatments. Therefore, our hypothesis was not supported since the soil legacy N pool was similar in magnitude in both burned and unburned prairies. However, annual prescribed fire did cause soil NP to recover faster, and drove greater fertilization sensitivity of ANPP and root C:N ratio, with subsequent recovery in root C:N ratio but not ANPP. Since fire suppression affected N- and C-cycling via different mechanisms that resulted in similar soil N recovery patterns, we suggest that similar soil N legacies can arise from different feedbacks that in turn affect an ecosystem's ability to

recover from N eutrophication, as well as the larger-scale consequences of ecosystem N loss during recovery.

Recovery of N availability incomplete, unaffected by fire

Inorganic N availability fell immediately in the first year of ceasing N fertilizer application, but it did not fully return to levels in control plots, showing that recovering soils in both fire treatments had sustained elevated rates of net N mineralization, as supported by previous studies at other grassland N-cessation experiments (Olff et al. 1994, Vinton and Burke 1995, Clark et al. 2009, O’Sullivan et al. 2011). Although this legacy is often explained by the low C:N ratio in litter (Clark et al. 2009, Liu et al. 2020), this cannot be the mechanism in burned prairies because fire consumes aboveground litter and root C:N ratio returned to values observed in never-fertilized conditions. Instead, a low C:N ratio in SOM may support greater net N mineralization rates (Frankenberger and Abdelmagid 1985, Manzoni et al. 2008), or greater N mineralization from faster growth and turnover of soil microbial communities may increase N availability (Hart et al. 1994, Blaško et al. 2013, Broderick et al. 2022a). In support of the latter possibility, N-fertilization increased the relative abundance of putative fast-growing microbial taxa (Carson and Zeglin 2018) that are associated with a smaller biomass pool, which was observed in recovering soils the first year post-cessation (Nieland et al. 2021). Until the SOM C:N ratio or the composition or function of microbial communities recover, elevated net N mineralization rates will likely persist along with altered N fluxes and other ecosystem processes.

Independent of fertilization effects, unburned prairies still supported higher soil N availability via greater net N mineralization likely, NP, DNP, root %N, and soil C and N stocks, and lower soil and root C:N ratios, than burned prairies. While these differences did not seem to

underpin the overall trajectory of ecosystem recovery, they do suggest different mechanisms of recovery, which are further explored below.

Incomplete recovery of N transformations

As predicted, NP in burned prairies recovered slightly faster after N-fertilization ceased than NP in unburned prairies and DNP in both fire treatments. Faster recovery of potential nitrification activity in burned prairies suggests nitrifiers were limited by substrate availability, due to possibly higher NH_4^+ assimilation by plants or immobilization by microbial heterotrophs (Hart et al. 1994, Schimel and Bennett 2004, Dell and Rice 2005). However, NP recovery was transient since this rate in recovering soils increased in 2021, suggesting that either nitrifier abundance must still be high, possibly due to higher abundance of archaeal ammonia oxidizers (Nielsen et al. 2021), or particular environmental conditions supported the of NH_4^+ . Therefore, our finding of incomplete recovery of potential nitrification activity is supported by other studies (Stienstra et al. 1994, Olff et al. 1994), such that we expect this N-cycling process to not fully recover until organic sources of ammonification are depleted. Importantly, the lack of recovery of NP in unburned prairies, coupled with the consistently elevated ratio of resin-sorbed NO_3^- : NH_4^+ in unburned prairies, suggests an excess of NO_3^- that could be lost from either through denitrification or leaching.

However, DNP did not recover differently between burned and unburned conditions. DNP is driven primarily by the ambient concentrations of dissolved organic C and NO_3^- for denitrification activity: While denitrifier abundance could also drive the recovery of this process (Wallenstein et al. 2006), DEA, which assays rates under non-limiting conditions and is hence an index of denitrifier abundance, was insensitive to N-fertilization and did not vary interannually. Rather, NO_3^- availability likely drove DNP recovery based on two lines of evidence. First, the

proportion of the DEA rate realized by DNP (i.e., DNP/DEA) increased across a gradient of NO_3^- availability (i.e., 4% under never-fertilized burned prairies and 55% under continuously fertilized unburned prairies); and second, DNP fell during the wet 2019 growing season when labile organic C availability was likely greatest because of high photosynthetic rates (Kuzyakov and Domanski 2000). The decrease in DNP during the wet year was quite surprising, since wetter soils should create more anoxic conditions that support the facultative denitrification process (Groffman 2012, Robertson and Groffman 2015), and $\text{NO}_3^-:\text{NH}_4^+$ ratio on resins was also an order of magnitude higher in this growing season, independent of fire or fertilization treatment. Instead of wet conditions promoting denitrification, the alleviation of water limitation in 2019 may have increased the demand and assimilation of NO_3^- by grassland plants (Broderick et al. 2022a) or increased NO_3^- leaching, thus limiting NO_3^- for denitrification. Similarly to nitrification, the long-term trajectory of potential denitrification recovery seems to depend on substrate availability, as affected by competition for NO_3^- with plants, not the size of the microbial denitrifier community.

Legacy on plant productivity led to modest increase in combustible N

At this grassland and others (Gough et al. 2000, Buis et al. 2009, Avolio et al. 2014, Ren et al. 2017), it is well established that N-fertilization increases ANPP, and this study further shows that ANPP remained elevated for at least five consecutive years after long-term fertilization ceased, in annually burned prairies but not unburned prairies. Herbaceous growth clearly supported this positive legacy of N-fertilization on ANPP (Figure 4-2, Sala et al. 2012, Reichmann et al. 2013), which is not surprising, since herbaceous species are usually more responsive to N-addition than woody species (Hall et al. 2011, Yahdjian et al. 2014). The smaller N-fertilization effect on herbaceous ANPP in unburned prairies was also expected (Seastedt et al.

1991) given litter reduces light availability before woody encroachment (Knapp and Seastedt 1986) and canopies in woody-encroached grasslands block light for understory plant communities (Ratajczak et al. 2011, Tooley et al. 2022). Although we previously found that ANPP did not decrease immediately after ceasing N-fertilization (Nieland et al. 2021), it is remarkable that ANPP still showed no recovery after five years, especially given the partial recovery of the associated soil N-cycling potential rates and pools. This study highlights the importance of monitoring ecosystem processes after reversing manipulation treatments (e.g., Broderick et al. 2022b) because their effects can persist after the treatment has ceased (Monger et al. 2015); in this case, we suggest the long life span (> 5 y) of perennial prairie grasses as a leading explanation for this strong positive legacy of herbaceous ANPP (Lauenroth and Adler 2008).

Interestingly, chronic fertilization has caused plant community turnover from dominance by big bluestem (*A. gerardii*) to dominance by switchgrass (*P. virgatum*) (Wilcox et al. 2016, Carson et al. 2019), a highly productive grass species (Collins et al. 2012, Knapp et al. 2012) which is notably flexible in its N demand physiology (Tilman 1982). The known plasticity of this dominant plant species helps to explain the increase of shoot and root C:N ratios within five years post-fertilization, despite no recovery in ANPP, and how persistently higher herbaceous ANPP only led to marginally higher excess potential N loss through combustion. The measured total pool of litter-N vulnerable to combustion before the 2021 prescribed fire was similar to our previous estimate ($2.1\text{--}3.7\text{ g N m}^{-2}\text{ y}^{-1}$; Nieland et al. 2021), and according to our simple estimates that account for interannual variability, a total of 21 g N m^{-2} in recovering prairies could be combusted since ceasing N-fertilization, which is in turn similar to the difference in belowground N stock between the continuously and previously fertilized treatments in burned

prairies (20 g N m^{-2}). It is unlikely that prescribed fires alone caused this decrease in belowground N stock in burned prairies, since perennial grasses retranslocate a large proportion (e.g., up to 70%) of aboveground N to belowground organs upon senescence (Hayes 1985, Knapp and Seastedt 1986), and ~10% of litter-N leaches and transforms into SOM over the winter (Cotrufo et al. 2015). Therefore, while combustion is the single largest N efflux in this ecosystem, we expect that other processes also contribute to N loss.

Partial recovery of soil N stocks, unaffected by fire

Independent of fire suppression, soils recovering from chronic fertilization had intermediate stocks of N relative to continuously fertilized and never-fertilized soils, showing that while fertilizer-N has been retained in the top 15 cm of soil in this ecosystem, soil N stock has also begun to decline in the five years post-fertilization. Our initial investigation, conducted the first year after ceasing N-fertilization, found that the ecosystem retained fertilizer-N, as there was a difference of 0.03 and 0.06 percentage points in soil %N between the previously fertilized and control soils in burned and unburned prairies, respectively (Nielsen et al. 2021). However, after four more years with no exogenous N inputs, overall fertilizer-N retention in the top 15 cm of soil was very low ($9 \pm 10\%$), based on the difference in total ecosystem N stock between recovering plots and the average in control plots relative to the cumulative amount of fertilizer-N added (Clark et al. 2009). This at first seems to contradict ^{15}N label studies that show high levels of N immobilization in both plant and soil microbial and total pools in unfertilized native prairie ecosystems (Dell and Rice 2005, Dell et al. 2005). However, those studies also showed that following eight years of fire suppression, total N retention decreased by 35% (Dell et al. 2005), showing that the retention capacity of this ecosystem can diminish over time. Because previously and continuously fertilized prairies had similar fertilizer-N retention in our study, this seems to

suggest that within 30 years, these N-enriched prairies reached their limit to hold more N (i.e., capacity N saturation) (Aber et al. 1998, Lovett and Goodale 2011), and that the remaining N sink potential in surface mineral soils may be low after thirty years of chronic fertilization.

The loss of a substantial amount of N from SOM in only five years after ceasing N-fertilization suggests an unprotected organic N pool may be the source. Either particulate or aggregate associated C could be vulnerable to loss during this recovery period. Perennial C₄ grasses at this tallgrass prairie depend on arbuscular mycorrhizal (AM) fungi to acquire phosphorus (P) from soil (Johnson et al. 2015), and, also at this site, AM fungi contribute to the formation of soil aggregates that protect a significant fraction of organic matter from microbial breakdown (Wilson et al. 2009). In the latter study, macroaggregate proportion increased after seventeen years of N fertilization, in tandem with enhanced P-limitation of plant production and AM colonization of roots, while six years of direct fungicide application significantly decreased macroaggregate proportions (Wilson et al. 2009). We do not know how quickly the AM association with plant roots is lost after ceasing chronic fertilization, but we do know that change in aggregate size distributions on decadal time scales is possible in these soils. While total root stocks did not change in this study, the potentially large amount of particulate OM associated with fine roots is difficult to measure directly, and may not be accounted for by our methods. SOM should be fractionated into individual pools to evaluate potential sources of this lost N (Six et al. 2002, Lavalley et al. 2020). Mineral-associated organic matter (MAOM) can persist in soil for decades to centuries (Von Lützow et al. 2007, Lavalley et al. 2020) due to its strong chemical bonding to silt and clay mineral surfaces (Kögel-Knabner et al. 2008), thus is unlikely to change in pool size over five years. Further, MAOM has a lower C:N ratio compared to other SOM pools because of its microbial origin (Cotrufo et al. 2015, Liang et al. 2017); hence, the lower

and stable soil C:N ratio in recovering plots provides evidence that MAOM retains N from the previous decades of fertilization.

The fate of this lost N from the topsoil may have been leaching as NO_3^- since NP rates were much higher than DNP and DEA rates, suggesting an excess supply of available NO_3^- for denitrification (Figure 4-1, Nieland et al. 2021). Further, while estimated combustion losses can account for the level of partial soil N pool recovery in burned prairies, back-of-the-envelope calculations using average growing season DNP rates to estimate gaseous N loss from unburned recovering prairies show that only 0.6 g N m^{-2} was lost cumulatively during the five growing seasons following fertilizer cessation, suggesting limited gaseous N loss. This estimate, combined with the observation of lowest DNP during the study year with highest NO_3^- and water availability, implicates leaching losses as an explanation for the weak soil N legacy in unburned prairie soils. Because NO_3^- or other dissolved organic N compounds could be stored in soil beyond the 15 cm depth and limit N leaching to groundwaters and downstream aquatic systems (Lovett et al. 2018, Yang et al. 2022), measurement of nitrate movement through soil would be useful. It is also possible that a significant amount of N has been retained in the unmeasured woody plant aboveground biomass in the unburned treatment. Still, our results suggest that fire remains the major mechanism of N loss from the ecosystem, but that after decades of fire suppression, N leaching, not denitrification, is more likely the dominant mechanism of N loss.

Contrasting effects of fire suppression and fertilization on ecosystem C

Overall, fire suppression resulted in similar ecosystem responses as N-fertilization in burned prairies (e.g., higher N availability and NP, decreased soil C:N ratio, etc.). However, important response differences in this study offer new insight into mechanisms of altered biogeochemistry in fire-suppressed mesic grasslands encroached by woody species. One key

distinction between N-fertilization and fire suppression was that the latter increased soil C stock, which is well supported from past research (McKinley and Blair 2008, Connell et al. 2020). If SOM decomposition does not change or increases slightly (e.g., higher C-acquiring activity; Nieland et al. *in review*), the increase in soil C must arise from higher litter input that adds particulate organic matter (POM) into the topsoil (Lavalley et al. 2020, Pellegrini et al. 2022). Although POM may not saturate in soils, manual removal of litter reduced POM over time in a restored prairie (Lajtha et al. 2014), revealing that POM is more vulnerable to decomposition or disturbance events (e.g., woody vegetation removal) than MAOM (Larson et al. 2019, Lavalley et al. 2020). Importantly, unburned prairies showed a compensatory decline in root biomass at the 0-15 cm depth (Appendix D Table D-2), and since unburned prairies tend to allocate proportionally more root growth at shallower depths than burned prairies (Kitchen et al. 2009), this suggests that total root biomass and root C may have overall decreased under fire suppression, with no increase in total belowground C under woody encroachment as a result. Therefore, these findings challenge a popular paradigm that a transition from grasslands to woodlands will increase belowground C storage (see Veldman et al. 2019). Even though total ecosystem C stock was underestimated in unburned prairies in this study since aboveground woody biomass was not estimated, we suspect that C storage will only drastically increase after woodlands reach a certain stand age when new feedbacks establish (McKinley and Blair 2008).

Although the unburned prairies now resemble woodland states in their plant community composition (Carson et al. 2019), we found that chronic N-fertilization did not increase soil C stock. Long-term addition of N in temperate forest ecosystems typically slows SOM decomposition as a result of lower oxidative enzyme activity associated with a decrease in ectomycorrhizal fungal abundance (Frey et al. 2004, Edwards et al. 2011). However, at our study

site and across other grasslands, the activity of oxidative enzymes that target lignin and other recalcitrant SOM pools does not change (Keeler et al. 2009, Riggs and Hobbie 2016, Nieland et al. *in review*), and the composition and function of soil fungal communities still resembles that of other grasslands, i.e., not dominated by Basidiomycetes like forest fungal communities (Carson et al. 2019). Additionally, though initial topsoil N concentrations would suggest that woody encroachment should increase the soil C sink potential (Hong et al. 2023), N-fertilization instead may result in these relatively young woodlands being C sources instead, as a potential threshold in soil N is surpassed (Hong et al. 2023). Thus, suppressing fire and adding supplemental N in ecosystems that typically experience fire will likely not sequester more C, hampering efforts to offset elevated CO₂ concentrations.

4.6 Conclusion

This study is one of the few that examines ecosystem recovery from N input by ceasing chronic N-fertilization and collecting evidence to discern which biotic mechanisms underpin soil N legacies. We showed that the similarity in total soil N recovery between burned and unburned prairies was likely from the same unprotected labile organic N pool, but not due to similar mechanisms (i.e., fire or NO₃⁻ leaching), highlighting the importance of studying different N loss pathways under contrasting land management. Moreover, because N-cycling potentials recovered at different rates, and in some cases because of annual prescribed fires, while ANPP remained elevated in burned prairies, this further suggests the need to monitor ecosystem processes after ceasing a treatment since ecosystem responses to N eutrophication may persist even after inputs decline or cease. Because N availability will continue to decrease in the future (Mason et al. 2022), it is important to consider the soil N legacy pool that can continue supplying

plants and soil microbes with N and the biotic mechanisms that that may be responsible for ecosystem recovery.

4.7 References

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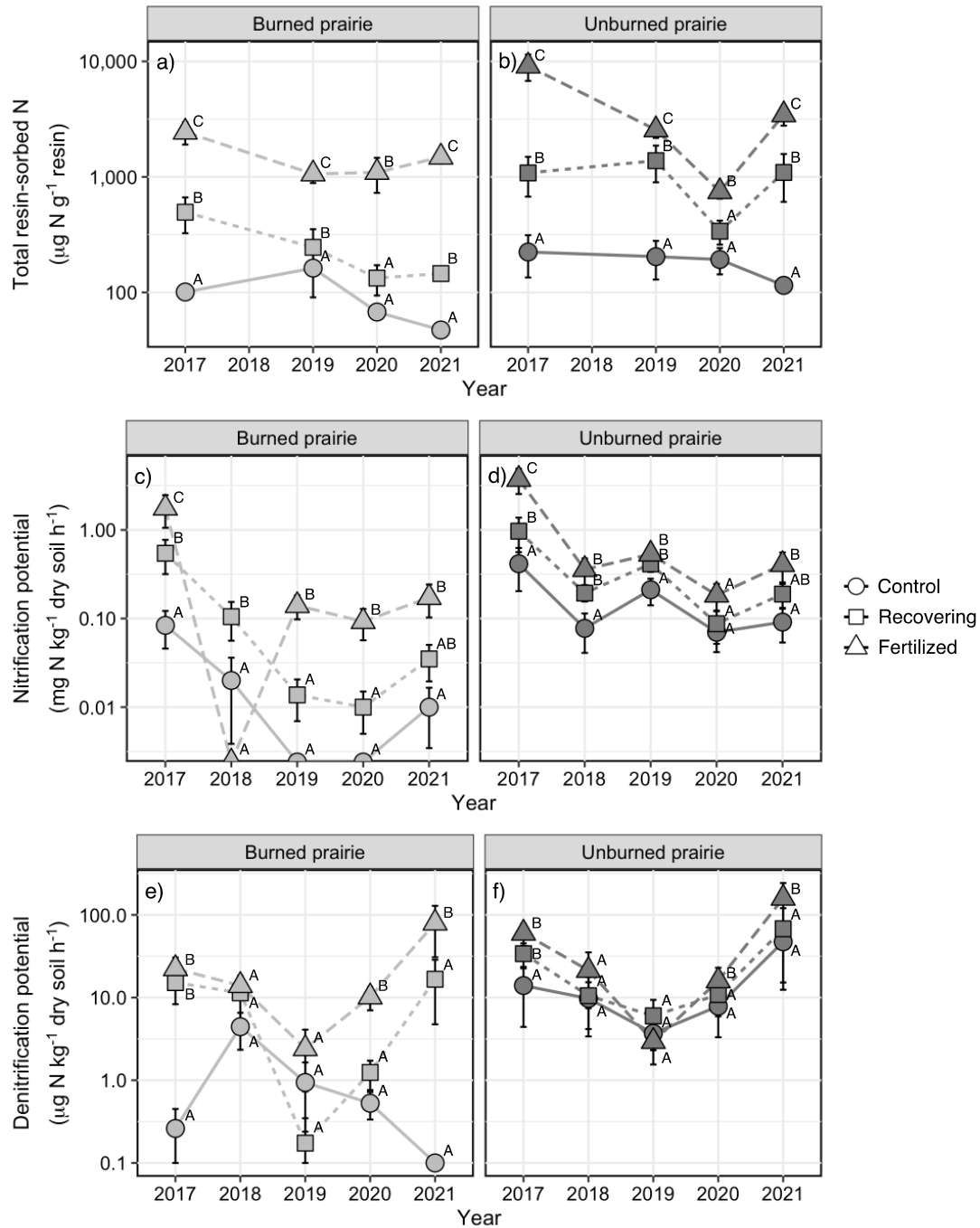


Figure 4-1. Total available inorganic N extracted from surviving resin bags during the growing season in 2017 and 2019-2021 (a, b), and nitrification potential (c, d) and denitrification potential (e, f) rates estimated twice during the growing season in 2017-2021, for both burned and unburned prairie treatments. Each point is the average annual value for the fertilization treatment, and the error bars are ± 1 standard error (SE). Letters denote significant differences between fertilization treatments within the year and fire treatment at $P \leq 0.05$.

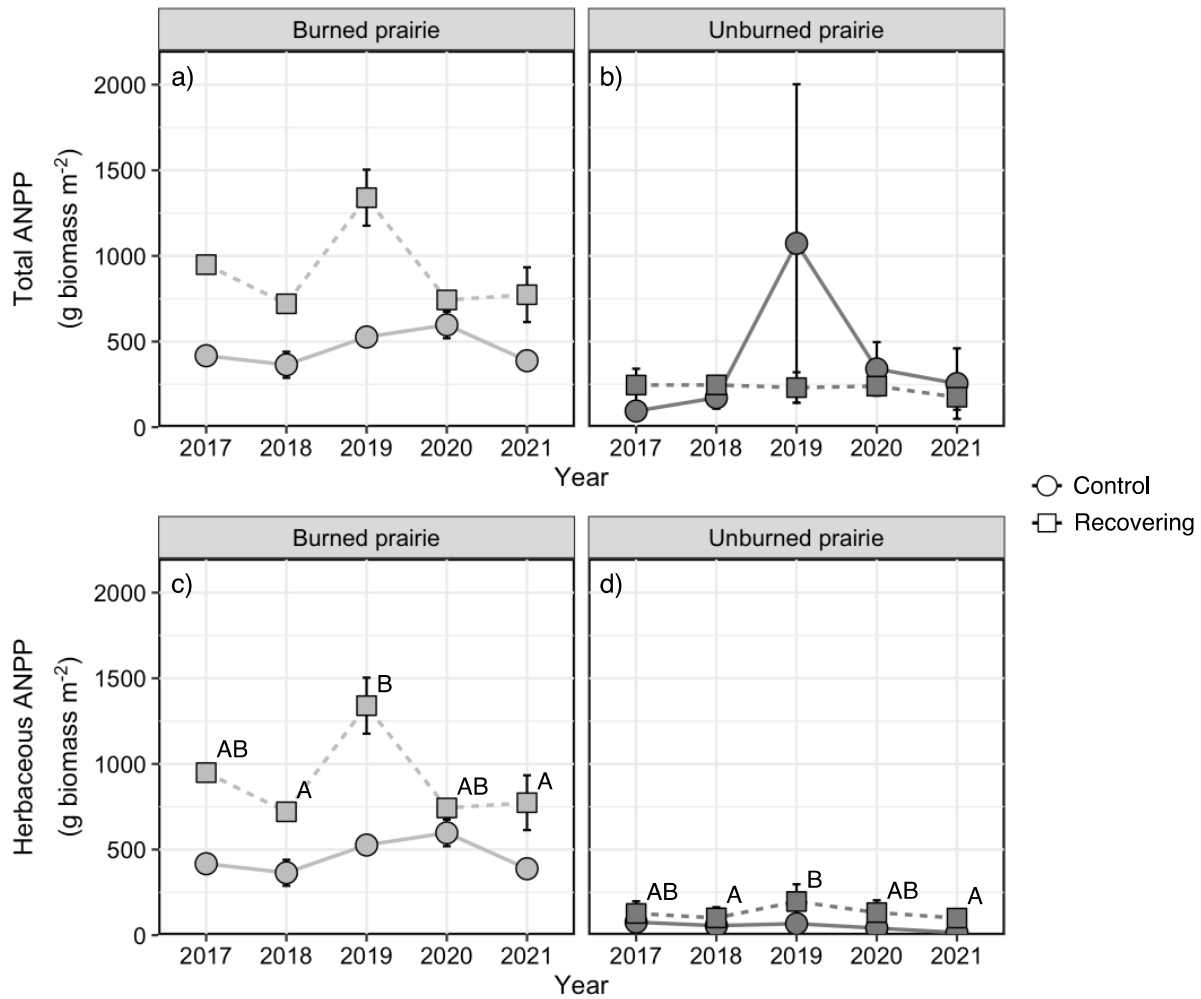


Figure 4-2. Total (a, b) and herbaceous (c, d) ANPP in 2017-2021 for both burned and unburned prairie treatments. Each point is the average rate for the fertilization treatment, and the error bars are ± 1 SE. Letters denote significant differences among years at $P \leq 0.05$.

