Grassland soil microbial responses to long-term management of N availability.

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Abstract

Anthropogenic actions have significantly increased biological nitrogen (N) availability on a global scale. In tallgrass prairies, this phenomenon is exacerbated by land management changes, such as fire suppression. Historically, tallgrass prairie fire removed N through volatilization, but fire suppression has contributed to increased soil N availability as well as woody encroachment. Because soil microbes respond to N availability and plant growth, these changes may alter microbial composition and important microbially-mediated functions. Grassland management affects the soil environment on multiple time scales including short (fertilization or fire event), seasonal (growing vs. non-growing season), and long-term (decadal plant turnover and nutrient accumulation), therefore my goal was to understand community variability at different time scales affecting the population and community dynamics of soil microbes. I predicted soil microbes would be sensitive to environmental changes at all time scales, seasonal variation would reflect increased plant rhizodeposit-supported populations during summer and decomposers during winter, and long-term fire suppression and chronic fertilization would drive soil microbial community turnover associated with accumulation of plant litter and N.

To address these predictions, soils were collected from the Belowground Plot Experiment (BGPE) at Konza Prairie Biological Station: a 30-y factorial field manipulation of N fertilization and burning. Surface soils (0-15 cm) were sampled monthly between Nov 2014 – Dec 2015, including one week post-fire (April) and post-fertilization (June). Genomic DNA was extracted from each sample for qPCR and PCR for Illumina MiSeq library sequencing of the prokaryotic 16S rRNA gene and fungal ITS, to estimate population and community dynamics of soil

microbes. Soil environmental characteristics and plant communities were measured in July 2015 to evaluate correlations between plant and microbial communities, and environmental variability.

Soil microbial responses to short-term fire/fertilization events were minimal, while microbial population sizes fluctuate seasonally and synchronously, and microbial community composition varied more with management history than at shorter time scales. Bacterial populations increased 10x during growing-season plant rhizodeposition, while fungal populations were less dynamic, but decreased in fall, possibly reflecting a shift to subsistence on soil organic matter. In contrast, microbial community composition was seasonally stable, but distinct between long-term management treatments, which may indicate accumulation of niche-defining plant or soil properties over decades. Prokaryotic communities responded to altered N availability via both fertilization and loss due to fire, with the highest abundance of "copiotrophic" (r-selected) taxa in unburned, fertilized soils. Fungal communities responded to N fertilization with higher abundance of arbuscular mycorrhizal fungi, pathogens, and saprotrophs, possibly due to changes in nutrient stoichiometry and litter availability in fertilized plots. However, fungal response to fire was largely independent of N availability, and plant community differences were correlated with fungal, but not bacterial, community composition, highlighting the likely nutritional codependence of fungi and plants, and fungal competitive advantages for plant litter substrates. The timing of changes in soil microbial communities is critical for plant nutrition and nutrient cycling in prairies, and this novel dataset on the temporal resolution of microbial responses to environmental variability contributes to the broader understanding of ecosystem responses to global change.

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Chapter 1 - Introduction

A central theme in ecological research relates to understanding, quantifying, and predicting ecosystem responses to anthropogenically-driven global change phenomena, such as alterations to biogeochemical cycles and modifications to land-use or management. These themes are especially important in threatened ecosystems where human activities have reduced the range and continuity of an ecosystem, such as the tallgrass prairie. Historically, tallgrass prairie stretched across the Great Plains ecoregion of the United States, but today, tallgrass prairie covers <5% of its original range due to land use conversions for agricultural or urban use (Samson and Knopf 1994). In general, tallgrass prairie was shaped and maintained by local climate, fire, and disturbance from ungulate grazing, but the removal of grazers and suppression of fire have led to widespread increases in woody plant cover and changes in nutrient availability (Parton et al. 1987, Knapp et al. 1998, Briggs et al. 2002, Veach et al. 2015). Grasslands are of vital conservation concern because they hold a significant portion of the world's biodiversity and contribute significantly to C sequestration (Scurlock and Hall 1998, Fierer et al. 2013). Therefore, ecologists and land managers recognize the need to properly manage the remaining tallgrass prairie, and understand the implications, intended results, and interactions of multiple management practices.