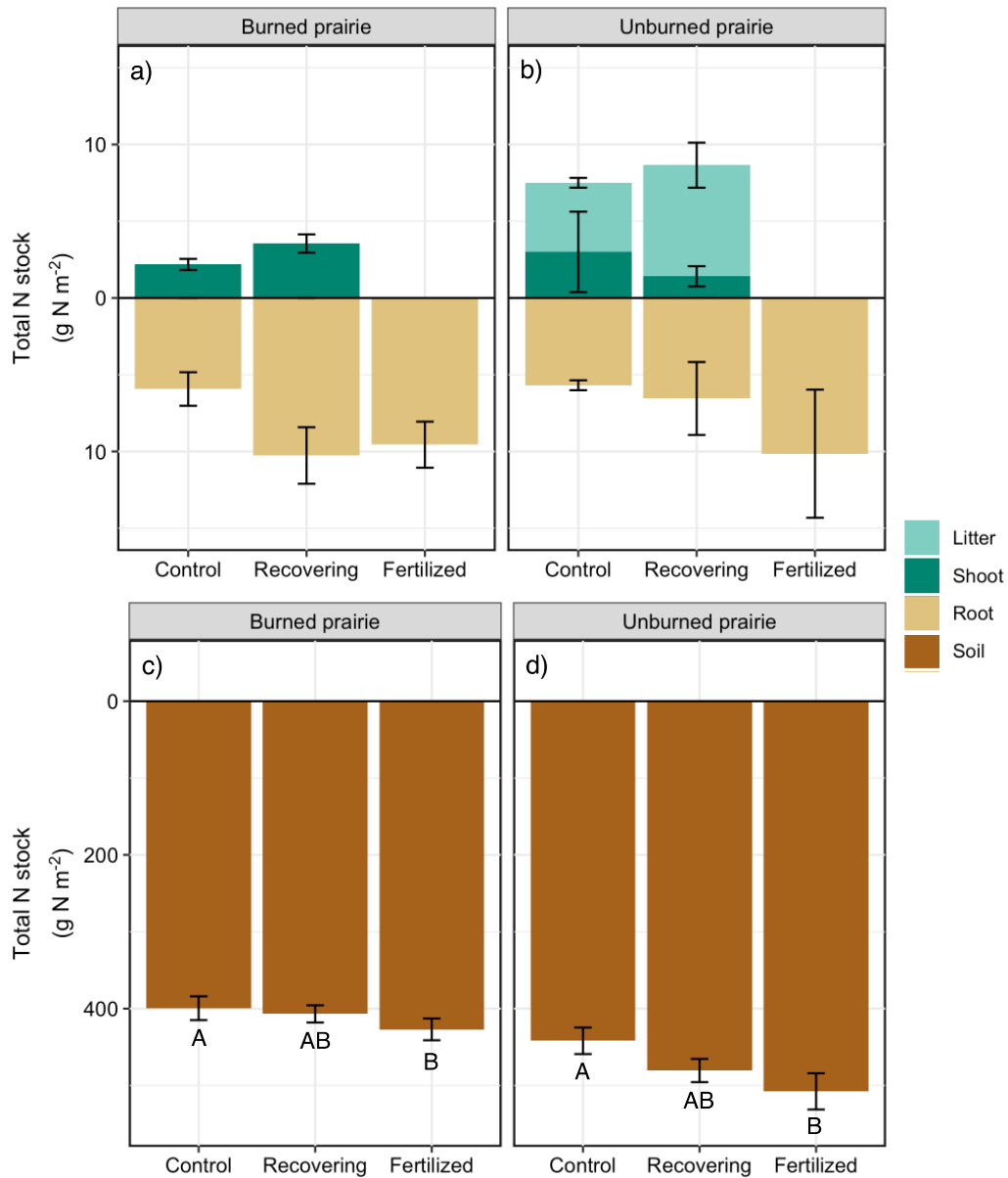


Figure 4-3. Plant (a, b) and soil (c, d) N stock in 2021 for both burned and unburned prairie treatments. Each bar is the average stock for the fertilization treatment, and the error bars are ± 1 SE. Capital letters denote significant differences among fertilization treatments at $P \leq 0.05$.

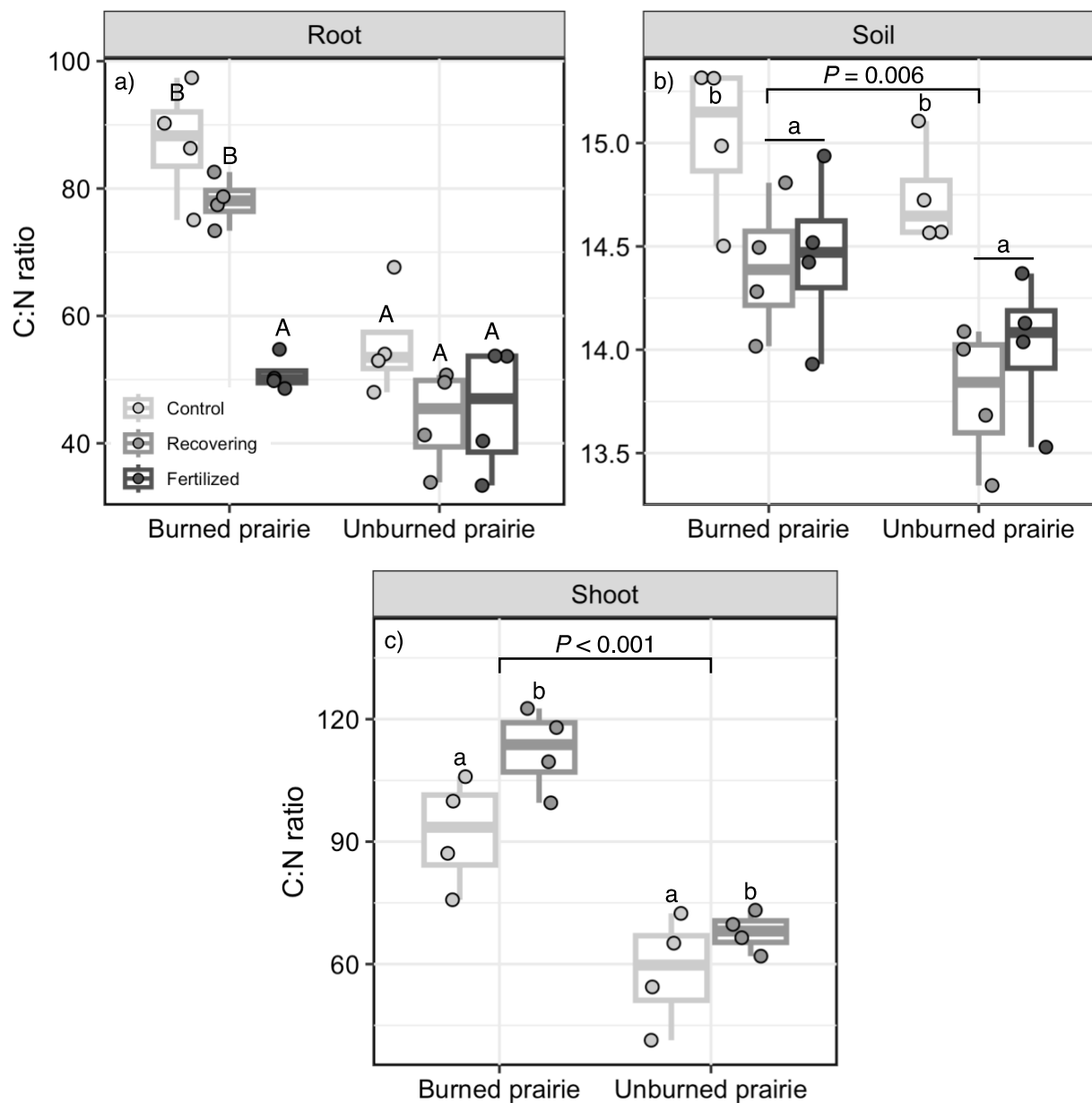


Figure 4-4. C:N ratio of shoots (a), roots (b), and soils (c) in burned and unburned prairies at the end of the 2021 growing season. Each point is the average C:N ratio for each individual plot corresponding to the fertilization treatment, with the different fertilization treatments denoted by color. Capital letters for root C:N denote significant differences between combined field treatments, while lowercase letters in soil C:N indicate significant differences between fertilization treatments, at $P \leq 0.05$.

Chapter 5 - Limited recovery of soil microbial communities reveals fertilization legacy in a burned and unburned grassland

5.1 Abstract

Ecosystem responses to disturbance could persist due to changes to the physical environment or taxonomic turnover. In soil microbiomes, structure and function are shown to be sensitive to short- and long-term disturbances, and recent work suggests functional legacies can emerge. However, whether a change in the soil microbial community is linked to sustained change in function after the perturbation ceases is unclear. We used a long-term nitrogen (N)-fertilization field experiment crossed with contrasting fire treatments to assess whether soil microbial community composition remains different after fertilization ceased, using 16S rRNA gene amplicon sequencing. Microbial community composition in previously fertilized soils still resembled communities in soils that continue to be fertilized for up to five years. We found that soil microbial communities with a history of fertilization in the fire-suppressed treatment, which also raised soil N availability, were more different from their control-reference communities than communities in the annually burned treatment, but resistance and resilience metrics disagree with this observation. To test whether microbial life history strategies explained these shifts, we defined taxa at the phylum and class levels and found no strong evidence that putative copiotroph relative abundance was persistently higher after fertilization ceased. Finally, we tested whether microbial community function remains altered from fertilization by measuring soil enzymatic activity and found no consistent patterns except for lower enzymatic investment for N relative to C in fire suppressed conditions. Collectively, these findings suggest that soil

microbial communities retain a memory of previous N-fertilization by altered community structure that may not necessarily reflect altered community function post-cessation.

5.2 Introduction

The soil microbiome is an important component of terrestrial ecosystems that supports services such as retaining and cycling nutrients, storing carbon, and promoting plant growth (Brussaard et al. 2007, Jansson and Hofmockel 2020). However, the eutrophication of terrestrial ecosystems by bioavailable nitrogen (N) from anthropogenic sources is a known driver of microbial community structure (i.e., taxonomic composition and relative abundance) and function that could disrupt the services they provide (Vitousek et al. 1997, Fixen and West 2002, Gruber and Galloway 2008, Ouyang et al. 2018). In most cases, soil microbial community structure is sensitive to both pulse (short) and press (chronic) disturbances and recovers more slowly than expected given microorganisms' high sensitivity (Shade et al. 2012). However, environmental change, including N-fertilization, may not affect microbial community function if it is insensitive to the change (i.e., has high resistance), or if redundancy rescues community function despite a change in structure; in contrast, the functioning of communities could recover slowly, if feedbacks that maintain the novel structure have taken effect (Allison and Martiny 2008, Shade 2023). Moreover, while biotic and abiotic feedbacks shape community function, land use change often co-occurs with N eutrophication to further affect community stability (Rillig et al. 2019, Suleiman et al. 2022). Therefore, discerning the mechanisms that underpin soil microbiome sensitivity and resilience to chronic N-fertilization, concomitant with land use change, may identify strategies to mediate or accelerate recovery to pre-disturbed microbial states.

One mechanism that may explain changes in taxonomic composition in response to elevated N availability is related to growth strategies of different microbial taxa. According to the copiotroph-oligotroph framework, microorganisms may be adapted to grow and reproduce quickly when nutrient availability is high (*r*-selected copiotrophs) or grow slowly and use resources efficiently to better compete when nutrients are scarce (*K*-selected oligotrophs) (Killham and Prosser 2015). This hypothesis predicts that the relative abundance of copiotrophic microbial populations should increase with N-fertilization and the relative abundance of putative oligotrophs should decrease (Ramirez et al. 2012), and has been supported across ecosystems (Ramirez et al. 2010, Leff et al. 2015). Although this hypothesis was intended to explain how communities might change under current N-addition treatments, it has not been evaluated as a mechanism of community structure or functional recovery after fertilization ceases. Moreover, the copiotroph-oligotroph framework has primarily been conceptualized and applied at a coarse taxonomic scale (e.g., Phylum or Class), but selection for optimal nutrient use for growth is likely to have occurred at finer taxonomic levels (Philippot et al. 2010, Stone et al. 2023); thus, classifying taxa at high taxonomic ranks could be a flawed strategy to assess recovery of microbial taxonomic composition from chronic N disturbances.

The copiotroph-oligotroph framework can also be used to explain a tradeoff between the investment to acquire resources and microbial growth (Ho et al. 2017). The copiotrophic hypothesis predicts that a shift towards dominance of copiotrophs will lower the synthesis, and thus activity, of extracellular enzymes used to acquire nutrients from soil organic matter (Ramirez et al. 2012). Though this growth-resource acquisition tradeoff has been observed in N-addition lab studies, there is little support for this tradeoff in field fertilization experiments (Riggs and Hobbie 2016, Keller et al. 2022), suggesting other mechanisms control microbial

community function, such as functional redundancy (Setälä and McLean 2004, Allison and Martiny 2008) or soil pH (Treseder 2008). Therefore, the balance between biotic and abiotic controls in community function could determine the magnitude and time of soil microbial function recovery from chronic N-fertilization. As such, a change in ecosystem function (e.g., decomposition) concomitant with changes to microbial community structure, measured by 16S rRNA gene data, that persists after ceasing chronic N-fertilization could provide evidence that the microbiome has shifted to an alternative or long transient state (Ratajczak et al. 2018, Shade 2023).

Like other terrestrial ecosystems, many grasslands are experiencing an increase in bioavailable N, and land management practices, such as fire, also impact N dynamics. Fire frequency in mesic grasslands plays an important role in N availability: A fire return interval of 1-2 years creates a persistently N-limited system due to combusting accumulated litter (Turner et al. 1997, Pellegrini et al. 2018) and favoring the productivity and dominance of C₄ grasses with inherently high nitrogen use efficiency (Tilman and Wedin 1991, Ojima et al. 1994), while infrequent fire return intervals (4-20 years), or suppressing fire altogether, increases biologically available N as litter decomposes and returns N belowground (Ojima et al. 1994, Blair 1997, Connell et al. 2020). Moreover, a switch from frequent to infrequent fire regimes can transition a mesic grassland to a woodland state over time (Briggs et al. 2005, Ratajczak et al. 2014, Collins et al. 2021). While soil microbial community structure is also sensitive to fire frequency (Carson and Zeglin 2018), it is not clear if their shifts are an outcome of altered soil chemistry, such as higher N availability, or changes in vegetation (Mino et al. 2021). As such, disentangling these mechanisms can improve our ability to predict soil microbial structure and function dynamics as

grasslands continue to experience changes in N availability and land use, including fire management.

In fire-adapted grasslands, current evidence shows equivocal responses to N-fertilization by soil microbial communities. In an annual Mediterranean grassland, both composition and function of soil microbial communities were more sensitive to recent and past fires (Yang et al. 2020) than to N-fertilization (Qin et al. 2020); yet, in tallgrass prairies, N-fertilization increased the relative abundance of putative copiotrophs and increased N-cycling rates to a higher magnitude in fire-suppressed (unburned) prairies than burned prairies (Carson and Zeglin 2018, Nieland et al. 2021). While little research has examined the recovery of soil microbial communities after N-fertilization ceases, the few studies that exist indicate moderate or incomplete recovery of microbial functions and population sizes (Bowman et al. 2018, Nieland et al. 2021), suggesting N-fertilization continues to affect soil microbial communities after the treatment ceases (i.e., causes a legacy effect). It is not clear whether soil microbial communities recover faster with fire relative to those in a fire-suppressed system, or if changes to microbial community structure and function caused by N-fertilization are due to competitive advantages of taxa (e.g., copiotrophic hypothesis).

To assess whether soil microbiomes recover from N-fertilization, bacterial and archaeal 16S rRNA gene community composition was collected from a thirty-year fertilization and fire experiment in a tallgrass prairie, for five consecutive years (2017-2021) after fertilization ceased in 2016. Moreover, soil extracellular enzyme activity was measured to assess microbial community function in the fifth year after fertilization ceased. If a copiotrophic state is driven by N-fertilization, we predicted that the soil microbial community would revert to a composition more similar to that of never-fertilized conditions following cessation of fertilizer addition. Thus,

soil extracellular enzyme activity and associated nutrient-limitation indices should collectively rebound if the copiotrophic hypothesis adequately explains tradeoffs between nutrient acquisition to growth. Also, because fire suppression increases N availability, we expected higher microbial community sensitivity to N-fertilization in unburned prairies, such that putative copiotroph and oligotroph differential abundance to be greater in magnitude. Finally, we predicted that soil microbial community structure and function would recover faster, or with a greater magnitude, in annually burned prairies because of more N-limited conditions for both plants and soil microbes.

5.3 Methods

Site description

The Konza Prairie Biological Station (KBPS; 39°N05' N, 96°35' W) is located in the Flint Hills region of eastern Kansas, USA, where the largest continuous tract of tallgrass prairie remains in North America. KBPS is a 3,487-ha tallgrass prairie with shallow soils in the uplands and deep, silty-clayey loams in the lowlands. At the field experiment site, the lowland soils belong to the Irwin series (fine, mixed, mesic, pachic Argiustolls) and have no organic horizon. Mean annual temperature at KPBS is 26.6°C and mean annual precipitation (1986-2021) is 849 mm (SD = 222) (Nippert 2023), with 75% of that precipitation typically falling during the growing season (April-September). However, during this study (2017-2021), cumulative growing season precipitation varied interannually such that it was one standard deviation under and over the mean (abnormally dry in 2018, and abnormally wet in 2019; Appendix E Figure E-1). Vegetation at KPBS is primarily covered by C₄ grasses, such as *Andropogon gerardii*, *Sorghastrum nutans*, *Panicum virgatum*, and *Schizachyrium scoparium*, and forbs that make up most of the species richness in frequently burned prairie (Towne 2002), while woody and

shrubby C₃ species, including *Juniperus virginiana*, *Cornus drummondii*, and *Rubus occidentalis*, dominate coverage in infrequently burned and unburned prairie (Carson et al. 2019).

Belowground plots experiment

The Belowground Plots (BGP) Experiment was started in 1986 and is a strip-plot field experiment that overlays different fire and fertilization treatments (Seastedt et al. 1991). There are eight experimental blocks: four blocks are burned early spring (burned prairie), and the other four blocks are not burned (unburned prairie). Within these blocks, we studied soil microbial communities in 12.5 × 12.5-m plots that were never fertilized (control treatment) and plots that were fertilized for 31 years (1986-2016) with N (10 g N m⁻² yr⁻¹ as NH₄NO₃) (recovering treatment). In 2017, we set up four 1 × 1-m subplots within the recovering plots and continued to fertilize them with N at the same rate (fertilized treatment) to continue assessing belowground sensitivity to N (Nieland et al. 2021). With this new fertilization treatment, there are a total of six field treatments, each with four replicate plots ($n = 4$).

Soil collection and processing

Soils for microbial community composition surveys were collected twice a year during the growing season in June, at least one week after fertilizer was applied, and in August, for five years (2017-2021). Four 2 cm diameter, 15 cm depth mineral soil cores were collected aseptically from each plot, or one core from each subplot, and were combined as a composite plot or subplot samples. Immediately after collection, samples were sieved through a 4 mm mesh to remove roots and rocks, and samples were flash frozen. Samples were stored at -80°C until processed.

Molecular microbial analysis

Genomic DNA (gDNA) was extracted using a modified CTAB method and precipitated overnight in 30% PEG 6000, followed by an ethanol wash the next day (DeAngelis et al. 2010). To measure bacterial and archaeal community composition, the V4 region of the 16S rRNA gene from gDNA was amplified by following an established one-step PCR protocol using 515F/806R primers fitted with adaptors for Illumina sequencing (Caporaso et al. 2011), except 25 PCR cycles were used instead of 35 to avoid PCR amplification bias (Zeglin et al. 2016). PCR products were normalized by concentration and pooled into two sequencing libraries that were cleaned with a gel-purification kit (Qiagen, Hilden, DE). Libraries were sequenced with 2 × 150 bp paired-end read Illumina MiSeq runs.

Raw sequence data were processed using the QIIME2 2022.8 bioinformatics pipeline (Bolyen et al. 2019). Sequence data were demultiplexed, and forward reads were truncated at the 150 bp position based on the lowest quality run. The DADA2 pipeline was used to denoise reads and to merge paired-end reads with default parameters (Callahan et al. 2016), and amplicon sequence variants (ASVs) were assigned taxonomically using a naïve Bayes classifier trained on the SILVA 138.1 16S rRNA gene database (Quast et al. 2012) with help from RESCRIPt (Robeson et al. 2021). ASVs were filtered out from further analyses if they met any of the following criteria: 1) they had less than three reads in the library (Bokulich et al. 2013), 2) they were associated with chloroplasts or mitochondria, or 3) they were at least 10× greater in extraction blanks or negative controls than in samples based on mean sequence reads. Following this quality control, the two libraries were merged.

Statistical analysis: Does fire accelerate microbial community diversity recovery from fertilization?

All statistical and visual analyses were conducted using R V4.2.1 (R Core Team 2022) in the RStudio environment V2022.07.0 (RStudio Team 2022), with assistance from *tidyverse* (Wickham et al. 2019). To evaluate how fire management affects soil microbial diversity responses to N-fertilization, we first trimmed samples with less than 19,000 sequence reads from the dataset (20 samples removed) using *phyloseq* (McMurdie and Holmes 2013), since sample sequence depth under this threshold fell precipitously (Appendix E Figure E-2). Afterwards, we rarefied samples to 19,000 sequences with replacement and calculated observed ASV richness, Shannon diversity, and evenness. Observed richness, Shannon diversity, and evenness were assessed using linear mixed effect models from the *lmerTest* package (Kuznetsova et al. 2017), with fire, fertilization, and year included as interacting fixed effects. To account for repeated sampling during the growing season, we included block and day of year (scaled to zero) as random intercepts in mixed models. After checking model assumptions, each model was assessed using analysis of variance (ANOVA), and Tukey's post-hoc analyses were conducted for significant effects or interactions with *emmeans* (Lenth 2016).

For beta diversity, we normalized the trimmed dataset by proportional transformation and then averaged ASV reads by combined field treatment for each month because it was a more conservative approach to detect community composition shifts. To test for fire, fertilization, and year effects on community composition, we implemented a permutational ANOVA (PERMANOVA) with the Bray-Curtis dissimilarity metric, from which pairwise PERMANOVAs were used because of significant interactions with *pairwiseAdonis* (Martinez Arbizu 2017). Further, we visualized microbial community structure using non-metric multidimensional analysis (NMDS) with the lowest stress value from twenty iterations, and plotted distance between NMDS ordination points across time to assess temporal stability based

on Lamothe et al.'s (2019) recommendations. Finally, to test if fire affected community sensitivity and recovery, we used resistance and resilience indices based on Bray-Curtis similarity from microbial community composition (Shade et al. 2011, Dacal et al. 2022). The resilience index represents the proportion of Bray-Curtis similarity recovered relative to the continuously fertilized treatment and was calculated using the equation

$$\text{Resilience} = \frac{S_{T_{RC}} - S_{T_{FC}}}{1 - S_{T_{FC}}}$$

where $S_{T_{FC}}$ is the Bray-Curtis similarity between fertilized and control treatments and $S_{T_{RC}}$ is the Bray-Curtis similarity between recovering and control treatments at time T . Resistance and resilience indices share similar scales, where 0 indicates community composition was not resistant (i.e., no similarity in composition) nor recovered from fertilization, while 1 means total resistance and/or recovery; however, the resilience index can also be negative, in which there was further community turnover after ceasing fertilization. Mixed effect models described above were used to analyze resistance and resilience, except that fertilization treatments were excluded.

Statistical analysis: Do microbial life history strategies explain changes to microbial community composition from fertilization?

To assess whether the copiotrophic hypothesis explained shifts in microbial community composition, we tested if common ASVs (i.e., ASVs detected in all combined field treatments at least once during the study) shifted in a predictable direction based on their phylum or class assignment. Phyla and classes were defined as putative copiotrophs or oligotrophs from previous studies (Ramirez et al. 2012, Leff et al. 2015). We analyzed differential abundance of ASVs from the trimmed community dataset using analysis of composition of microbiomes with bias correction (ANCOM-BC) (Lin and Peddada 2020) with the control treatment as reference. ANCOM-BC accounts for compositionality of high throughput sequence data and is an

improvement from previous methodologies of differential abundance testing by estimating unknown sampling fractions from data and correcting for bias. We ran ANCOM-BC analyses separately for fire and fertilization treatments each year, averaging ASV log-fold changes for each combined field treatment, and tested phylum and class shifts using *t*-tests. Finally, we assessed the number of unique and shared ASVs in the trimmed dataset between field treatments using the *MicrobiotaProcess* package (Xu et al. 2023).

Statistical analysis: Does soil microbial function recover five years after ceasing fertilization?

To test if soil microbial community function differed between fire and fertilization treatments, we measured soil extracellular enzyme activities (EEAs) and microbial biomass from a new batch of soil samples collected in September 2021. Briefly, four 5 cm diameter, 15 cm depth soil cores from each plot or subplot were collected and stored at 5°C until sieved and processed as composites. Soil EEAs were measured using short-term, room temperature fluorescent assays (Saiya-Cork et al. 2002, Zeglin et al. 2013). Activities of β -glucosidase (BG; EC 3.2.1.21), cellobiohydrolase (CBH; EC 3.2.1.91), *N*-acetylglucosaminidase (NAG; EC 3.2.1.14), leucyl aminopeptidase (LAP; EC 3.4.11.1), acid phosphatase (PHOS; EC 3.1.3.1), and phenol oxidase (PHX; EC 1.10.3.2) were measured in 96-well plates that included blanks and quench controls with a FilterMax F5 Multimode Microplate Reader (Molecular Devices, San Jose, CA, USA). We assessed relative microbial investment to acquire certain nutrients by calculating ratios between enzymes for C relative to N, C relative to P, and N relative to P. We measured microbial biomass C and N by chloroform fumigation extraction (Brookes et al. 1985, Vance et al. 1987), and quantified dissolved organic C and N using a Shimadzu TOC-L with an attached TN analyzer (Shimadzu Scientific Instruments Inc., Columbia, MD, USA). ANOVAs were used to test for differences in EEAs, EEA ratios, and microbial biomass C and N.

5.4 Results

Does fire accelerate microbial community recovery from fertilization?

In each fire treatment, soil microbial community composition differed between control soils and both previously and continuously fertilized soils, which were similar to each other but well separated from the control treatment in NMDS space (Figure 5-1). The separation between control and fertilized treatments was stronger in the unburned prairie than burned prairie (Figure 5-1). However, there was no evidence of community composition recovery following cessation of fertilization in either fire treatment (Figure 5-1), and according to resistance and resilience indices based on Bray-Curtis similarity, microbiomes in burned prairies were neither more resistant nor resilient to fertilization than unburned prairies (Appendix E Table E-1, Appendix E Figure E-3). Indeed, pairwise PERMANOVAs confirmed that community composition in recovering and continuously fertilized soils were not different (Appendix E Table E-2), though fire, fertilization, and treatment interactions explained a significant proportion of the overall variation (13.9%, 16.5%, and 9.5%, respectively; Table 5-1). Soil microbial community composition also changed interannually, explaining 16.8% of the variation. The NMDS showed that community composition shifted along mostly independent trajectories in response to field treatments (Axis 1) and through time (Axis 2) (Figure 5-1). Notably, the temporal trajectory in community composition reversed on the same path for all fertilization treatments in 2020 (Appendix E Figure E-4).

There were no consistent alpha diversity responses to continuous fertilization (Figure 5-2), yet fertilization did affect observed ASV richness, Shannon diversity, and evenness, but always with a temporal interaction (Appendix E Table E-1). The number of observed ASVs was lowest in 2017 for all fertilization treatments, but the 16S rRNA gene richness range in

continuously fertilized soils (449 observed ASVs in 2017 to 1012 in 2020) was wider than other fertilization treatments. Similarly, continuously fertilized soils had the lowest and highest Shannon diversity (5.29 in 2017 to 5.99 in 2020). Though evenness decreased during the wet 2019 growing season in the control and continuously fertilized soils, Shannon diversity and evenness were less temporally variable in recovering soils, and both did not change in 2019.

Do microbial life history strategies explain changes to microbial community composition from fertilization?

ASVs defined as putative copiotrophs or oligotrophs according to their phylum or class provided limited support for the copiotrophic hypothesis (Figure 5-3). ASV abundance responses to fertilization varied widely among ASVs, and when aggregated at broad taxonomic levels, nine of the 32 group comparisons had significant mean log fold changes in ASV abundance (Appendix E Table E-3), in which unburned prairie soils possessed eight of the significant differences. Contrary to predictions supporting the copiotrophic hypothesis and previous research at this site (Carson and Zeglin 2018, Nieland et al. 2021), all significant oligotroph population shifts increased with fertilization ($P \leq 0.006$). Putative copiotroph and oligotroph grouping was a poor predictor of the direction ASVs shifted (Appendix E Figure E-5). Further, most ASVs in fertilized treatments were differentially abundant for only one growing season, although ASV log-fold changes between fertilized-control and recovering-control treatments fell along the 1:1 line. However, recovering and continuously fertilized soils together harbored over 40% of unique ASVs in the entire dataset (Appendix E Figure E-6), indicating that taxonomic turnover, rather than shifts in relative abundance of putative copiotrophs and oligotrophs, had a strong role in soil microbial community structure change following chronic N-fertilization.

Does soil microbial function recover five years after ceasing fertilization?

Contrary to previous findings reported by Nieland et al. (*in review*), there was no decrease in N-acquiring NAG and LAP activities under fire suppressed or chronic N-fertilized conditions (Appendix E Table E-4, Appendix E Figure E-7); thus, microbial enzymatic investment for C relative to N ($\ln(\text{BG}):\ln(\text{NAG}+\text{LAP})$) was not higher (i.e., less N-limited) from N-fertilization (Figure 5-4). However, fire suppression increased C-acquiring CBH activity (Appendix E Table E-4) and microbial enzymatic investment for C relative to N (Appendix E Table E-4), indicating consistent microbial responses to higher N availability from fire suppression (Figure 5-4). Notably, compared to never-fertilized soils, soil CBH activity decreased in recovering soils (post-hoc $P = 0.017$). However, biomass-specific CBH activities were not different between fertilization treatments, hinting that the slight decrease in microbial biomass after ceasing N-fertilization affected soil cellulolytic activity.

5.5 Discussion

Chronic N-eutrophication, due to increased atmospheric deposition and mineral N-fertilization, can negatively affect terrestrial ecosystems (Vitousek et al. 1997, Schlesinger 2009). While soil microorganisms are sensitive to this press disturbance, the soil microbiome may mediate total ecosystem recovery if its structure and/or function is resilient (Nieland et al. 2021). However, our results show that the soil microbial community structure in this mesic grassland did not recover in the five years after chronic N-fertilization ceased. This is the first study, to our knowledge, that investigated soil microbial community responses after long-term N-fertilization ceased. It adds to the growing list of studies that show soil microbiomes are often sensitive, yet not resilient, to chronic disturbances (e.g., Allison and Martiny 2008, Shade et al. 2012).

Our hypothesis that annual prescribed fire would decrease community structure sensitivity and increase resilience was only partially supported. Although microbial community structure in burned prairies was less sensitive to N-fertilization as indicated by the NMDS, the resistance index showed that soil microbial communities in burned and unburned prairies were equally sensitive to N-fertilization during the study. Further, as evident by the NMDS and resilience index, there was no difference in microbial community structure recovery from N-fertilization between the different fire treatments. Therefore, the absence of recovery suggests that feedbacks from the soil environment, such as sustained higher levels of bioavailable N from N mineralization (Clark and Tilman 2008, O’Sullivan et al. 2011, Nieland et al. 2021), may slow or prevent recovery of community structure (Ratajczak et al. 2017).

In contrast, Shannon diversity and evenness of microbial communities in recovering soils were different from the continuously fertilized soils, indicating slight changes to community dynamics after ceasing N-fertilization. The higher variability in observed ASV richness and Shannon diversity in continuously fertilized soils could result from chronic N-fertilization lowering microbial community stability, which may also affect functional stability (Collins and Xia 2015, Koerner et al. 2016). Although alpha diversity metrics tend to be poor measures of community dynamics (Wilsey et al. 2005, Jones et al. 2017, Hillebrand et al. 2018), alpha diversity in this study signaled small changes in community dynamics between fertilization treatments across time. For example, alpha diversity responded negatively to the wet 2019 year and then recovered in 2020, suggesting that the water stress may have resulted in a microbial mortality event (Veatch and Zeglin 2020). Thus, it is important to consider microbial community structure recovery at different timescales from pulse and press disturbances.

The copiotrophic hypothesis was not supported in this study despite its popularity in explaining changes to soil microbial community structure following N-fertilization (e.g., Yao et al. 2014, Leff et al. 2015, Grant et al. 2022), including at this site (Carson and Zeglin 2018, Nieland et al. 2021). Rather than putative copiotrophs increasing and oligotrophs decreasing in relative abundance with N-fertilization, we found positive and negative responses among ASVs within these groups. This mixed set of responses are consistent with a continuum, rather than a dichotomous, framework of copiotroph-oligotroph life history (Stone et al. 2023). Therefore, growth strategies of microbial taxa, and their functional tradeoffs, may not be reliably predictable at high taxonomic ranks; instead, finer taxonomic levels (e.g., family or genus) likely serve as better conduits for predicting life history strategy (Morrissey et al. 2016, 2019).

Importantly, soil microbial enzymatic investment for C relative to N appeared to decrease (i.e., increased demand for N) slightly in burned prairies in the fifth year after ceasing N-fertilization. This change in relative microbial investment to acquire N suggests that soil microbial cells have become more N-limited since fertilization ceased, supporting a cellular level feedback associated with the product-inhibition hypothesis (Olander and Vitousek 2000, Schimel and Weintraub 2003, Allison and Vitousek 2005). If the cellular level feedback is the mechanism as a response to the decline in extracellular N availability, and enzymatic investment for C relative to N continues to decrease, then this suggests that the function of soil microbial communities is recovering in burned prairies. Thus, current or historic prescribed fires may increase resilience of the soil microbiome from N-fertilization (Allison and Martiny 2008, Shade et al. 2012), at least in terms of function, assuming activities of soil extracellular enzymes reflect aggregate community function.

In contrast, fire suppression may have constrained the recovery of the soil microbiome from N-fertilization. In particular, there was no change to extracellular enzyme activities and enzymatic investment for C relative to N did not decrease, suggesting that either N availability still remains high, or that the soil microbiome has passed a ‘threshold’ from an environmental driver and has undergone a regime shift (Andersen et al. 2009, Scheffer et al. 2015, Shade 2023). In support of the former possibility, inorganic N availability remained an order of magnitude higher in recovering soils than control soils (Nieland and Zeglin *in prep*). Fire suppression increases N availability as litter decomposes and resupplies soil microorganisms with N (Ojima et al. 1994, Blair 1997), as was observed in this experiment (Nieland and Zeglin *in prep*). Notably, soil microbial community structure in a temperate steppe changed significantly at a high threshold of N addition along an experimental gradient (Liu et al. 2020). This N-addition threshold suggests that N-fertilization and fire-suppression together at this tallgrass prairie site could have pushed soil microbial communities to a new state that are reinforced with new feedbacks. Although soil pH can decrease under N-fertilization (Ouyang et al. 2018, Jia et al. 2020) and consequently affect soil microbial community structure (Widdig et al. 2020), the limestone parent material and near saturation of base cations (Ransom et al. 1998) at this site buffer soil pH, making pH shifts weaker here than at other chronic fertilization experiments. Hence, N-availability, both from direct N addition and from fire suppression, are more likely drivers of the observed shift in the soil microbiome.

In conclusion, chronic N fertilization shifted soil microbial communities to alternative states that persisted after the treatment ceased, but the fire-mediated removal of ecosystem N via pyrovolatilization did not accelerate the recovery of community structure. In addition, the copiotrophic hypothesis, a dichotomous response model used to explain microbial community

structure shifts from N-fertilization, was not supported. Rather, a continuum along a copiotroph-oligotroph framework better explained common ASV responses to chronic N-fertilization (Stone et al. 2023). These findings reinforce the idea that taxonomically classifying microbes at finer taxonomic ranks are more reflective, and relevant, of their responses to environmental changes (Philippot et al. 2010). Therefore, future studies should investigate whether differential abundances of soil microbes classified at the order or genus level, instead at the phylum or class level, are consistent across varying ecosystems in response nutrient addition. Moreover, different ecological drivers of community assembly, notably changes to N availability and water stress, may have affected community dynamics at different time scales (i.e., interannual and decadal). Finally, while testing for ecological thresholds was beyond the scope of this study, soil microbial function, measured by soil EEAs, did not recover in previously fertilized unburned prairies. In this context, future research should assess whether N-fertilization shifts soil microbiomes to alternative stable states by manipulating interacting drivers and surveying soil microbial dynamics across time, as well attempting to ‘push’, or ‘rescue’, microbiomes back to reference states.

5.6 References

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Table 5-1. Permutational analysis of variance (PERMANOVA) results for soil microbial community composition different treatments and time.

Fixed effects	Degrees of freedom	Pseudo- <i>F</i>	<i>R</i> ²	<i>P</i>
Fire (B)	1	16.1	0.139	0.001
Fertilization (F)	2	9.6	0.165	0.001
Year (Y)	4	4.9	0.169	0.001
B × F	2	5.5	0.095	0.001
B × Y	4	1.0	0.033	0.570
F × Y	8	1.2	0.080	0.167
B × F × Y	8	0.9	0.059	0.845
Residuals	30		0.259	

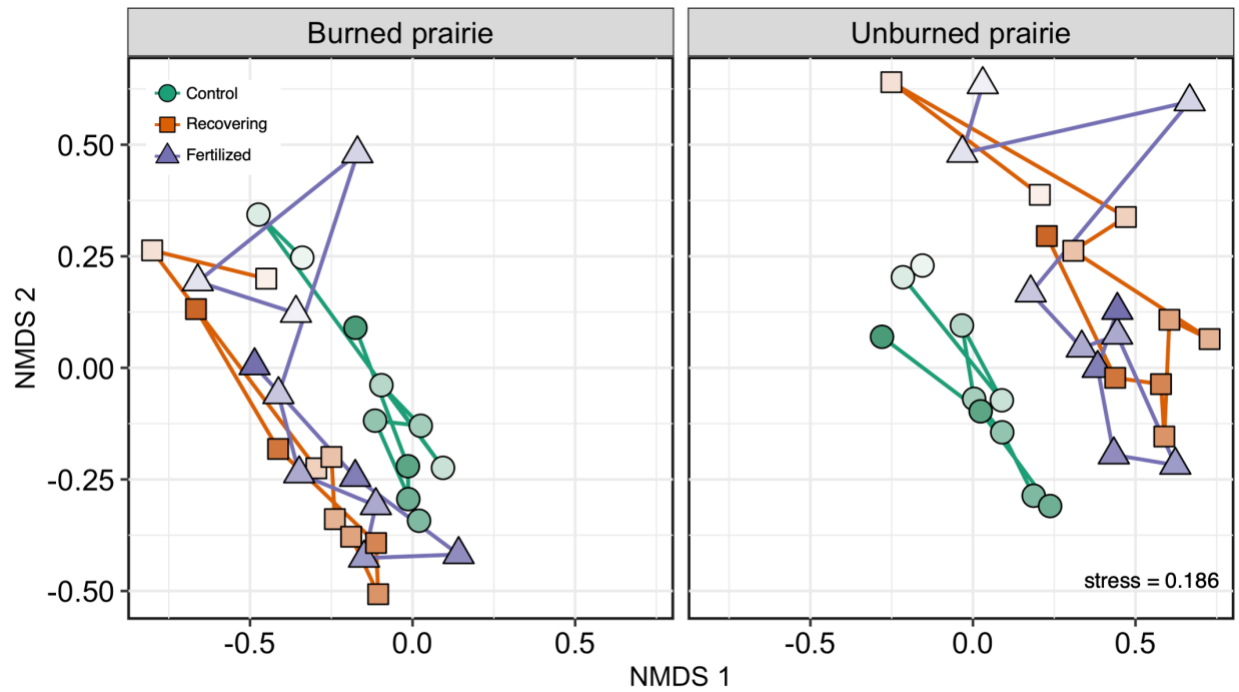


Figure 5-1. NMDS of soil microbial communities along field treatments at the BGP experiment. Each ordination point represents a single time point, and lines connect the samples across time. The gradient of color intensity reflects sample collection date, with the lightest points reflecting the 2017 soil microbial communities. The ordination is split by fire management treatment for clarity purposes.

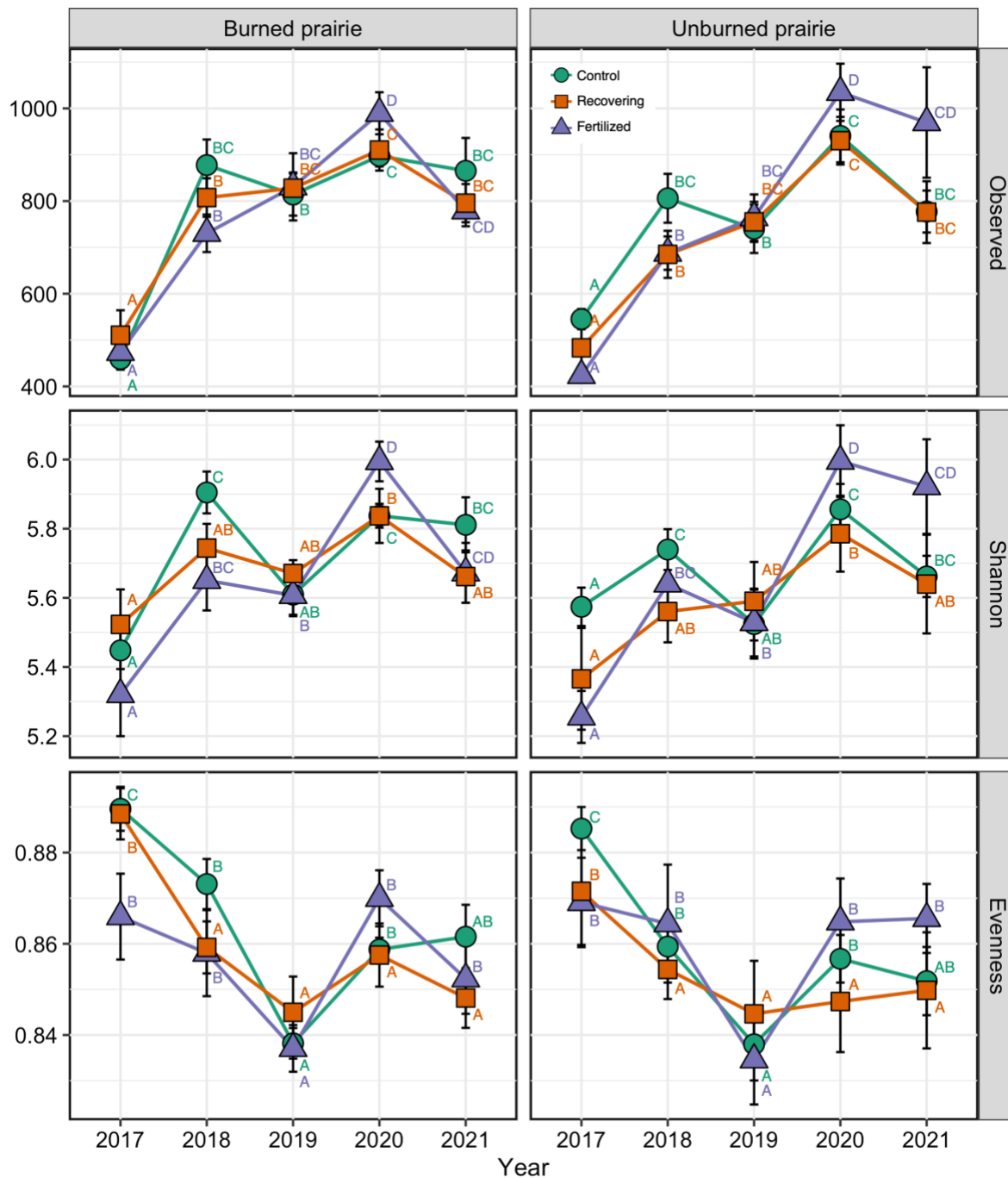


Figure 5-2. Observed ASV richness, Shannon diversity, and evenness of soil microbial communities during the growing season from 2017-2021. Each point is the average alpha diversity metric for the fertilization treatment, split by the burned and unburned prairies, and the error bars are ± 1 standard error (SE). Letters near points denote significant differences between years within the fertilization treatment at $P \leq 0.05$.

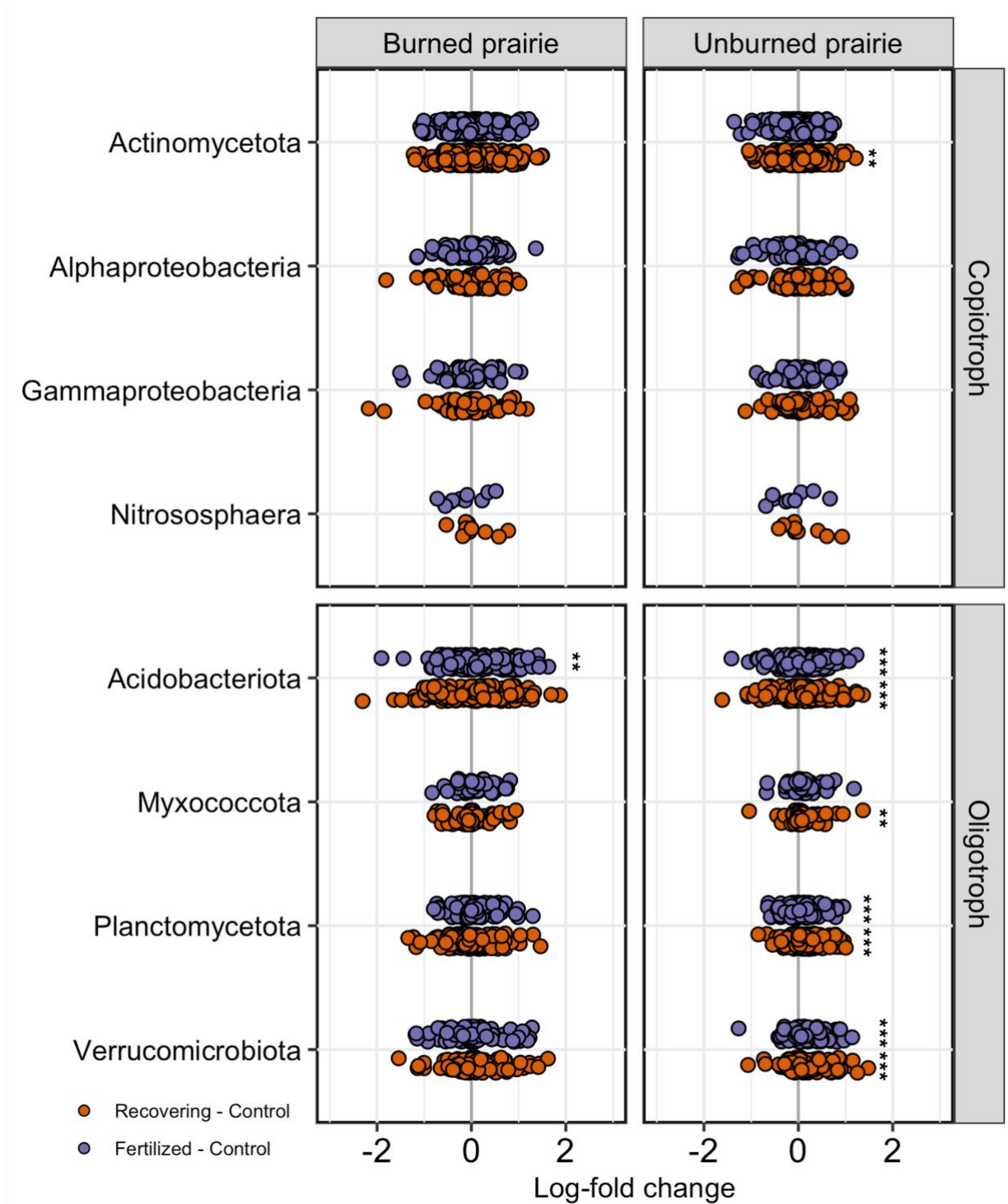


Figure 5-3. Average ASV log-fold changes from previous and continuous N-fertilization, relative to ASV abundance in the control treatment, binned by phyla and classes defined as putative copiotrophs (top facet) and oligotrophs (bottom facet). Asterisks near points indicate phylum or class is statistically higher than zero at $**P < 0.01$, and $***P < 0.001$ thresholds.

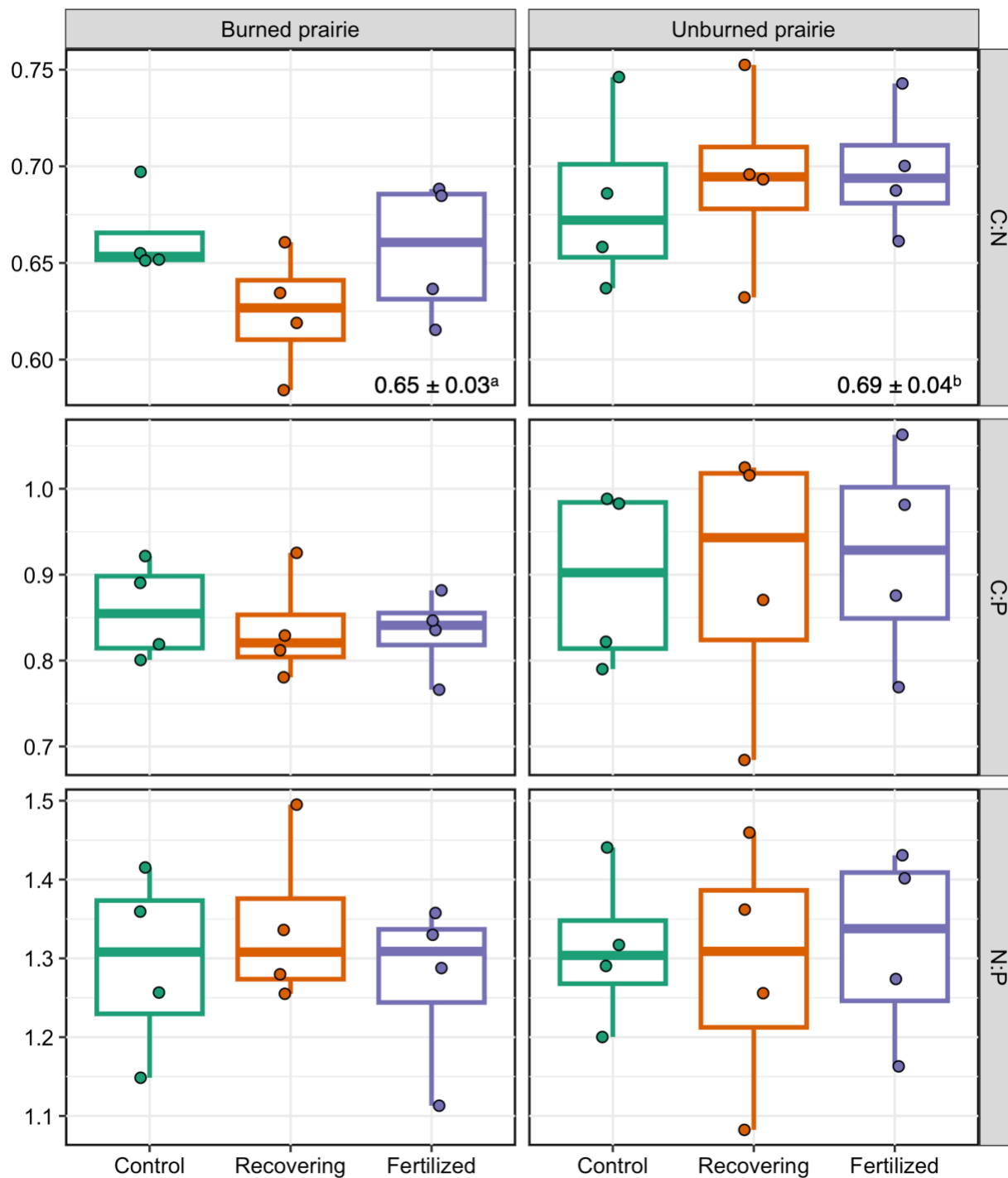


Figure 5-4. Microbial enzymatic investment for C relative to N (C:N), C relative to P (C:P), and N relative to P (N:P) summarized by boxplots. Each point is the enzymatic ratio for each plot corresponding to the fertilization treatment. Letters next to the mean $\pm$ standard deviation in C:N plots denote significant difference between fire treatments at $P \leq 0.05$.

Chapter 6 - Conclusions

The industrialization of modern dominant human societies has altered the global nitrogen (N) cycle by increasing biologically available N in terrestrial ecosystems (Vitousek et al. 1997, Galloway et al. 2008, Schlesinger 2009). Although increased N availability has allowed plants in some ecosystems to fix more carbon (C) (Gough et al. 2008, LeBauer and Treseder 2008), this has lowered plant diversity (Stevens et al. 2004, Suding et al. 2005) and caused negative consequences to soils, such as acidification and loss of essential plant nutrients like calcium (Aber et al. 1998, Lovett and Goodale 2011). However, while the effects of increased N availability have been well documented across ecosystems, little research exists on the effects of historically elevated N conditions after exogenous N input decreases or ceases altogether (Stevens 2016, Gilliam et al. 2019), or on the mechanisms that underlie ecosystem recovery. Since ecosystems have strong potential to be sinks and thus retain large stocks of anthropogenically-derived N in their plant and soil pools, exogenous N from the past may become a source for future ecosystem communities and modify ecosystem processes later (Van Meter et al. 2016, Nieland et al. 2021). On the other hand, changes to plant and microbial community structure by previous high N input could alter ecosystem functioning to future conditions. Therefore, terrestrial ecosystems may recover slowly after cessation of exogenous N supply, and the speed and attributes of ecosystem recovery will depend on the rate and mechanism of ecosystem N loss, or until community structure recovers.

The recovery of tallgrass prairies from N eutrophication may depend on land management, such as fire. Fire removes ecosystem N by combusting organic N stored in litter (Ojima et al. 1994, Blair 1997). In contrast, fire suppression promotes accumulation of soil N by allowing litter to decompose and return N belowground. Hence, tallgrass prairie sensitivity from

chronic N-fertilization should reflect fire history: Repeated removal of fertilizer-N by fire curbs N accrual and ecosystem responses to N-addition should be constrained, while fire suppression permits more fertilizer-N to be retained, and on top of the litter-N, should increase sensitivity of ecosystem functions (except when other resources become limiting such as light; Blair 1997). In addition, recovery may mirror sensitivity due to similar plant and soil microbial feedbacks. This dissertation aimed to increase our understanding of initial ecosystem responses to the cessation of chronic N-fertilization and the mechanisms that underpin these responses to improve predictability of ecosystem recovery.

Through this work, I found that chronic N-fertilization, both N-fertilization and fire suppression independently decreased N-acquiring enzyme activities and microbial enzymatic investment for C relative to N, suggesting lower soil microbial N-limitation (Chapter 2). This finding is consistent with a product-inhibition feedback (Olander and Vitousek 2000, Schimel and Weintraub 2003, Allison and Vitousek 2005), in which the extracellular concentration of an assimilable nutrient up- or down-regulates the synthesis of enzymes responsible for releasing that nutrient from soil organic matter (SOM). I also found that dual fertilization (N and P) most strongly increased the relative abundance of putative copiotrophs, but a concomitant decrease in all soil extracellular enzyme activities was not found; therefore, there was no support for the copiotrophic hypothesis (Fierer et al. 2007, Ramirez et al. 2012), in which a tradeoff exists between microbial population growth and resource acquisition. In addition, I found no support for a soil pH-related feedback (Treseder 2008, Riggs and Hobbie 2016), where a decrease in pH decreases enzyme activities. However, I found that hydrolytic enzyme activities declined in unburned prairies soils fertilized with N only, suggesting that a threshold in N availability to alter belowground functions may exist. Collectively, this chapter shows that changes to soil enzymatic

activities are independent of changes to soil microbial community composition, and that fire suppression does increase N availability to soil microbes, but possibly due to different mechanisms.

In the first year of fertilization cessation, I found that inorganic N availability dropped immediately, nitrification potential recovered more than denitrification potential, and nitrifier and denitrifier populations had different sensitivities and recoveries (Chapter 3). I also found that soil microbial community composition did not recover, as well as aboveground net primary productivity (ANPP) where it remained elevated. Moreover, soil N pools (inorganic and total) and fluxes were always higher in unburned prairies. Altogether, this chapter suggests that the differential recovery of biotic populations and processes may potentially shape future outcomes of soil N loss and recovery of the tallgrass prairie from chronic N-fertilization.

By continuing to measure N pools, N fluxes, and ANPP over the next four growing seasons, I found that N availability did not recover any further, that nitrification potential recovered faster in burned prairies than denitrification potentials, regardless of the fire treatment, and that ANPP never recovered in burned prairies (Chapter 4). I also found that the potential combustible N pool in burned prairies were only slightly higher in the previously fertilized plots. Additionally, I found that soil N stock in previously fertilized prairies were intermediate between soil N stocks in control and continuously fertilized prairies in both fire treatments; however, shoot and root C:N stocks in burned prairies recovered, but soil C:N ratios did not recover in either fire treatment. Collectively, this chapter finds that plants in burned prairies are highly effective in assimilating or retranslocating N to maintain ANPP and limit N loss likely due to the turnover of dominance by *Andropogon gerardii* to *Panicum virgatum* (Hayes 1985, Knapp and Seastedt 1986, Tilman and Wedin 1991), and substrate limitation is a similar mechanism for the

recovery of nitrification and denitrification. Further, nitrate leaching from topsoil may have contributed to significant soil N loss from the unburned prairie.

Finally, I found that soil microbial community structure (composition, relative abundance, and diversity) did not recover from chronic N-fertilization, and that the copiotrophic-oligotrophic life history framework was a poor mechanism to explain microbial community shifts, at least at coarse taxonomic resolution (Chapter 5). I also found that soil microbial community structure was more sensitive to fertilization under fire suppression. Moreover, I found that microbial enzymatic investment for C relative to N slightly decreased, although insignificantly, in the burned prairies but not for the unburned prairies, suggesting this community is starting to become N-limited. Together, this chapter demonstrates that soil microbial communities exist in alternative states due to the chronic N-fertilization, in which communities in unburned prairies may have passed an environmental threshold that restricts microbial community recovery.

Altogether, this dissertation points to important biotic mechanisms that underpin soil N legacies in mesic grasslands managed differently by fire. First, soil microbial community responses to changes in N availability occur at the cellular level, meaning shifts to aggregate microbial community function are not necessarily connected to shifts in overall microbial community structure. Second, N-fertilization supplies tallgrass prairie with a substantial amount of available N, but changes to soil chemistry (e.g., C:N ratio) or microbial community activities, potentially through faster growth and turnover, can support higher net N mineralization even after input ceases. This mechanism will constrain N-cycling recovery. Third, primary productivity may not recover immediately after ceasing N-fertilization. Plants can effectively assimilate or retranslocate N to sustain this process. Finally, fire suppression, though it may

similarly increase N availability, imposes mechanisms that alter the structure and function of tallgrass prairies. Namely, the input of particulate organic matter will continue to support higher N availability relative to burned prairies, but this additional source of N and C may not contribute to greater soil stocks. Instead, in our study, previous fertilization may have saturated this ecosystem, making N more likely to leach from the unburned prairie as a pollutant.

How long will the recovery from long-term N-fertilization be for these ecosystems? Based on the first-year responses to the cessation of N-fertilization, Nieland et al. (2021) predicted that the removal of exogenous N from the ecosystem could take several decades for burned prairies if only considering the fire loss pathway, but only about half a decade for unburned prairies because of high rates of N-cycling potentials. However, physiochemical differences between SOM pools can allow anthropogenic-N to persist in terrestrial ecosystems well after ceasing N-fertilization. As highlighted in Chapter 4, soils in burned and unburned prairies lost a similar amount of N during the five year recovery periods, suggesting that the N lost from the ecosystem came from a similar source – an unprotected, labile organic N pool. Thus, early N losses may be high, but ephemeral, as this pool is depleted. After this initial stage of recovery, N losses will likely be slower as the next possible N source, the aggregate-protected SOM pool, will depend on the turnover of soil aggregates to make this N accessible for plants and soil microbes. Finally, N stored in microbial necromass that is sorbed to soil minerals (i.e., mineral-associated organic matter (MAOM)) will take the longest to recover, as this N pool has very long residence times. This final stage of N may take centuries for the exogenous N to leave from the MAOM pool and the ecosystem. Ultimately, N loss from SOM may well be non-linear, with each recovery stage reflecting the pool that is the predominant source for the lost N, and with subsequent stages increasing in duration. However, the magnitude and time for each stage

will be ecosystem-context as feedbacks and land management will vary between sites. Therefore, it is difficult to predict how long it will take for this ecosystem to recover from long-term N-fertilization, and it remains possible that the system will not return to a pre-fertilized state.

Importantly, the removal of exogenous N only reflects one aspect of ecosystem recovery: The return of plant and soil microbial communities to those that resemble unfertilized conditions, which may not parallel with SOM recovery. As shown in Chapter 4, the increase in plant C:N ratio in burned prairies reveals that the shift in dominance from *A. gerardii* to *P. virgatum* is likely underpinning the positively ANPP legacy in burned recovering prairies, which is not directly related to the partially recovered level of N availability. If feedbacks reinforce the composition of plant communities in these recovering prairies, then ANPP may not recover and not align with decreases in ecosystem N. In parallel, soil microbial communities, as shown in Chapter 5, showed no recovery, despite reduced N availability and soil N stocks. Though it is possible that the unprotected organic N pool, or the higher proportion of N in the SOM, could be supporting alternative microbial states, it is also possible that soil microbial community dynamics have decoupled from their primary N supply and are sustaining greater net N mineralization due to faster microbial growth and death. For both the plant and soil microbial communities, their legacies may enable ecosystems to retain this memory of previous long-term N-fertilization. Collectively, both material (i.e., SOM) and information (i.e., plant and soil microbial communities) legacies are evident as this tallgrass prairie ecosystem undergoes recovery from chronic N addition (Johnstone et al. 2016), with differences in feedbacks between the burned and unburned prairies driving the legacy of soil N.

6.1 References

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Appendix A - Supplementary tables and figures for Chapter 2

Appendix A Table A-1.

<i>F, P</i>	Burn (B)	Nadd (N)	Padd (P)	Month (M)	Burn*N	Burn*P	N*P	B*N*P	Burn*M	N*M	P*M	B*N*M	B*P*M	N*P*M	B*N*P*M
%C	1.4, 0.25	1.4, 0.24	0.0, 0.83	.	3.8, 0.06	1.2, 0.29	0.1, 0.78	1.1, 0.30	.	.	.	.	.	.	.
%N	2.9, 0.11	4.2, 0.05	0.0, 0.88	.	3.7, 0.07	1.1, 0.31	0.0, 0.84	0.5, 0.51	.	.	.	.	.	.	.
Soil C:N	6.2, 0.02	23.7, <0.001	4.23, 0.05	.	0.4, 0.51	0.4, 0.52	0.4, 0.55	5.2, 0.03	.	.	.	.	.	.	.
pH	0.3, 0.57	0.8, 0.40	0.1, 0.71	.	1.6, 0.22	0.1, 0.78	0.1, 0.71	0.8, 0.39	.	.	.	.	.	.	.
GWC	39.2, <0.001	0.3, 0.62	1.5, 0.23	59.0, <0.001	0.1, 0.74	0.5, 0.47	0.9, 0.35	0.7, 0.43	8.1, <0.001	1.5, 0.14	2.9, 0.003	0.8, 0.59	0.9, 0.50	1.4, 0.18	1.1, 0.39
BG	10.0, 0.02	0.30, 0.59	0.82, 0.38	12.2, <0.001	3.3, 0.09	0.001, 0.97	1.1, 0.30	2.5, 0.13	1.4, 0.18	1.0, 0.48	2.6, 0.008	0.4, 0.93	0.8, 0.65	0.5, 0.89	0.7, 0.76
[†] CBH	30.5, 0.001	1.2, 0.28	3.7, 0.07	16.0, <0.001	1.5, 0.24	0.3, 0.60	1.5, 0.24	0.16, 0.69	1.8, 0.08	1.4, 0.18	7.9, <0.001	1.0, 0.46	1.4, 0.17	0.7, 0.74	1.0, 0.46
POX	0.2, 0.68	0.9, 0.34	1.7, 0.19	12.2, <0.001	2.6, 0.11	1.2, 0.27	0.01, 0.93	0.1, 0.82	0.9, 0.52	1.3, 0.26	2.1, 0.03	0.5, 0.85	1.3, 0.24	0.5, 0.89	0.7, 0.68
[†] NAG	1.4, 0.28	7.8, 0.01	0.53, 0.48	4.0, <0.001	2.0, 0.17	0.0, 0.98	1.8, 0.20	3.9, 0.07	1.1, 0.34	0.71, 0.68	4.9, <0.001	0.5, 0.87	0.4, 0.90	0.7, 0.65	0.5, 0.83
LAP	7.4, 0.03	9.3, 0.007	0.0, 0.93	19.5, <0.001	1.2, 0.29	0.3, 0.59	0.6, 0.45	3.0, 0.10	1.0, 0.41	0.6, 0.81	2.3, 0.02	0.3, 0.96	1.5, 0.16	1.1, 0.37	0.4, 0.92
[†] PHOS	0.1, 0.76	1.4, 0.25	0.6, 0.45	13.5, <0.001	0.3, 0.62	0.0, 0.90	0.1, 0.74	0.1, 0.71	1.2, 0.32	0.6, 0.79	3.2, 0.001	2.2, 0.02	0.8, 0.63	0.4, 0.92	0.6, 0.81
C:N	10.6, 0.02	5.1, 0.04	0.3, 0.62	8.4, <0.001	0.0, 0.85	0.2, 0.65	0.0, 0.85	0.2, 0.65	1.3, 0.22	0.6, 0.79	2.8, 0.006	0.3, 0.95	0.2, 0.98	0.6, 0.74	0.1, 1.00
[†] C:P	4.4, 0.08	5.1, 0.02	2.2, 0.14	2.9, 0.003	1.1, 0.29	0.8, 0.39	2.5, 0.12	2.6, 0.11	1.9, 0.05	0.9, 0.56	5.0, <0.001	0.5, 0.84	0.6, 0.81	0.5, 0.89	1.4, 0.20
N:P	12.4, <0.001	21.4, <0.001	0.3, 0.62	5.6, <0.001	0.9, 0.35	0.8, 0.37	0.4, 0.51	2.1, 0.15	0.4, 0.93	0.8, 0.64	4.2, <0.001	0.6, 0.78	0.3, 0.98	0.9, 0.49	0.2, 0.99

[†]1.5× IQR rule applied to remove outliers.

Appendix A Table A-2. Results of T-tests on log response ratios of extracellular enzyme activities and major bacterial Phyla. LRR and response statistics are *italicized* if $P < 0.05$.

	Burned +N		Burned +P		Burned +N+P	
	<i>LRR</i>	<i>t, P</i>	<i>LRR</i>	<i>t, P</i>	<i>LRR</i>	<i>t, P</i>
BG	0.07	0.5, 0.63	0.06	0.5, 0.67	0.09	0.8, 0.49
CBH	0.05	0.3, 0.82	0.07	0.4, 0.74	0.14	0.8, 0.48
POX	-0.07	-0.3, 0.76	0.35	1.3, 0.22	0.25	0.9, 0.40
NAG	-0.05	-0.5, 0.67	-0.09	-0.8, 0.48	-0.12	-0.9, 0.43
LAP	-0.06	-0.8, 0.51	0.01	0.2, 0.84	-0.11	1.6, 0.20
PHOS	0.13	0.6, 0.59	-0.02	-0.1, 0.91	0.08	0.4, 0.69
	Unburned +N		Unburned +P		Unburned +N+P	
	<i>LRR</i>	<i>t, P</i>	<i>LRR</i>	<i>t, P</i>	<i>LRR</i>	<i>t, P</i>
BG	-0.22	-4.2, 0.02	-0.08	-1.0, 0.30	-0.06	-0.6, 0.60
CBH	-0.30[†]	-4.5, <0.001	-0.03	0.3, 0.79	-0.07 [†]	-0.6, 0.57
POX	0.34	1.3, 0.22	0.01	0.0, 0.99	0.35	0.8, 0.49
NAG	-0.31	-4.3, 0.02	-0.17	-2.2, 0.04	-0.23	-1.8, 0.18
LAP	-0.29	-2.1, 0.13	-0.08	-0.7, 0.55	-0.15	-3.0, 0.42
PHOS	-0.0 [†]	-0.2, 0.86	-0.12 [†]	-1.4, 0.16	-0.02 [†]	-0.1, 0.91
	Burned +N		Burned +P		Burned +N+P	
	<i>LRR</i>	<i>t, P</i>	<i>LRR</i>		<i>LRR</i>	<i>t, P</i>
Actino	-0.05	-0.63, 0.58	0.14	Actino	-0.05	-0.63, 0.58
Alpha	0.03	0.67, 0.55	-0.10	Alpha	0.03	0.67, 0.55
[†] Gamma	-0.01	-0.09, 0.93	0.04	[†] Gamma	-0.01	-0.09, 0.93
[†] Thaum	0.52	0.90, 0.44	0.25	[†] Thaum	0.52	0.90, 0.44
Acido	-0.05	-1.27, 0.29	0.07	Acido	-0.05	-1.27, 0.29
[†] Delta	-0.20	-3.7, 0.03	-0.05	[†] Delta	-0.20	-3.7, 0.03
[†] Plancto	-0.20	-2.4, 0.10	0.02	[†] Plancto	-0.20	-2.4, 0.10
Verruco	0.11	1.4, 0.27	-0.14	Verruco	0.11	1.4, 0.27
	Unburned +N		Unburned +P		Unburned +N+P	
	<i>LRR</i>	<i>t, P</i>	<i>LRR</i>		<i>LRR</i>	<i>t, P</i>
[†] Actino	0.11	1.7, 0.18	0.11	[†] Actino	0.11	1.7, 0.18
[†] Alpha	0.07	1.6, 0.20	-0.14	[†] Alpha	0.07	1.6, 0.20
Gamma	0.38	1.4, 0.26	0.45	Gamma	0.38	1.4, 0.26
[†] Thaum	0.58	2.7, 0.08	0.16	[†] Thaum	0.58	2.7, 0.08
[†] Acido	-0.07	-2.2, 0.04	0.02	[†] Acido	-0.07	-2.2, 0.04
[†] Delta	-0.41	-3.7, 0.04	-0.14	[†] Delta	-0.41	-3.7, 0.04
Plancto	-0.13	-1.3, 0.30	0.11	Plancto	-0.13	-1.3, 0.30
Verruco	-0.08	-1.1, 0.36	-0.22	Verruco	-0.08	-1.1, 0.36

[†] denotes if model excluded data that fell outside the 1.5× interquartile range from the 25 and 75% quartiles.

+N, nitrogen-alone; +P, phosphorus-alone; +N+P, nitrogen- and phosphorus-fertilized

Appendix A Table A-3. Correlation tests between soil EEAs and edaphic properties.

R^2, P	%C	%N	Soil pH	GWC
BG	0.106, 0.09	0.178, 0.03	0.001, 0.84	0.032, 0.001
CBH	0.077, 0.14	0.106, 0.09	0.094, 0.10	0.006, 0.19
[†] PHX	0.003, 0.78	0.076, 0.60	0.344, 0.30	0.001, 0.84
NAG	0.022, 0.45	0.024, 0.42	0.062, 0.19	0.026, 0.006
LAP	0.086, 0.12	0.099, 0.10	0.002, 0.80	0.006, 0.20
PHOS	0.040, 0.30	0.028, 0.38	0.131, 0.05	0.090, <0.001

[†]Removed all data points that fell below detection limit

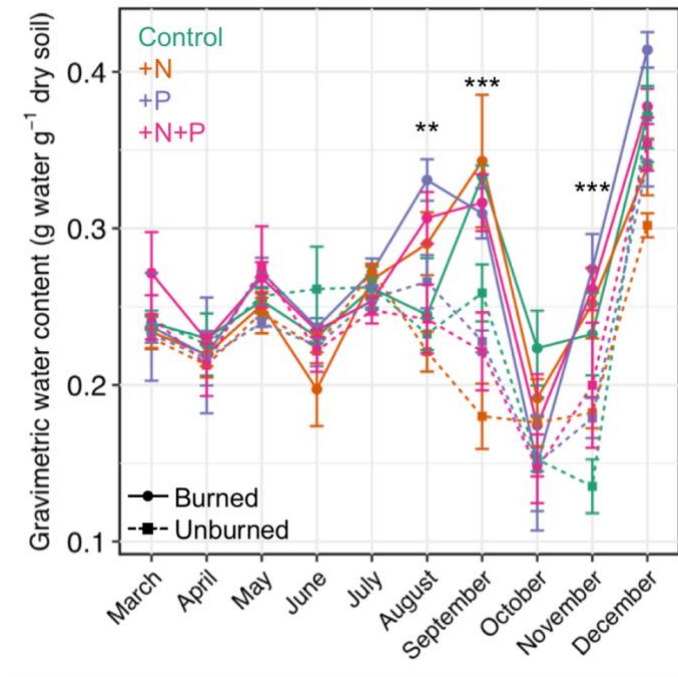
Appendix A Table A-4. Pairwise PERMANOVA results for microbial community composition between different field treatments.

Fire	Fertilization	Pseudo- <i>F</i> , <i>R</i> ²						
		Burned prairie			Unburned prairie			
		+N	+P	+N+P	Control	+N	+P	+N+P
Burned	Control	5.34,	4.58,	5.26,	1.63,	2.36,	8.64,	6.00,
		0.064***	0.057***	0.063***	0.020	0.029*	0.101***	0.074***
	+N		10.80,	5.73,	5.18,	6.41,	14.85,	5.79,
			0.127***	0.070***	0.062***	0.077***	0.165***	0.074***
	+P			3.92,	6.26,	8.34,	8.64,	9.70,
Unburned	Control			0.050***	0.076***	0.100***	0.106***	0.120***
					5.52,	6.63,	8.53,	4.57,
	+N				0.066***	0.079***	0.102***	0.059***
						2.84,	7.63,	5.94,
	+P					0.035**	0.090***	0.073***
							9.43,	4.92,
							0.110***	0.062***
								9.07,
								0.112***

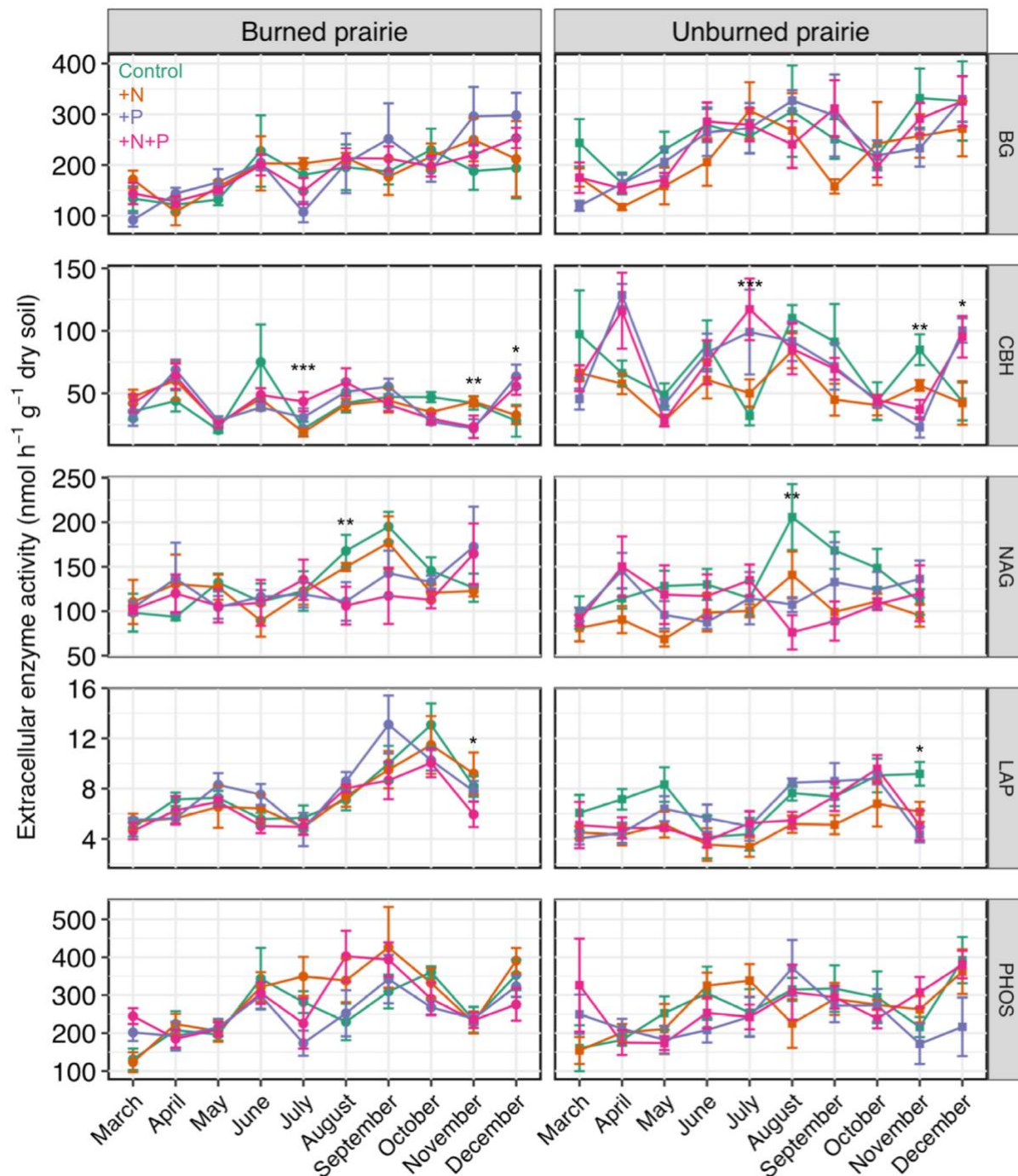
+N, nitrogen-alone; +P, phosphorus-alone; +N+P, nitrogen- and phosphorus-fertilized

P* ≤ 0.05, *P* < 0.01, and ****P* < 0.001

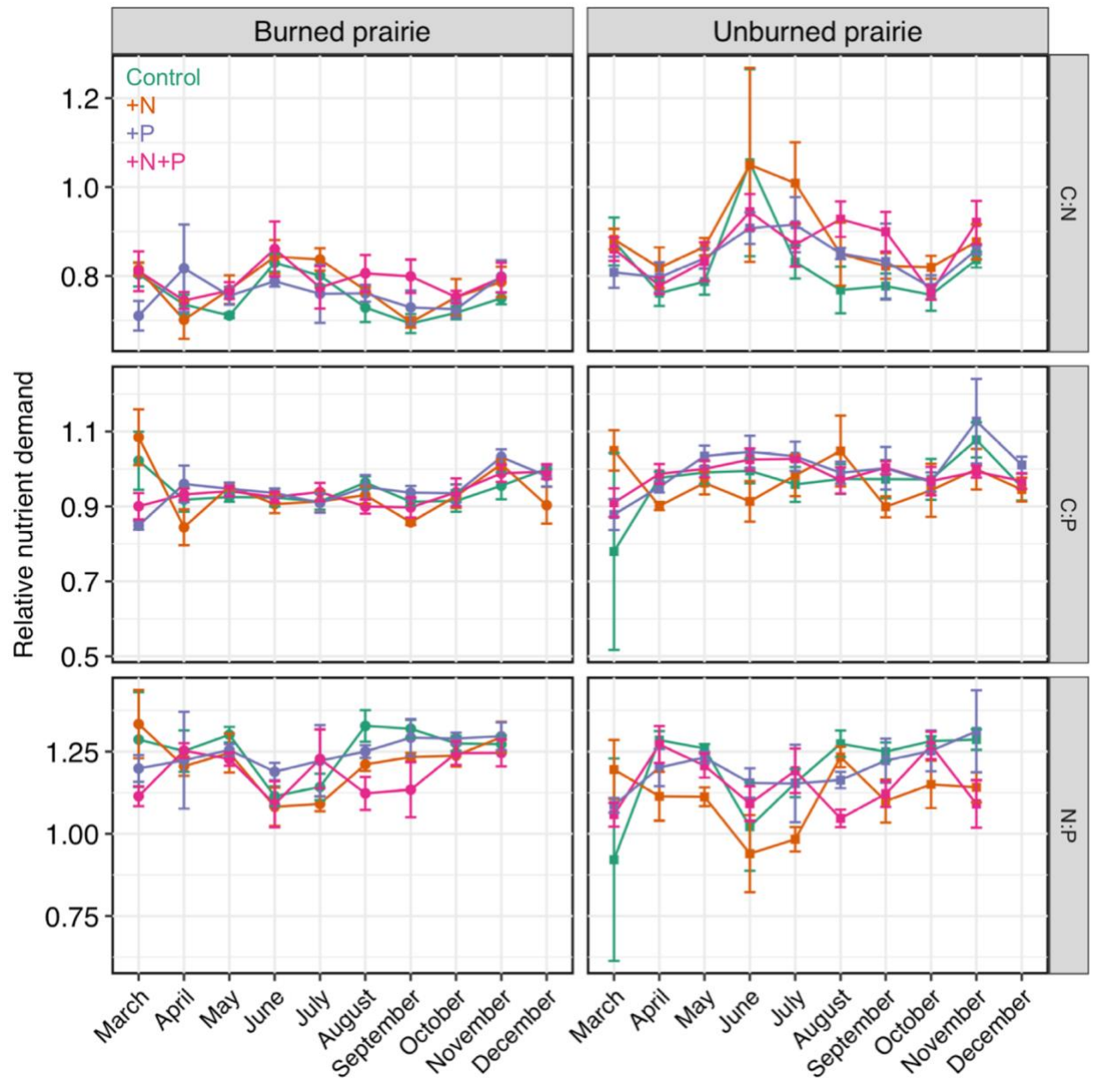
Appendix A Figure A-1. Gravimetric water content (GWC) during the study time course, split between burned and unburned prairies and colored by the fertilization treatment (+N, N-alone; +P, P-alone; +N+P, N- and P-fertilized). Each point is the mean GWC for the fertilization treatment during the sample month, and the error bars are ± 1 standard error. Significant post-hoc differences in GWC between burned and unburned prairie soils at a particular month are defined as $*P \leq 0.05$, $**P < 0.01$, and $***P < 0.001$.



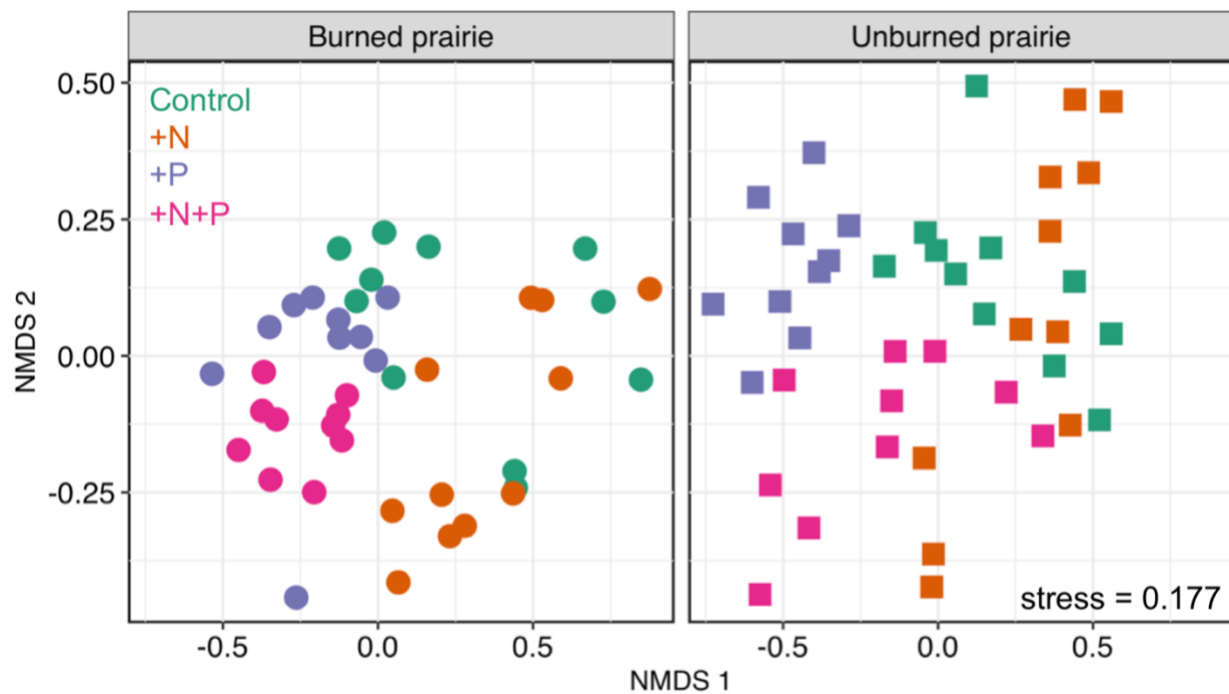
Appendix A Figure A-2. Soil extracellular enzyme activities during the study time course, split between burned and unburned prairies and colored by the fertilization treatment (+N, N-alone; +P, P-alone; +N+P, N- and P-fertilized). Each point is the mean potential activity for the fertilization treatment during the sample month, and the error bars are ± 1 standard error. Due to missing data, soil NAG and LAP activities for December are excluded. Significant post-hoc differences in enzyme activities between no-P and P-fertilized soils at a particular month are defined as $*P \leq 0.05$, $**P < 0.01$, and $***P < 0.001$.



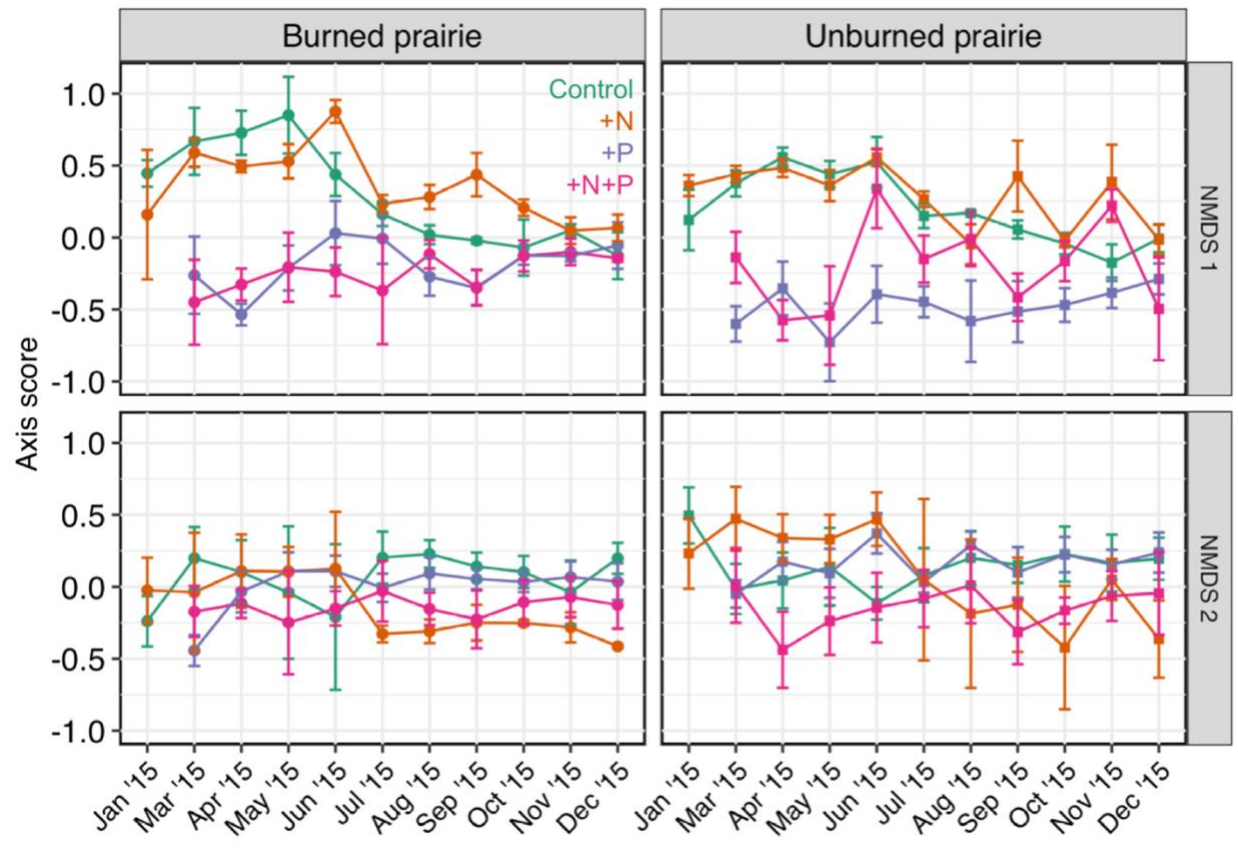
Appendix A Figure A-3. Relative soil microbial demand between C:N, C:P, and N:P during the study time course shown with time on the x-axis. Each point is the mean relative nutrient demand for the fertilization treatment (+N, N-alone; +P, P-alone; +N+P, N- and P-fertilized) during the sample month, and error bars are ± 1 standard error.



Appendix A Figure A-4. Non-metric multidimensional scaling (NMDS) ordination of the Bray-Curtis dissimilarities of soil microbial community composition, separated by burned and unburned prairies. Points are centroids for each field combination at each sample month. The NMDS was performed with all data together; thus, the burned and unburned prairie plots are scaled similarly and separated for clarity. +N, N-alone; +P, P-alone; +N+P, N- and P-fertilized.



Appendix A Figure A-5. Non-metric multidimensional scaling (NMDS) ordination of the Bray-Curtis plotted monthly. Points are mean ordinates for each field combination at each sample month, and error bars are ± 1 standard error. +N, N-alone; +P, P-alone; +N+P, N- and P-fertilized.



Appendix B - Detailed methods for Chapter 3

Soil chemistry

Resin bags were used to estimate the amount of inorganic nitrogen (N) available for plants and microbes during the growing season. The bags were prepared by adding 5 g each of the cation and anion beads (Dowex® 50WX8 hydrogen form, 50-100 mesh and Dowex® 1X8 chloride form, 50-100 mesh) in nylon bags (Baer and Blair 2008). Four resin bags were randomly carefully installed ~10 cm below the mineral soil surface to avoid extraneous disturbance within each plot, while one resin bag was installed for each fertilized subplot. The resin bags were installed at the beginning of June 2017 after subplots were fertilized and left in until September 2017. Adsorbed ions were removed from the resin surfaces by shaking each bag in 100 ml of 2 M KCl for 2 hr. Concentrations of NH_4^+ -N and NO_3^- -N in the extractants were quantified using a modified indophenol and VCl_3 /Griess reagent method, respectively (Hood-Nowotny et al. 2010) and measured spectrophotometrically with a FilterMax F5 Multimode Microplate Reader (Molecular Devices, San Jose, CA, USA).

Soil %C and %N from the September 2017 samples were measured at the Kansas State University Soil Testing Laboratory in Manhattan, KS, USA. 0.35 g of oven-dried soils were combusted using a LECO TruSpec CN Combustion Analyzer (LECO Corporation, St. Joseph, MI, USA), and the %C and %N of soil were reported on a weight percentage basis. From these values, the molar C:N ratio of soil were calculated. Soil pH from the September samples was measured by shaking ~15 g of field-moist soil with an equivalent amount of DI H_2O and recording the pH of the soil slurry (Robertson et al. 1999). Gravimetric water content (GWC) of

soil was estimated by drying ~5 g of soil at 105 °C overnight and calculating the loss of water mass.

Soil microbial biomass C (MBC) and N (MBN) were estimated using the chloroform fumigation-extraction method. For each soil sample, two subsamples of ~10 g soil were either fumigated with chloroform for 24 h in an air-tight container or left unfumigated (Brookes et al. 1985, Vance et al. 1987, Zeglin and Myrold 2013). Both subsamples were then shaken in 0.5 M K₂SO₄ for 1 h. Following filtration through Whatman Paper (Grade 1 – 11 µm filter mesh), the DOC was quantified via combustion using a Shimadzu TOC analyzer (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA), and the difference in DOC between the fumigated and unfumigated was assumed to be liberated C from MBC. Fumigated solution was digested with a persulfate solution to convert organic-N to NO₃⁻-N. Similar to resin bag extracts, the TIN from the fumigated and unfumigated subsamples were quantified using the modified indophenol and VCl₃/Griess reagent methods as noted above with the difference between the two subsamples assumed to come from MBN.

Microbial N-cycling assays

Nitrification potential (NP) assays were run on fresh soil collected within 48 hours. In a specimen cup with 50 ml of 1 mM phosphate buffer solution (pH 7.2), 2.5 g of soil was amended with 250 µl of 100 mM (NH₄)₂SO₄ and shaken at 150 rpm for 24 h (Taylor et al. 2010, Petersen et al. 2012) with a perforated lid to allow for aeration. Subsamples of the soil slurry were aliquoted at 15 min and 24 h, centrifuged to remove any soil, and stored at -20 °C until further processing. NO₃⁻-N concentrations were quantified using the VCl₃/Griess reagent method as mentioned above. NP was calculated as the rate at which a saturating level of NH₄⁺ was oxidized completely to NO₃⁻; thus the NO₃⁻-N concentration at 24 h were subtracted by the NO₃⁻-N

concentration at 15 min. NP rates were normalized to dry soil mass and recorded as mg N kg^{-1} dry soil h^{-1} .

Denitrification enzyme activity (DEA) and potential (DNP) assays were performed concurrently with fresh soil collected within 48 hours. For both DEA and DNP assays, 5.0 g of soil were placed in a 125 ml serum bottle amended with 10 ml of buffer solution containing $0.8 \mu\text{M}$ chloramphenicol to inhibit further enzyme synthesis (Groffman et al. 1999). The buffer solution for DEA assays additionally contained 7.1 mM KNO_3 and 2.8 mM glucose to supply denitrifiers with N and C. In contrast, no nutrients supplemented the substrate-limited DNP assays. Serum bottles were sealed, evacuated of atmospheric gas, and flushed with N_2 . 10 ml of acetylene gas was immediately added to prevent the enzymatic reduction of N_2O to N_2 , and the anaerobic bottles were shaken at 150 rpm. Headspace gas subsamples were collected at 30 min and 1.5 h for the DEA assays, and at 1 h and 4 h for the DNP assays. N_2O -N concentrations were measured with gas chromatography using a Shimadzu 2014 GC analyzer (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). DEA and DNP were calculated as the rate of N_2O accumulation; therefore, the concentration of N_2O -N from the later subsample gas extractions were subtracted by the concentration collected earlier. Both DEA and DNP rates were normalized and recorded as $\mu\text{g N}_2\text{O-N kg}^{-1}$ dry soil h^{-1} .

Molecular microbial analysis

Genomic DNA (gDNA) was extracted from each soil sample using a modified CTAB extraction approach (DeAngelis et al. 2010). 0.5 g of soil underwent two rounds of physical lysis in 0.5 ml of 10% CTAB with 0.25 M phosphate and 1 M NaCl solution and $50 \mu\text{l}$ of 0.1 M ammonium aluminum sulfate solution for 30 s (5.5 m s^{-1} on a MP Biomedicals FastPrep instrument with lysis bead matrix E). gDNA was purified with phenol:chloroform:isoamyl

alcohol (25:24:1) and chloroform, and the gDNA was precipitated overnight in 1 ml of 30% PEG 6000 and 1.6 M NaCl solution. The precipitate was washed with cold 70% (v/v) ethanol, dried, and resuspended in 50 µl of 100 µM Tris buffer pH 8. gDNA yields were estimated using Quant-iT PicoGreen assay kits (Life Technologies, Grand Island, NY, USA) and normalized by dry soil mass (Zeglin et al. 2016).

Quantitative polymerase chain reactions (qPCR) were used to estimate the abundance of selected N-cycling functional genes. These genes included the archaeal and bacterial *amoA* (Rotthauwe et al. 1997, Francis et al. 2005, Mosier and Francis 2008), the *nosZ* clade I and II (Henry et al. 2006, Sanford et al. 2012), and the bacterial 16S rRNA (Fierer et al. 2005, Zeglin et al. 2016). Standard concentrations for the N-cycling functional genes were prepared by amplifying the genes with PCR from soil clones (archaeal and bacterial *amoA*), *Bradyrhizobium japonicum* USDA 110 (*nosZ* clade I), and *A. dehalogenans* 2CP-1 (*nosZ* clade II). The amplicons were inserted into plasmids and later taken up by *Escherichia coli* competent cells using a TOPO-TA kit (Invitrogen, Carlsbad, CA, USA). *E. coli* DNA from the newly modified strains, as well as *E. coli* ATCC 25922 (16S rRNA), was extracted using a DNeasy PowerSoil Kit (Qiagen, Hilden, DE). DNA yields were quantified as noted above and used to calculate the gene copy number for standard curves using the mass of the DNA and genome sizes of the strains. Three replicates of serially diluted standards and diluted environmental samples (25 µl and 20 µl for the functional and 16S rRNA genes, respectively) were used for qPCR. All reactions were performed with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA) in a Bio-Rad CFX CONNECT system. Reaction specifics and thermal cycler settings can be found in Appendix B Table B-1 for further detail. No-template controls and melting curves were included for all qPCR plates to check for background contamination

and non-target amplification, neither of which were detected. Gene abundance was calculated from the standard curves and normalized by DNA yield.

PCR was used to amplify the bacterial and archaeal V4 region of the 16S rRNA gene for downstream Illumina MiSeq sequencing (Caporaso et al. 2012). Three replicates of ~50 ng of gDNA sample were added to separate 25 µl reactions with a final concentration of 1 × PCR buffer (Promega, Madison, WI, USA), 0.2 µM of 515F and 806R primers each, 0.2 µM of dNTPs, 1 U of GoTaq polymerase (Promega), and 0.1% (w/v) of bovine serum albumin (Zeglin et al. 2016). PCR was carried out with an established protocol using primers with unique barcodes and sequencing adapters already attached (Caporaso et al. 2012) with the exception that only 25 cycles were applied instead of 35 (Zeglin et al. 2016, Carson and Zeglin 2018). Following PCR, the replicates were consolidated, and ExoSAP-IT (Affymetrix, Santa Clara, CA, USA) was added to remove small nucleic acids. PCR products were pooled together and gel purified using a QIAquick Gel Extraction kit (Qiagen). Sequencing was performed with Illumina MiSeq Technology (Illumina, San Diego, CA, USA) using 2 × 150 paired end cycles with a 15% PhiX spike at the Kansas State University Integrated Genomics Facility.

QIIME 1 bioinformatics software was used to process the raw data from the Illumina sequencing run (Caporaso et al. 2010). Raw data were demultiplexed, joined, and denoised prior to assigning the ~250 bp length sequences to operational taxonomic units (OTUs) set at 97% similarity with an open-reference database (Reeder and Knight 2010, Edgar 2010). OTUs were aligned using the GreenGenes v.13.8 16S rRNA gene reference database (DeSantis et al. 2006, Wang et al. 2007, McDonald et al. 2012). Chimeric sequences were identified with ChimeraSlayer (Haas et al. 2011) and were removed; non-aligned OTUs and sequences with less than 3 total reads were removed from further analysis. This resulted in a dataset with a total of

3,983,461 sequences and 70,024 OTUs. Due to the uneven distribution of sequence reads in each sample, 10,000 sequences were randomly subsampled in all samples for community analysis.

Statistical analysis

All soil chemistry, microbial, and plant biomass response data and models were assessed for statistical assumptions. First, the normality of data distribution was checked with the Shapiro-Wilk test, using the function *shapiro.test*, to determine whether the null assumption of normal distribution should be rejected at a $P \leq 0.05$ cutoff. If the null hypothesis was rejected, we $\log_{10}$ transformed the data and re-tested the normality assumption; the only exception was to plant biomass which was square root transformed. We also checked normality using QQ-plots. Second, after creating the models detailed below, we assessed the assumption of homoskedasticity by plotting the models' residuals with the fitted values to check whether distribution (i.e., spread) changed. The assumptions were valid for all statistical models prior to further analysis.

We tested the statistical hypothesis that the contrasting fertilization and burning treatments, as well as their interactions, differentiated the responses of variables. Monthly contrasts were also hypothesized to occur. To test this alternative hypothesis against the null expectation that these experimental treatments did not change responses, as well as the absence of temporal differences within the growing season, we created linear mixed-effect models and tested their fixed effects (burning regime, fertilization treatments, and month of collection) with analysis of variance (ANOVA). A plot within block random effect was included in the mixed-effect models to account for spatial variation of the Belowground Plot experiment. Thus, in RStudio version 1.2.1335 (RStudio Team 2018), most models and ANOVAs were coded as

```
> lm.variable = lmer(variable ~ Burn*NewFertTrt*Month + (1|Block_Number/Plot_Number),
data=QPCR,na.action=na.exclude)

> anova(lm.variable)
```

using the lme4 package (Bates et al. 2015). Soil %C, %N, and pH were also tested with mixed-effect models, but the fixed effect ‘Month’ was excluded. In a case where the ANOVA suggested the fixed effects, or their interactions, had statistically significant differences ($P \leq 0.05$), we performed Tukey’s honest significant difference post-hoc tests with the significant fixed effects along with the random effects using the lsmeans package (Lenth 2016).

Indirect ordination and permutational ANOVA (PERMANOVA) were used to compare bacterial and archaeal 16S rRNA gene composition of the contrasting fertilization treatments crossed with different burning regimes. Bray-Curtis dissimilarity of the sampled communities were calculated with non- $\log_{10}$ transformed data using the function *vegdist* in vegan package (Oksanen et al. 2019). Dissimilarity between communities were visualized by performing ten separate iterations of multivariate non-metric multidimensional scaling (NMDS) analysis. The iteration with the best apparent fit (stress = 0.1409) was further scrutinized by examining the goodness of fit using the function *stressplot* (non-metric $R^2 = 0.98$). Bray-Curtis dissimilarity of the recorded communities was also used to test the null hypothesis that centroids, dispersing distances, or both of the composition data were not significantly different between the treatments. PERMANOVA was used to test this null by using the *adonis* function by permutating null communities 999 times. Significance between factors, as well as their interactions, was set at $P \leq 0.05$.

Appendix B Table B-1.

Genes	Reaction concentrations	Protocol	Gene copy range per reaction, Efficiency, R ²
Archaeal <i>amoA</i>	25 µl reaction: - 1 × SsoAdvanced supermix - 0.32 µM Arch amoA_F - 0.32 µM Arch amoA_R - 0.032% BSA - 2 µl DNA - H ₂ O	94.0°C for 5 min 40 cycles of: - 94.0°C for 30 sec - 56.0°C for 30 sec - 72.0°C for 30 sec - Plate read 95.0°C for 15 sec Melt curve	1.3 × 10 ⁶ – 1.3 × 10 ⁰ copies rxn ⁻¹ Efficiency: 87.5% - 95.0% R ² : 0.990-0.999
	25 µl reaction: - 1 × SsoAdvanced supermix - 0.32 µM amoA_1F - 0.32 µM amoA_2R - 0.04% BSA - 2 µl DNA - H ₂ O	95.0°C for 5 min 40 cycles of: - 94.0°C for 45 sec - 56.0°C for 30 sec - 72.0°C for 1 min - Plate read 95.0°C for 15 sec Melt curve 95.0°C for 5 min 5 touchdown cycles of: - 95.0°C for 15 sec - 65.0°C for 30 sec (-1°C after cycle) - 72.0°C for 30 sec - Plate read 35 cycles of: - 95.0°C for 15 sec - 60.0°C for 30 sec - 72.0°C for 30 sec - Plate read - Melt curve	1.16 × 10 ⁶ – 1.16 × 10 ⁻¹ copies rxn ⁻¹ Efficiency: 98.1% - 100.4% R ² : 0.993-0.998
<i>nosZ</i> clade I	25 µl reaction: - 1 × SsoAdvanced supermix - 0.24 µM NosZ2F - 0.24 µM NosZ2R - 0.032% BSA - 2 µl DNA - H ₂ O	95.0°C for 15 min 40 cycles of: - 94.0°C for 30 sec - 56.0°C for 30 sec - 72.0°C for 30 sec - Plate read 95.0°C for 15 sec Melt curve	1.7 × 10 ⁵ – 1.7 × 10 ⁰ copies rxn ⁻¹ Efficiency: 88.5% - 93.0% R ² : 0.985-1.000
<i>nosZ</i> clade II	25 µl reaction: - 1 × SsoAdvanced supermix - 1 µM AdehalonosZ 2CPC256F - 1 µM AdehalonosZ 2CPC739R - 0.04% BSA - 2 µl DNA - H ₂ O	95.0°C for 15 min 40 cycles of: - 94.0°C for 30 sec - 56.0°C for 30 sec - 72.0°C for 30 sec - Plate read 95.0°C for 15 sec Melt curve	1.68 × 10 ⁶ – 1.68 × 10 ⁰ copies rxn ⁻¹ Efficiency: 95.3% - 100.3% R ² : 0.995-1.000
Bacterial 16S rRNA	20 µl reaction: - 1 × SsoAdvanced supermix - 0.08 µM 338F - 0.08 µM 518F - 0.026% BSA - 1 µl DNA - H ₂ O	95.0°C for 15 min 40 cycles of: - 95.0°C for 15 sec - 55.0°C for 30 sec - 72.0°C for 30 sec - 80.0°C for 30 sec - Plate read Melt curve	3.68 × 10 ⁶ – 3.68 × 10 ³ copies rxn ⁻¹ Efficiency: 104.0% - 105.2% R ² : 0.999

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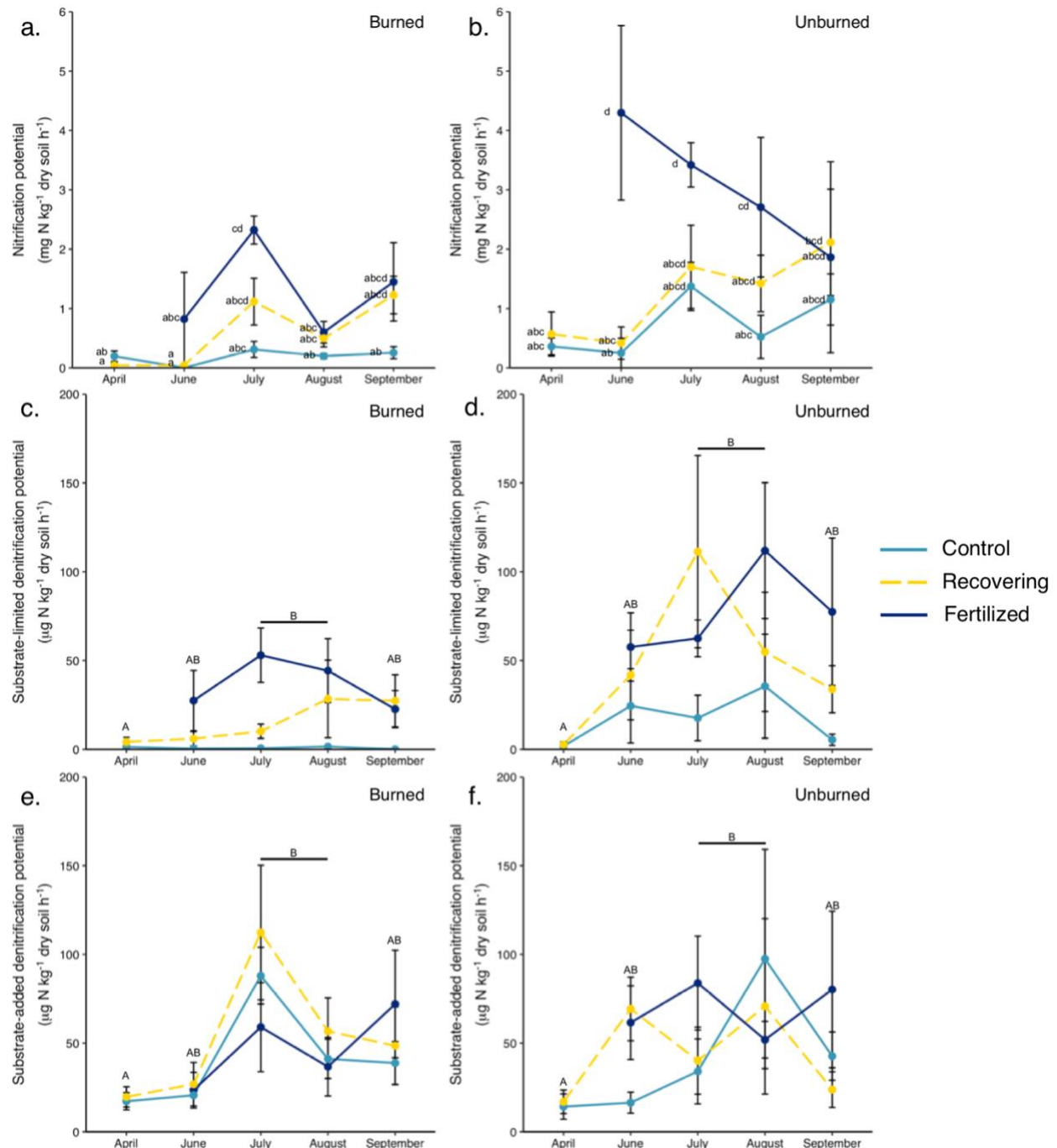
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Appendix C - Supplementary tables and figures for Chapter 3

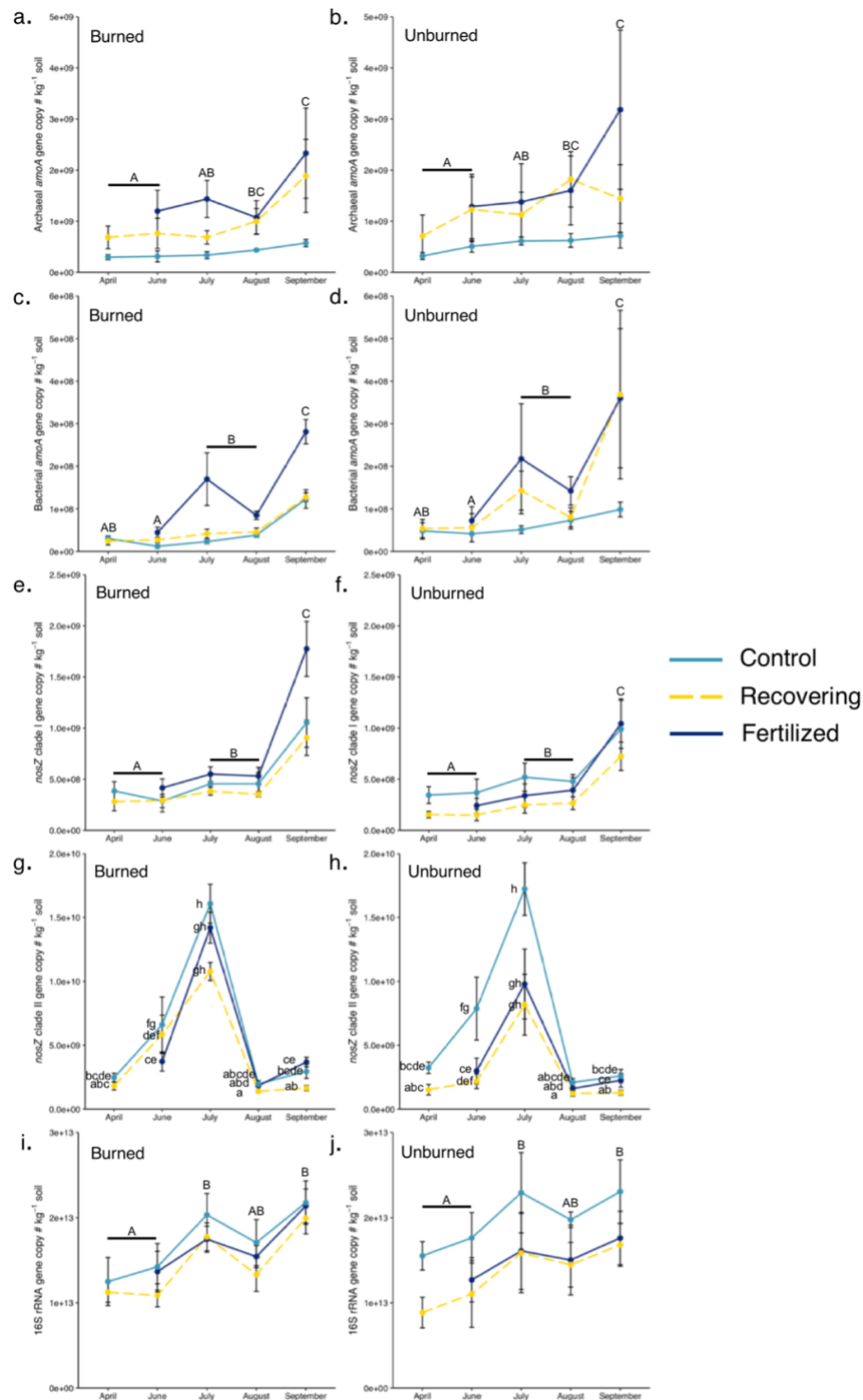
Appendix C Table C-1. Mean relative abundance of common microbial phyla and classes whose relative abundance was $\geq 0.10\%$ on average throughout the sampling season. Relative abundances are separated by burning (“Burned” and “Unburned”) and fertilization (“Control,” “Recovering,” and “Fertilized”) treatments. Names of microbial taxa with the superscript “^l” denote $\log_{10}$ transformation before statistical analyses. Superscripts near the relative abundances in a row signify Tukey’s statistical significance (set at $P \leq 0.05$) indicated by Tukey’s post-hoc tests between the fertilization treatments for the (sub)phyla. The last column lists the P values from the two-way ANOVA tests for each microbial taxa; rows with “-” indicate a significant Burn $\times$ Fertilization effect, in which the superscripts near the relative abundances indicate statistical significance between all the combined treatments.

Microbial taxa	Mean relative abundance $\pm$ SD (%)						<i>P</i> value between burning treatments
	Burned			Unburned			
	Control	Recovering	Fertilized	Control	Recovering	Fertilized	
Acidobacteria	14.52 $\pm$ 2.16 ^b	13.36 $\pm$ 1.62 ^a	12.81 $\pm$ 1.77 ^a	13.72 $\pm$ 1.85 ^b	11.52 $\pm$ 1.99 ^a	11.50 $\pm$ 1.77 ^a	0.003
Actinobacteria	23.27 $\pm$ 3.85	22.74 $\pm$ 3.09	23.46 $\pm$ 4.70	23.42 $\pm$ 3.25	26.21 $\pm$ 2.98	26.48 $\pm$ 4.84	0.016
Alphaproteobacteria ^l	11.42 $\pm$ 1.11 ^a	12.09 $\pm$ 1.25 ^{ab}	11.40 $\pm$ 1.41 ^a	11.41 $\pm$ 0.80 ^a	14.17 $\pm$ 2.10 ^c	13.44 $\pm$ 2.18 ^{bc}	-
Armatimonadetes	0.62 $\pm$ 0.17	0.67 $\pm$ 0.20	0.64 $\pm$ 0.14	0.41 $\pm$ 0.11	0.45 $\pm$ 0.14	0.41 $\pm$ 0.11	< 0.001
Bacteroidetes	3.67 $\pm$ 0.84 ^b	3.18 $\pm$ 0.53 ^a	3.34 $\pm$ 0.95 ^a	3.83 $\pm$ 0.98 ^b	3.02 $\pm$ 1.41 ^a	2.91 $\pm$ 0.94 ^a	0.502
Betaproteobacteria ^l	2.33 $\pm$ 0.50	2.93 $\pm$ 0.82	2.53 $\pm$ 0.61	2.61 $\pm$ 0.65	2.64 $\pm$ 0.96	2.50 $\pm$ 0.59	0.836
Chloroflexi	4.76 $\pm$ 0.71	4.76 $\pm$ 0.60	4.95 $\pm$ 0.94	4.58 $\pm$ 0.48	4.10 $\pm$ 0.59	4.17 $\pm$ 0.63	0.006
Crenarchaeota ^l	0.29 $\pm$ 0.14 ^a	0.67 $\pm$ 0.34 ^b	0.87 $\pm$ 0.48 ^b	0.51 $\pm$ 0.15 ^a	0.87 $\pm$ 0.53 ^b	1.12 $\pm$ 0.67 ^b	0.008
Cyanobacteria ^l	0.34 $\pm$ 0.23	0.27 $\pm$ 0.23	0.31 $\pm$ 0.17	0.15 $\pm$ 0.10	0.17 $\pm$ 0.14	0.14 $\pm$ 0.09	< 0.001
Deltaproteobacteria	6.63 $\pm$ 0.71 ^b	5.31 $\pm$ 0.82 ^a	4.99 $\pm$ 0.56 ^a	6.31 $\pm$ 0.84 ^b	4.50 $\pm$ 1.01 ^a	4.53 $\pm$ 0.89 ^a	0.005
Firmicutes ^l	0.44 $\pm$ 0.15	0.60 $\pm$ 0.31	0.61 $\pm$ 0.28	0.58 $\pm$ 0.40	0.56 $\pm$ 0.37	0.59 $\pm$ 0.23	0.984
Gammaproteobacteria ^l	1.59 $\pm$ 0.36	2.14 $\pm$ 1.15	1.74 $\pm$ 0.26	1.97 $\pm$ 0.64	2.99 $\pm$ 1.04	2.90 $\pm$ 0.75	< 0.001
Gemmatimonadetes ^l	2.01 $\pm$ 0.34 ^a	2.84 $\pm$ 0.79 ^c	2.48 $\pm$ 0.51 ^b	1.97 $\pm$ 0.51 ^a	2.89 $\pm$ 0.58 ^c	2.51 $\pm$ 0.51 ^b	0.894
Nitrospirae	2.03 $\pm$ 0.58 ^b	1.56 $\pm$ 0.74 ^a	1.52 $\pm$ 0.53 ^a	2.15 $\pm$ 0.61 ^b	1.02 $\pm$ 0.64 ^a	1.08 $\pm$ 0.55 ^a	0.014
Planctomycetes	6.65 $\pm$ 0.62	6.38 $\pm$ 0.85	6.38 $\pm$ 0.55	5.78 $\pm$ 0.65	5.26 $\pm$ 0.69	5.42 $\pm$ 0.56	< 0.001
Proteobacteria (other) ^l	0.57 $\pm$ 0.12 ^b	0.69 $\pm$ 0.12 ^a	0.69 $\pm$ 0.16 ^a	0.56 $\pm$ 0.10 ^b	0.65 $\pm$ 0.15 ^a	0.64 $\pm$ 0.13 ^a	0.177
Verrucomicrobia ^l	17.49 $\pm$ 3.69	18.14 $\pm$ 4.00	19.85 $\pm$ 5.31	18.73 $\pm$ 3.57	17.78 $\pm$ 4.70	18.48 $\pm$ 5.32	0.879
WS3	0.58 $\pm$ 0.11 ^b	0.49 $\pm$ 0.15 ^b	0.55 $\pm$ 0.15 ^b	0.55 $\pm$ 0.14 ^b	0.24 $\pm$ 0.15 ^a	0.32 $\pm$ 0.20 ^a	-

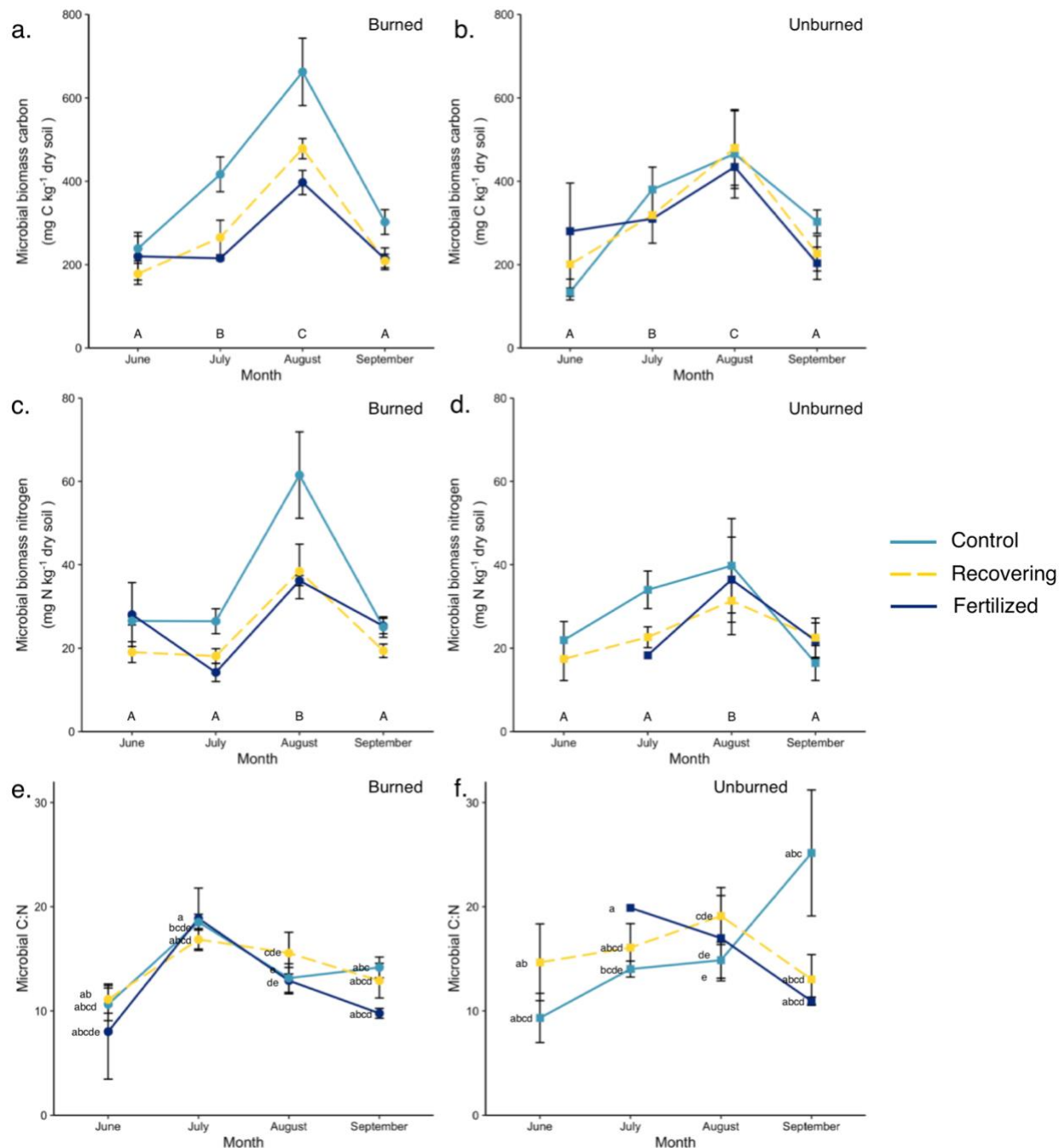
Appendix C Figure C-1. Estimated NP, DNP, and DEA rates in the burned (left column) and the unburned (right column) soils distinguished by the fertilization treatments for each month. Each point is the monthly mean of the plots ($n = 4$) for each treatment combination. Error bars are ± 1 SE. Uppercase letters above points (c – e) show statistically significant differences ($P < 0.05$) between months, while lowercase letters near points (a – b) show the statistical differences between the Burn $\times$ Fertilization $\times$ Month, with similar letters (either month or Burn $\times$ Fertilization $\times$ Month) between means indicating no statistical difference. Differences between the burned and unburned soils were included for clarity, but only the DNP rates had significant differences between burning treatment (Table 3-1).



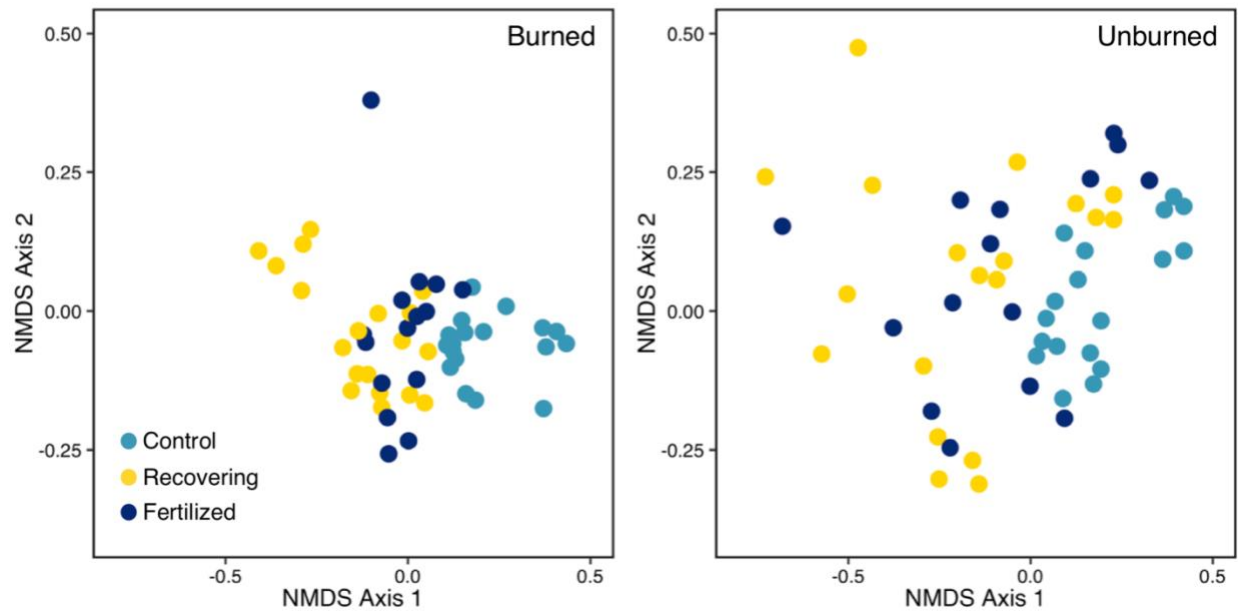
Appendix C Figure C-2. The copy number of genes in soils with contrasting fertilization and burning treatments. Plots are separated by burning treatments, indicated by the text in the upper left-hand of each graph. Monthly means for each treatment combination are shown as points with the error bars representing ± 1 SE. The uppercase letters (not present in g and h) demonstrate the differences in copy number between months, while the lowercase letters (g and h) are used for the Fertilization $\times$ Month, as suggested by Tukey's HSD the post-hoc tests.



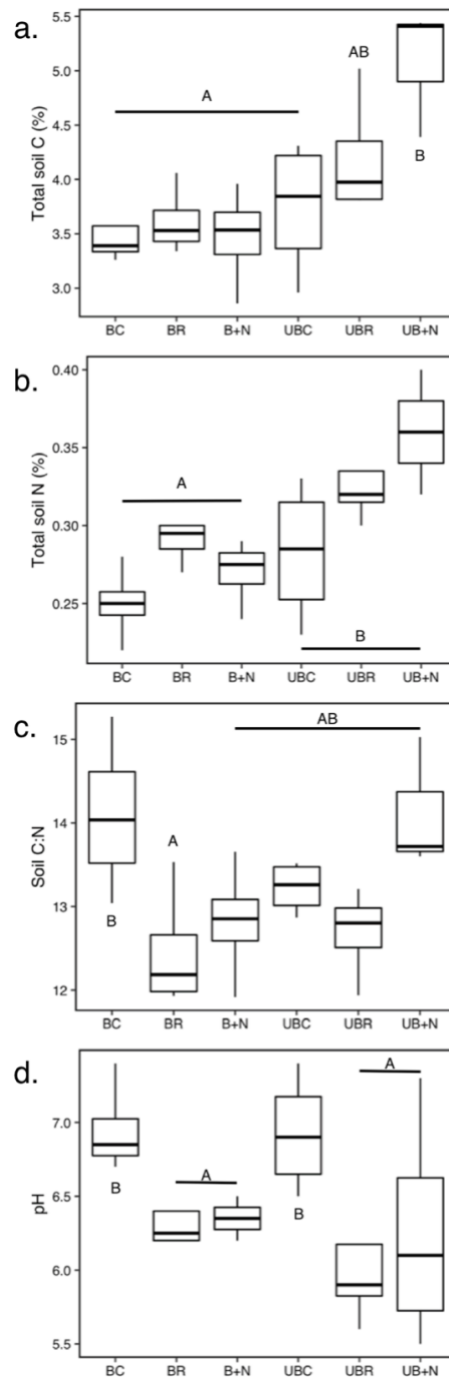
Appendix C Figure C-3. Monthly time series of the microbial biomass carbon (MBC), microbial biomass nitrogen (MBN), and the microbial C:N ratio. Left and right columns are separated by burned and unburned soils, respectively (although there was no significant difference between burning treatments). Points on each graph are the monthly mean for each treatment combination, along with error bars that are ± 1 SE. Uppercase letters (a – d) above the months signify the significant differences between months, and the lowercase letters (e and f) near the points signify the differences between the fertilization $\times$ month effects, with similar letters indicating similar responses.



Appendix C Figure C-4. Non-metric multidimensional scaling (NMDS) ordination comparing the composition of the microbial communities from soils with different burning and fertilization treatments. Both (a) burned and (b) unburned ordination plots are scaled similarly. The fertilization treatments contrast by the color of the points.



Appendix C Figure C-5. Edaphic responses to long-term management practices. The middle line in the boxplot represent the median values for each treatment combination, and the lines above and below the median are calculated as the upper and lower quartiles of the data, respectively. Vertical lines extending out of the box are either the upper and lower fences of the data or the furthest data point. For total soil C (a) and soil C:N (c), the letters were drafted from the burning $\times$ fertilization Tukey's HSD post-hoc analysis tests, while the letters for total soil N (b) and soil pH (d) come from the post-hoc analysis for burning and fertilization, respectively. Dissimilar letters signify differences among the treatments ($P < 0.05$).



Appendix D - Supplementary tables and figures for Chapter 4

Appendix D Table D-1.

<i>F, P</i>	Burn (B)	Fert (F)	Year (Y)	B×F	B×Y	F×Y	B×F×Y	*Marginal R^2 , Conditional R^2
Total Resin N	21.9, 0.003	148.9, < 0.001	10.6, < 0.001	2.6, 0.083	0.9, 0.47	2.6, 0.027	1.8, 0.115	0.830, 0.849
NO₃:NH₄ Ratio	11.8, 0.014	2.9, 0.061	41.5, < 0.001	5.2, 0.008	0.2, 0.910	1.2, 0.297	0.7, 0.643	0.674, 0.700
Nitrification Potential	11.2, 0.015	72.2, < 0.001	1.0, 0.473	0.1, 0.869	4.7, 0.001	5.8, < 0.001	3.9, < 0.001	0.456, 0.771
Denitrification Potential	3.8, 0.100	16.3, < 0.001	3.2, 0.118	2.9, 0.057	2.4, 0.048	3.9, < 0.001	0.4, 0.931	0.331, 0.438
Denitrification Enzyme Activity	0.1, 0.755	2.7, 0.070	4.1, 0.077	1.4, 0.239	0.2, 0.956	1.8, 0.072	0.5, 0.869	0.180, 0.227
Herbaceous ANPP	36.6, < 0.001	79.4, < 0.001	6.4, < 0.001	12.3, < 0.001	1.2, 0.332	2.1, 0.098	2.1, 0.099	0.790, 0.925
Woody ANPP	3.8, 0.099	4.5, 0.038	1.8, 0.138	2.0, 0.164	1.2, 0.309	0.6, 0.633	0.2, 0.951	0.311, 0.865
Total ANPP	45.2, < 0.001	19.5, < 0.001	1.3, 0.265	8.6, 0.005	1.1, 0.369	1.2, 0.317	0.2, 0.958	0.627, 0.680
Combustible Dead Plant Biomass	.	6.4, 0.045	.	.	.	.	.	0.516, .
Combustible N	.	5.1, 0.064	.	.	.	.	.	0.461, .
Dead Plant Biomass C:N Ratio	.	4.2, 0.052	.	.	.	.	.	0.040, 0.781
Dead Plant Biomass %N	.	5.3, 0.033	.	.	.	.	.	0.042, 0.811

* R^2 or adj. R^2 for linear models

$\alpha = 0.05$

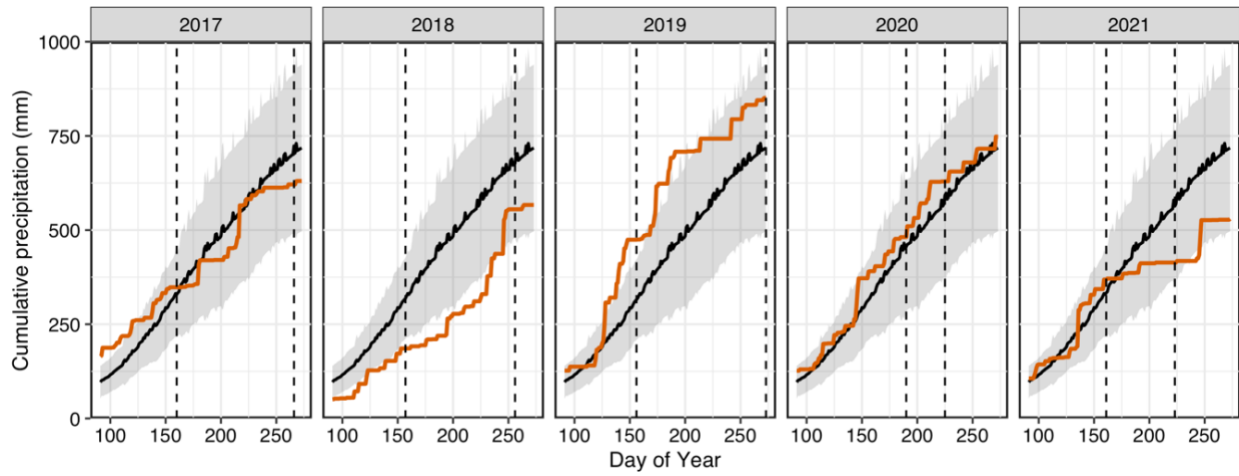
Appendix D Table D-2.

<i>F, P</i>	Burn (B)	Fert (F)	B×F	*Marginal R^2 , Conditional R^2
Shoot dry mass	10.5, 0.007	1.1, 0.316	0.1, 0.761	0.366, .
Shoot %N	21.0, < 0.001	0.0, 0.980	0.6, 0.445	0.392, 0.685
Shoot C:N ratio	50.2, < 0.001	7.2, 0.020	0.9, 0.352	0.787, .
Shoot N stock	4.2, 0.063	0.4, 0.517	0.1, 0.819	0.101, .
Litter dry mass (standing, Sept)	.	0.4, 0.519	.	0.030, .
Litter %N	.	0.5, 0.491	.	0.082, .
Litter C:N ratio	.	0.9, 0.369	.	0.136, .
Litter N stock	.	0.6, 0.449	.	0.042, .
Root dry mass	7.6, 0.013	0.0, 0.959	1.2, 0.323	0.180, .
Root %N	24.0, < 0.001	9.7, 0.001	1.5, 0.242	0.643, .
Root C:N ratio	59.2, < 0.001	19.2, < 0.001	8.7, 0.002	0.827, .
Root N stock	1.4, 0.254	1.5, 0.252	0.8, 0.482	0.037, .
Plant C stock	7.3, 0.020	<i>4.0,</i> <i>0.067</i>	3.5, <i>0.086</i>	0.440, .
Plant N stock	1.9, 0.198	3.5, <i>0.088</i>	1.3, 0.281	0.193, .
Soil %N and N stock	23.5, < 0.001	4.0, 0.037	0.8, 0.484	0.549, .
Soil %C and C stock	14.8, 0.001	1.8, 0.191	0.5, 0.643	0.384, .
Soil C:N ratio	9.8, 0.006	11.7, < 0.001	0.5, 0.642	0.560, .

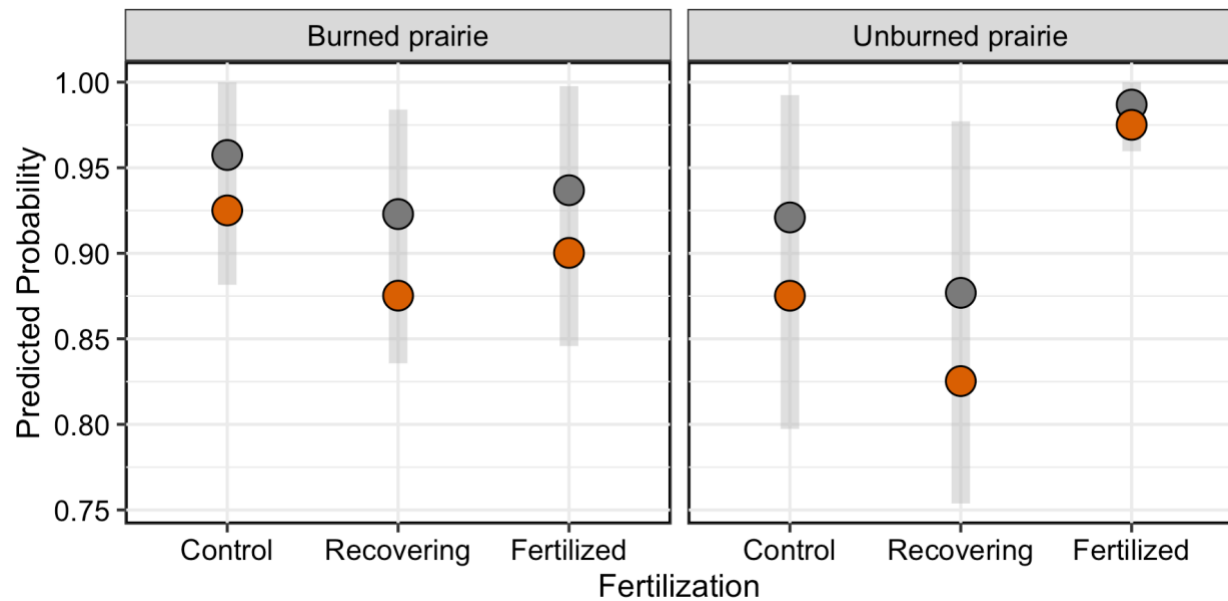
* R^2 or adj. R^2 for linear models

$\alpha = 0.05$

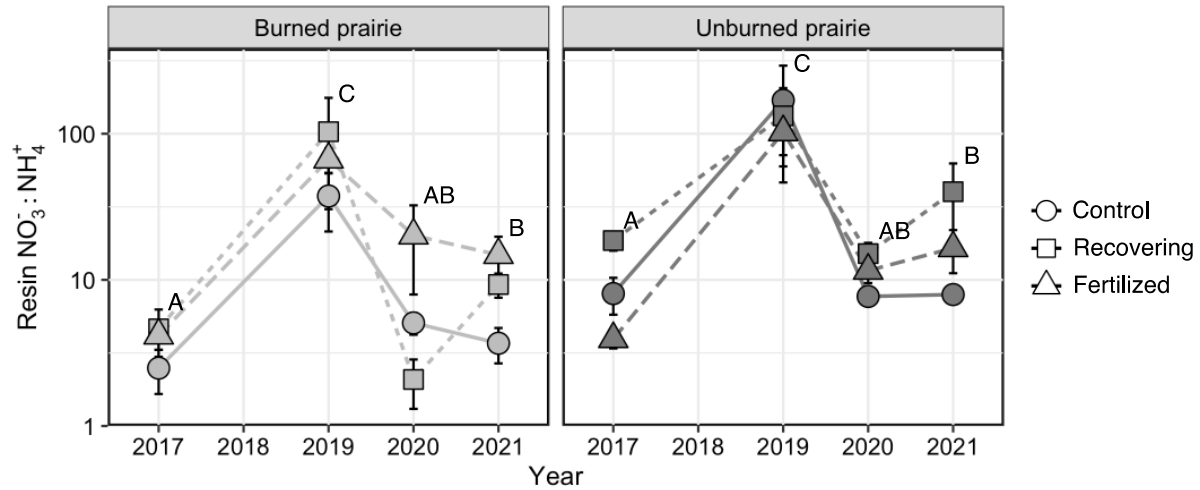
Appendix D Figure D-1. Cumulative precipitation during the growing season (April-September) collected near the BGP experiment at KBPS in 1986-2021, shown yearly during the study period (2017-2021). Black lines are the average cumulative precipitation, and the shaded area is ± 1 SD. Orange lines are the cumulative precipitation for each growing season. Vertical dashes show the dates when soil samples were collected for microbial N-cycling potentials.



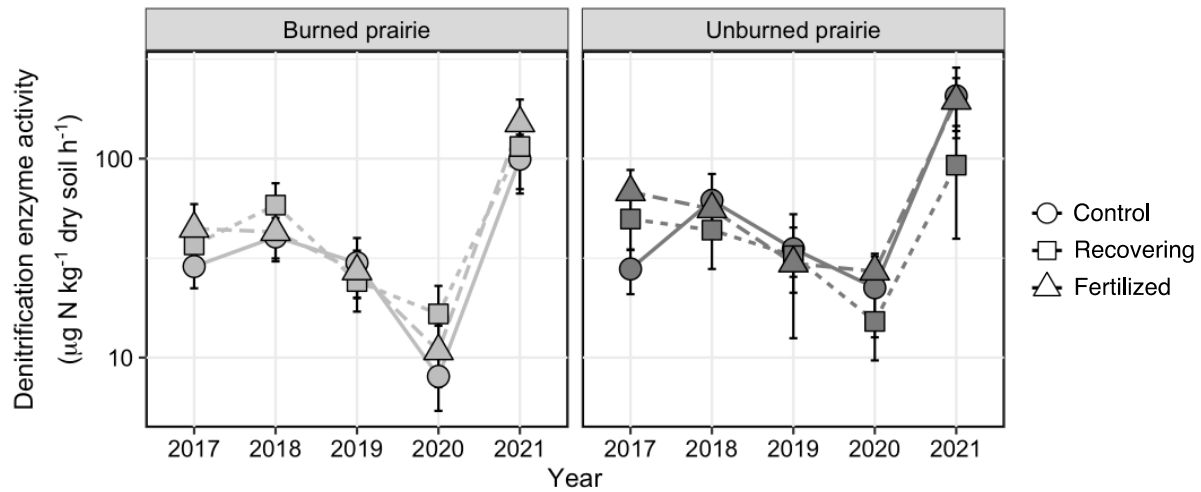
Appendix D Figure D-2. Modeled probability of observing a non-zero denitrification enzyme activity using a generalized linear model with binomial distribution. Orange points are the average probability for each combined fire and fertilization treatment where year was modeled as a fixed effect. Grey points are the mean probability for the combined field treatments where year was modeled as a random intercept. Grey bars are the 95% confidence intervals estimated by bootstrapping.



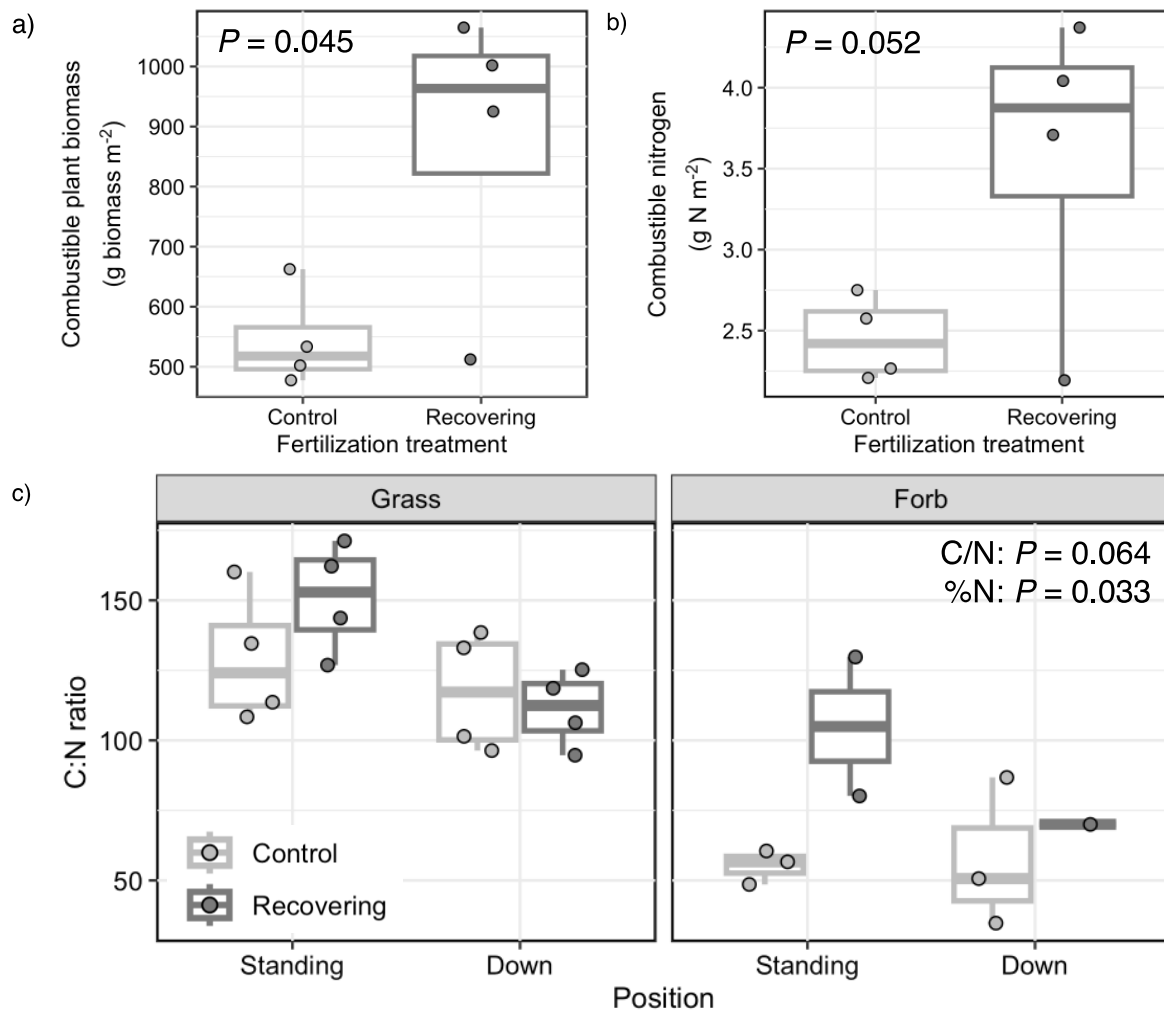
Appendix D Figure D-3. Ratio of $\text{NO}_3^- \text{-N} : \text{NH}_4^+ \text{-N}$ sorbed from surviving resin bags during the growing season in 2017 and 2019-2021. Each point is the average ratio for the fertilization treatment, split by the burned and unburned prairies, and the error bars are ± 1 standard error (SE). Letters denote significant differences among years at $P \leq 0.05$.



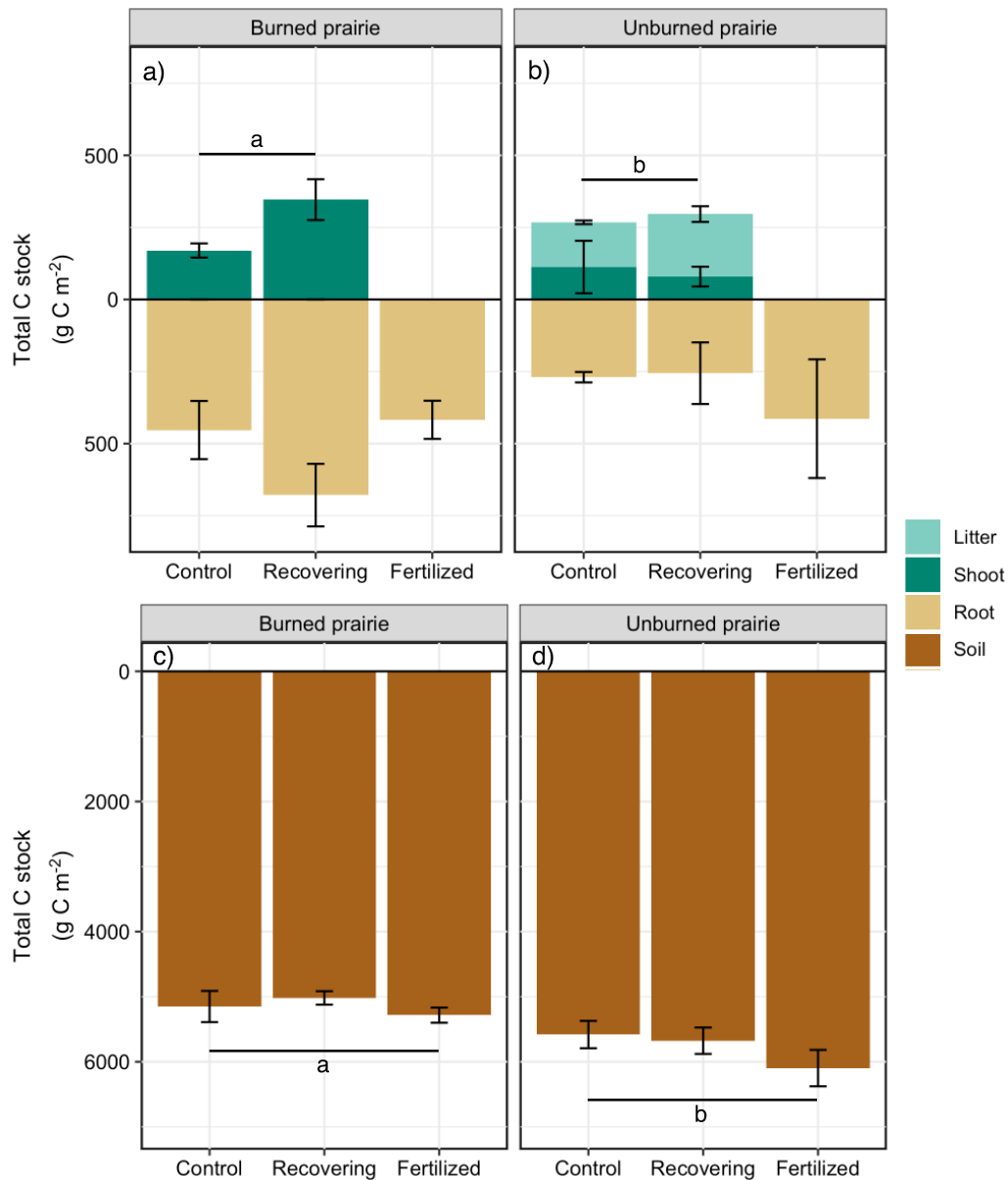
Appendix D Figure D-4. Denitrification enzyme activity (DEA) estimated twice during the growing season in 2017-2021. Each point is the average potential rate for the fertilization treatment, split by the burned and unburned prairies, and the error bars are ± 1 SE. DEA was not statistically different between fire and fertilization treatments or growing seasons.



Appendix D Figure D-5. Combustible plant litter (A), total combustible N in litter (B), and grass and forb litter C:N ratio (C) quantified before the 2021 prescribed fire in burned prairies, summarized by boxplots. Each point is the average quantity for each individual plot corresponding to the fertilization treatment.



Appendix D Figure D-6. Plant (a, b) and soil (c, d) C stock in 2021 for both burned and unburned prairie treatments. Each bar is the average stock for the fertilization treatment, and the error bars are ± 1 SE. Lowercase letters denote a significant difference in soil C stock between burned and unburned prairies at $P \leq 0.05$.



Appendix E - Supplementary tables and figures for Chapter 5

Appendix E Table E-1. ANOVA outputs from linear mixed effect models for alpha diversity and resistance and resilience indices.

<i>F, P</i>	Burn (B)	Fert (F)	Year (Y)	B×F	B×Y	F×Y	B×F×Y	Marginal R^2 , Conditional R^2
Observed	0.3, 0.633	0.6, 0.600	51.9, < 0.001	0.9, 0.393	1.9, 0.105	2.0, 0.045	1.0, 0.442	0.584, 0.646
Shannon diversity	0.3, 0.631	1.4, 0.247	33.6, < 0.001	1.5, 0.229	0.7, 0.586	3.1, 0.002	0.9, 0.508	0.462, NA
Pielou's evenness	0.2, 0.707	1.1, 0.346	25.6, < 0.001	1.4, 0.259	0.3, 0.885	2.3, 0.023	0.4, 0.907	0.378, NA
Resistance index	0.11, 0.768	.	0.7, 0.605	.	0.9, 0.495	.	.	0.083, 0.843
Resilience index	0.2, 0.684	.	0.7, 0.632	.	1.0, 0.429	.	.	0.109, 0.536

NA indicates conditional R^2 could not be computed since random effects overfitted mixed effect models.

Appendix E Table E-2. Pairwise PERMANOVA for treatment combinations.

Fire	Fertilization	Pseudo- <i>F</i> , <i>P</i>				
		Burned prairie		Unburned prairie		
		Recovering	Fertilized	Control	Recovering	Fertilized
Burned	Control	10.6, 0.001	8.2, 0.001	4.6, 0.001	11.7, 0.001	8.4, 0.001
	Recovering		1.5, 0.058	8.6, 0.001	9.2, 0.001	8.2, 0.001
	Fertilized			6.8, 0.001	8.3, 0.001	6.8, 0.001
Unburned	Control				9.3, 0.001	6.1, 0.001
	Recovering					1.4, 0.069

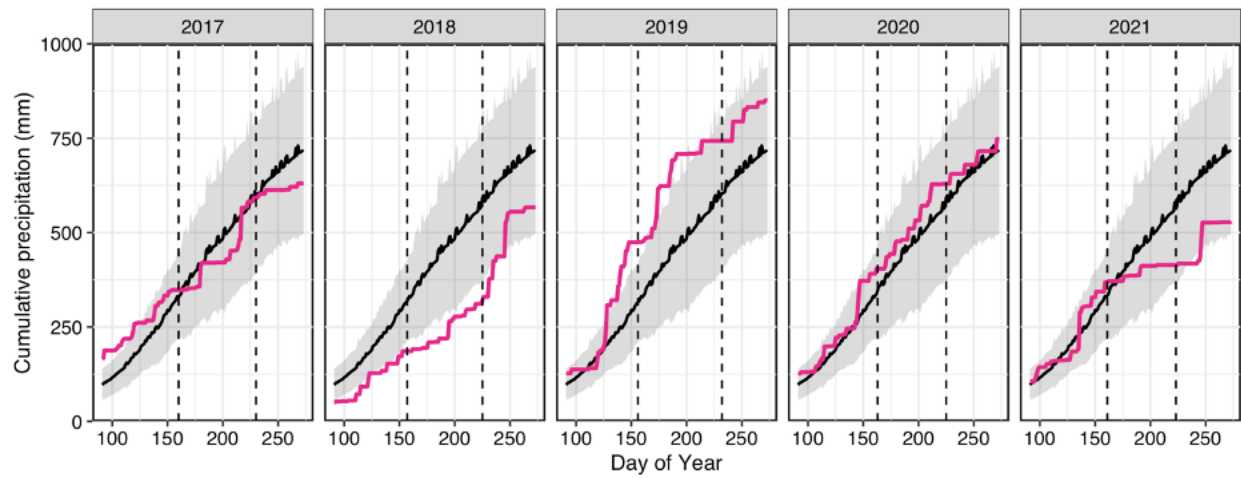
Appendix E Table E-3. Descriptive and hypothesis testing statistics for log-fold changes among phyla and classes defined as putative copiotrophs and oligotrophs according to previous studies. Bolded cells indicate statistically significant differences from zero at $P \leq 0.05$. Taxonomy labels are shortened for clarity: Actino, Actinomycetota (formerly Actinobacteria); Alpha, Alphaproteobacteria; Gamma, Gammaproteobacteria; Nitroso, Nitrososphaera (formerly Thaumarchaeota); Acido, Acidobacteriota (formerly Acidobacteria); Delta, Deltaproteobacteria; Planco, Planctomycetota (formerly Planctomycetes); Verruco, Verrucomicrobiota (formerly Verrucomicrobia).

Putative life history strategy	Phylum/Class	Mean log-fold change, <i>t</i> , <i>P</i>			
		Burned prairie		Unburned prairie	
		Fertilized	Recovering	Fertilized	Recovering
Copiotroph	Actino	-0.006, -0.27, 0.785	0.027, 0.93, 0.355	-0.010, -0.57, 0.567	0.057, 2.97, 0.003
	Alpha	0.009, 0.23, 0.818	0.012, 0.28, 0.78	-0.006, -0.16, 0.873	0.055, 1.37, 0.173
	Gamma	-0.021, -0.43, 0.669	-0.035, -0.6, 0.55	0.028, 0.70, 0.484	0.095, 1.96, 0.054
	Nitros	-0.147, -1.14, 0.282	0.060, 0.49, 0.634	-0.129, -0.99, 0.348	0.087, 0.65, 0.532
Oligotroph	Acido	0.099, 2.80, 0.006	0.070, 1.71, 0.088	0.103, 4.20, < 0.001	0.157, 5.40, < 0.001
	Myxo	0.043, 1.13, 0.264	0.005, 0.10, 0.919	0.069, 1.83, 0.073	0.118, 2.78, 0.007
	Plancto	0.043, 1.82, 0.071	0.008, 0.27, 0.785	0.079, 3.90, < 0.001	0.133, 6.15, < 0.001
	Verruco	0.063, 1.21, 0.229	0.095, 1.68, 0.097	0.180, 4.89, < 0.001	0.244, 5.68, < 0.001

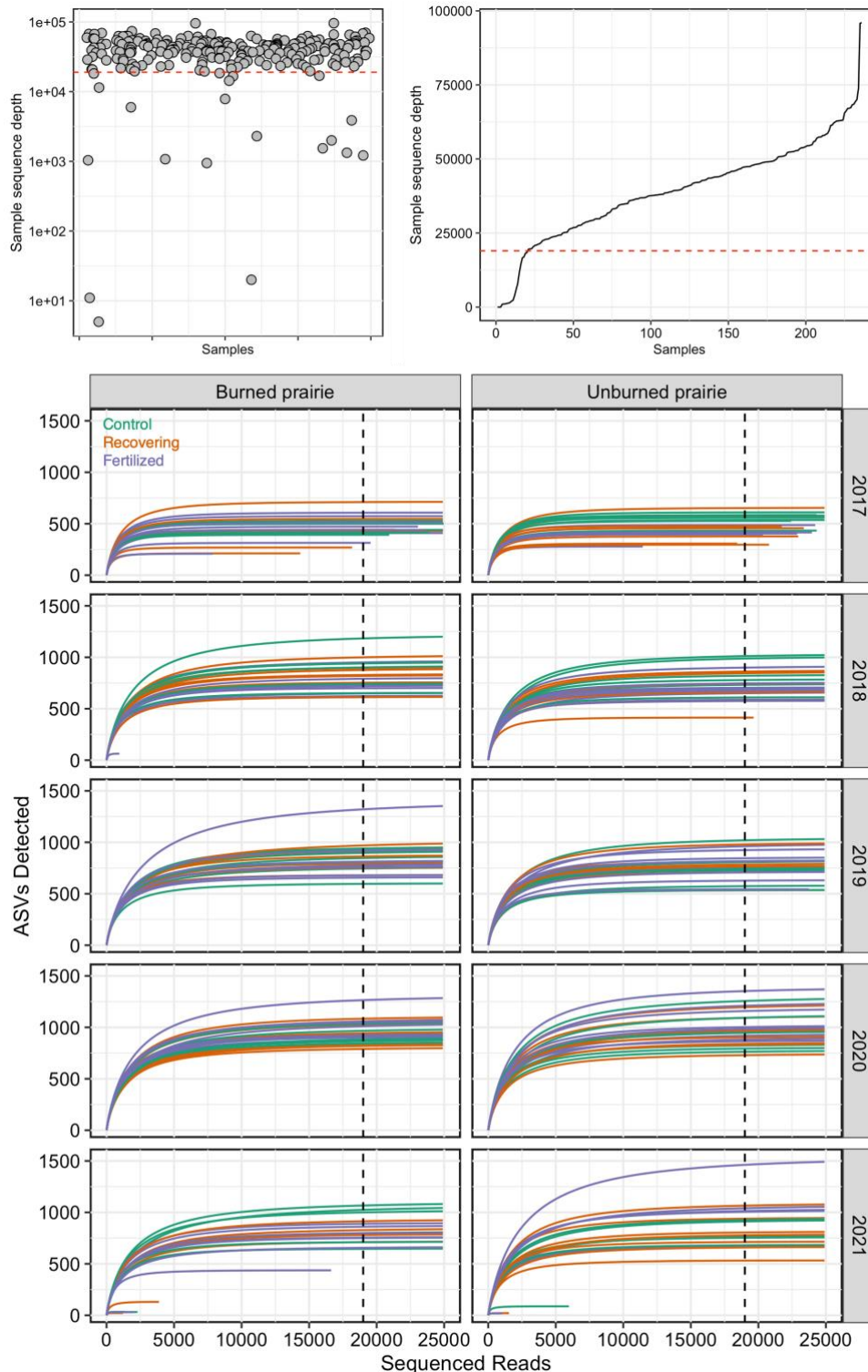
Appendix E Table E-4. ANOVA outputs for soil extracellular enzyme activities (EEAs) and microbial biomass C and N. Note that EEAs labeled with “_MBC” were normalized by microbial biomass C.

<i>F, P</i>	Burn (B)	Fert (F)	B×F	Adj. <i>R</i> ²
BG	2.9, 0.106	1.7, 0.216	1.3, 0.300	0.143
CBH	5.8, 0.028	4.8, 0.021	2.3, 0.127	0.395
NAG	2.2, 0.157	0.2, 0.785	0.6, 0.554	-0.051
LAP	1.5, 0.241	0.2, 0.815	1.4, 0.282	-0.018
PHOS	0.8, 0.373	0.1, 0.939	0.6, 0.550	-0.139
PHX	0.7, 0.425	0.0, 0.970	0.7, 0.526	-0.146
C:N	7.7, 0.013	0.5, 0.617	0.9, 0.425	0.192
C:P	2.3, 0.146	0.0, 0.978	0.1, 0.879	-0.116
N:P	0.0, 0.943	0.1, 0.942	0.3, 0.720	-0.224
MBC	0.0, 0.913	0.4, 0.660	0.3, 0.777	-0.187
MBN	2.0, 0.179	2.1, 0.157	0.3, 0.729	0.069
Microbial biomass C:N	1.2, 0.282	1.3, 0.309	0.3, 0.752	-0.031
BG_MBC	1.4, 0.251	0.6, 0.582	1.4, 0.276	0.012
CBH_MBC	4.5, 0.049	2.8, 0.084	3.2, 0.062	0.337
NAG_MBC	2.1, 0.167	0.1, 0.875	0.9, 0.410	-0.035
LAP_MBC	1.3, 0.272	0.2, 0.846	1.2, 0.326	-0.045
PHOS_MBC	0.8, 0.370	0.0, 0.960	0.9, 0.442	-0.115
PHX_MBC	0.7, 0.427	0.0, 0.964	0.7, 0.529	-0.147
C:N_MBC	4.8, 0.041	0.3, 0.762	0.3, 0.750	0.041
C:P_MBC	2.0, 0.171	0.2, 0.843	0.4, 0.666	-0.084
N:P_MBC	0.0, 0.953	0.3, 0.734	0.4, 0.687	-0.186

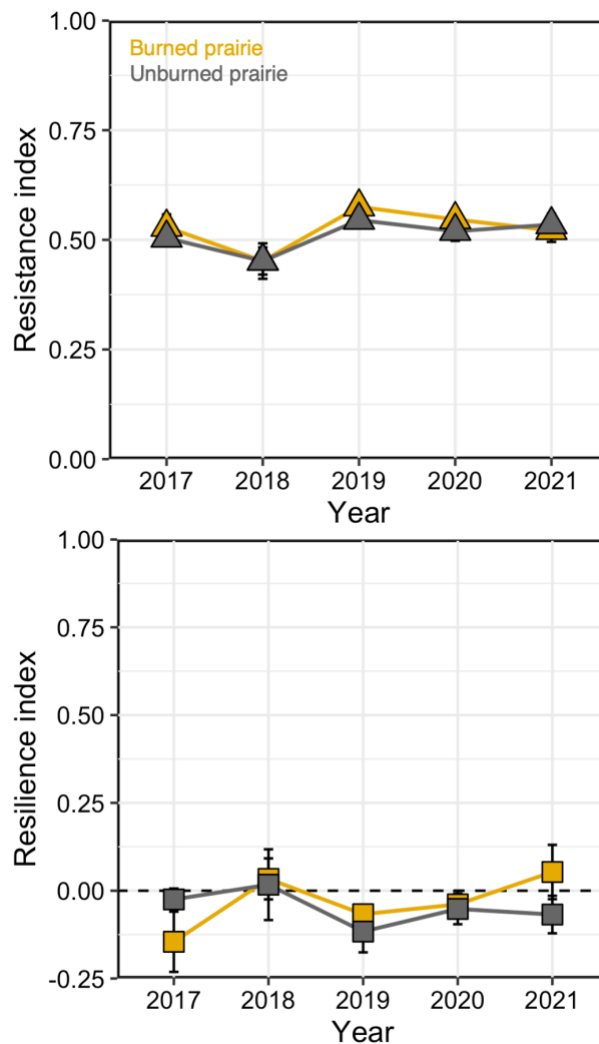
Appendix E Figure E-1. Cumulative precipitation during the growing season (pink line) compared to the average cumulative precipitation (black line) and ± 1 standard deviation (shaded bar) from 1986-2021. Dashed lines represent the dates soil samples were collected.



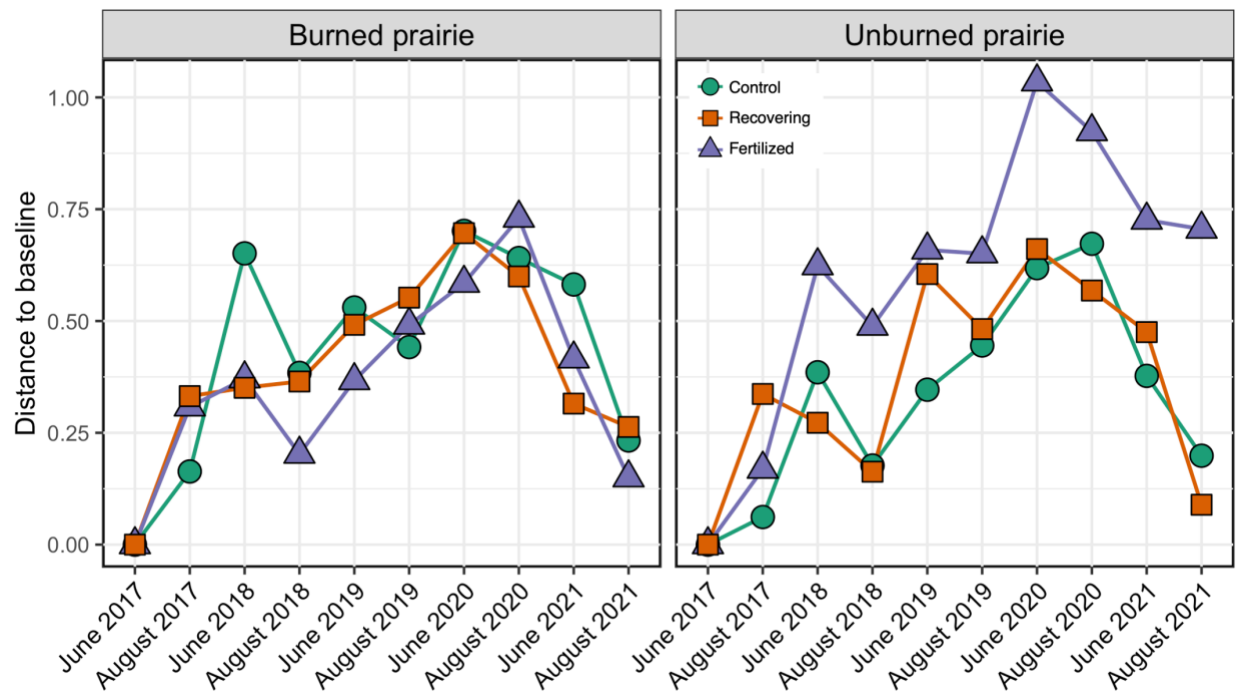
Appendix E Figure E-2. Sequence depth of samples (top figures), where the horizontal red dashed line is the 19,000-sequence read threshold. Rarefaction curves (bottom), split by field treatment and year, show good overall coverage of detected ASVs. The vertical black dashed line is the 19,000-sequence read threshold. For clarity, the x-axis was limited to the first 25,000 sequences.



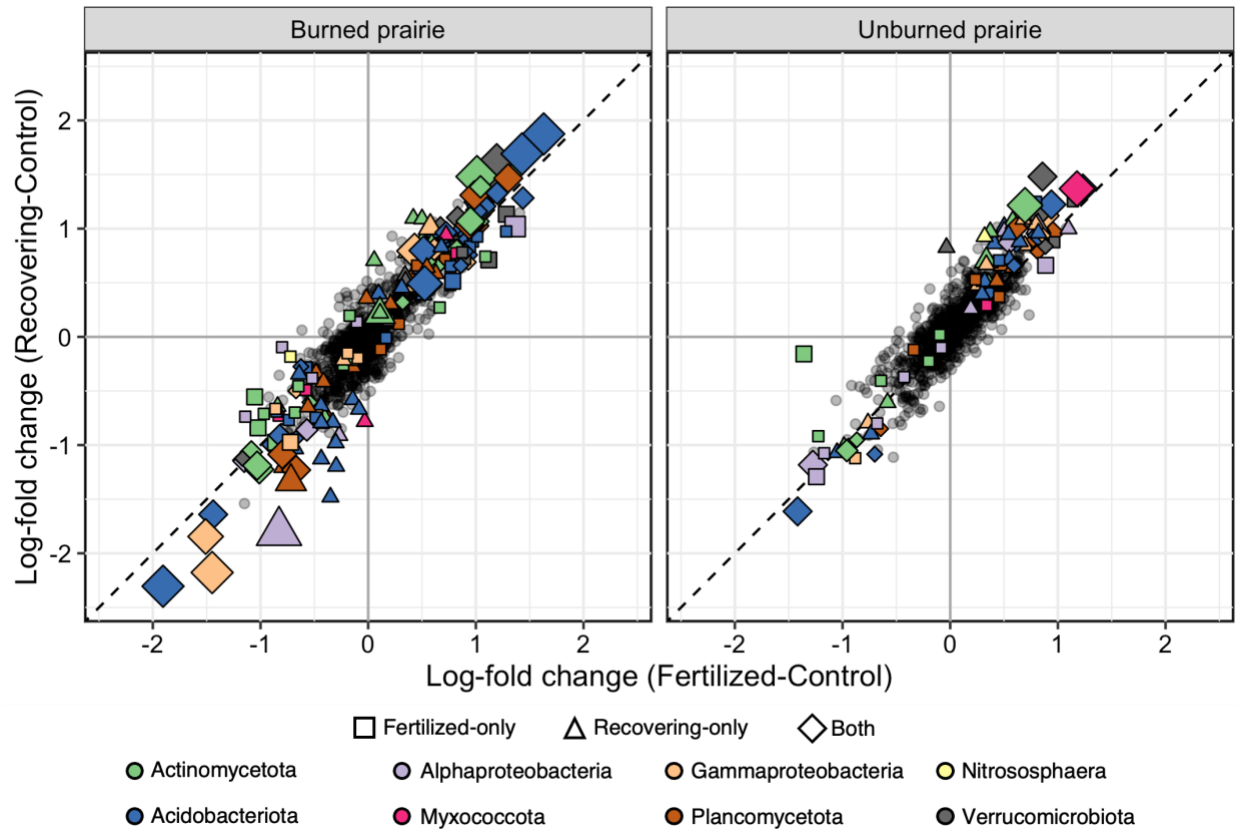
Appendix E Figure E-3. Resistance (top) and resilience (bottom) indices across years, split by burned and unburned prairies. A resistance or resilience index of 1 indicates complete resistance or recovery from N-fertilization, respectively. A negative resilience index represents greater taxonomic turnover of soil microbial communities after ceasing N-fertilization, relative to fertilized and control treatments for that given year.



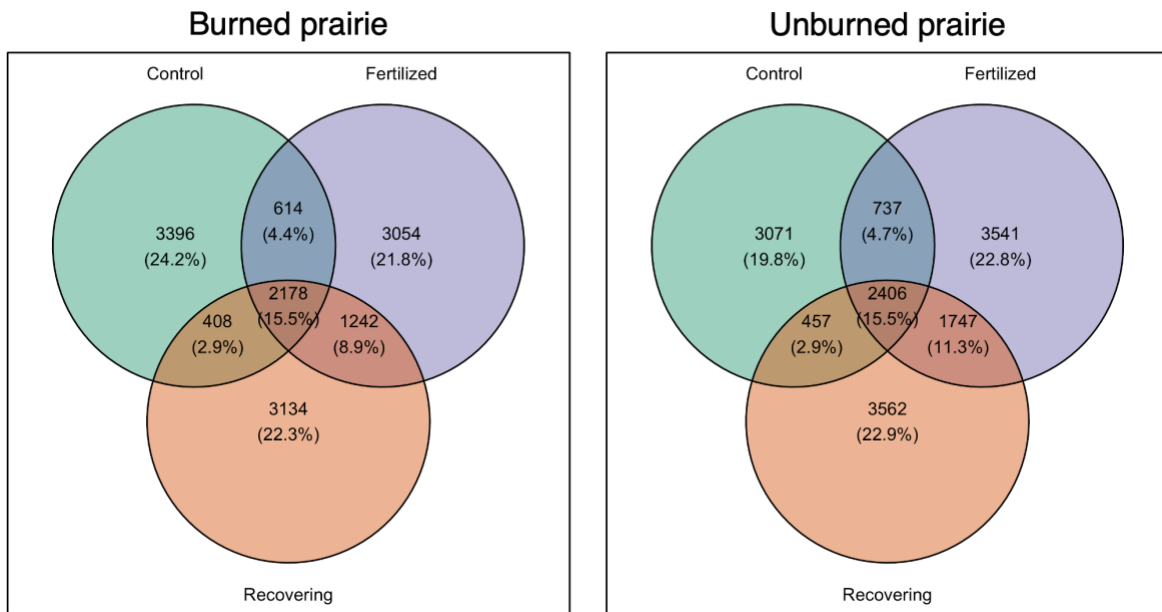
Appendix E Figure E-4. Distances between NMDS ordination points relative to the reference point, defined as the June 2017 samples. NMDS was used among all combined field treatments, but burned and unburned prairies are split for clarity.



Appendix E Figure E-5. Average log-fold changes between continuously fertilized-control treatments and recovering-control treatments, split by burned and unburned prairies. Gray dashed line represents the 1:1 ratio (i.e., no difference between fertilization treatments). Black translucent points are ASVs that were not differentially abundant at bias-corrected $P \leq 0.05$, while colored points are ASVs that were significantly different at least once during the study where larger points indicate ASV abundance was different for a great number of years. Point shapes represent which fertilization treatment resulted in differentially abundant ASVs.



Appendix E Figure E-6. Venn diagrams of unique and shared ASVs among fertilization treatments between burned and unburned prairies.



Appendix E Figure E-7. Soil extracellular enzyme activities (EEAs), normalized by g of dry soil, measured in September 2021. Each point is the measured enzyme activity for each plot corresponding to the fertilization treatment. Letters next to the mean $\pm$ standard deviation in CBH plots denote significant difference between fire treatments at $P \leq 0.05$.