The ecology of microorganisms has become central to the discussion surrounding ecosystem management and change, because microbial communities are well-known to impact ecosystem functionality (Wagg et al. 2014, Delgado-Baquerizo et al. 2016). For example, soil microbes in tallgrass prairie have been shown to drive plant production and diversity (Reynolds et al. 2003, Schnitzer et al. 2011), regulate transformations, retention, and cycling of nutrients (Coleman et al. 1983, Ojima et al. 1994), and make critical contributions to carbon (C)

sequestration (Waldrop et al. 2004, Six et al. 2006). Furthermore, microbial populations within the soil community are diverse in their life-history strategies, physiology, and morphology, at coarse (e.g. bacteria vs. fungi) and finer taxonomic scales (specific phyla within the Bacteria and Fungi) of resolution, and these differences have variable effects on ecosystem processes (Torsvik and Ovreas 2002, de Boer et al. 2005). Furthermore, bacteria and fungi differ in their growth habits (small single cell vs. large hyphal bodies, respectively), phylogenetic domains (prokaryotic vs. eukaryotic), and nutrient acquisition strategies (e.g. dominantly saprophytic vs. mycorrhizal), therefore we can expect bacteria and fungi to be differentially impacted by changes in the soil habitat (Adu and Odes 1978, Six et al. 2006, Strickland and Rousk 2010). In addition to coarse differences mentioned above, defined taxa of soil bacteria and fungi vary in their ecological life-history strategies at finer scales, which can ultimately alter microbial community composition and function under varying environmental disturbance regimes, management, or global change phenomena. Therefore, a comprehensive understanding of the soil microbial community can aid in understanding ecosystem processes.

Fire is a natural disturbance that is central to the development and maintenance of the tallgrass prairie ecosystem and plant community structure (Hulbert 1969). Grassland fires are generally swift and there is little heat penetration into soil, but fires indirectly impact grassland biogeochemical cycles by decreasing nitrogen (N) availability through removal of aboveground vegetation and reduction of available nitrogen and soil organic matter (SOM) inputs from plant litter (Hobbs et al. 1991, Johnson and Matchett 2001), while increasing plant C and N allocation to root biomass (Kitchen et al. 2009). For management of tallgrass prairies, fires are arguably most important for maintaining herbaceous plant communities by preventing the establishment of woody vegetation. In grasslands where fire has been suppressed, plant communities tend to

feature more woody plants such as Eastern Red Cedar (*Juniperus virginiana*) and Roughleaf Dogwood (*Cornus drummondii*), which alters tallgrass prairie biodiversity and soil properties, and therefore could have cascading effects on soil microbial communities (Dooley and Treseder 2011, Ratajczak et al. 2012, Ratajczak et al. 2016). Much of the literature surrounding fire and soil microbes has focused on significant changes in bacterial community structure in forest ecosystems where fires are hotter and have a more direct influence on soil properties (Dooley and Treseder 2011). However, microbial communities are tightly coupled with soil properties and plant community composition, and we expect that soil microbial communities may change in response to the presence of long-term fire history. Because soil microbes have pivotal roles in ecosystem function, understanding microbial responses to long-term fire management can provide insight into best management practices for the remaining tallgrass prairie ecosystem.

Tallgrass prairie biogeochemical cycles are also impacted by increased N availability though atmospheric N deposition. Over the past 100 years, anthropogenic alterations of the N cycle have led to nearly doubling the amount of biologically available N in the atmosphere through increased fossil fuel use and increased use of fertilizers in industrial agriculture (Galloway 2004, Riggs et al. 2015). In grassland ecosystems, N is generally a limiting nutrient and N enrichment is known to have variable effects on the soil environment (Neff 2002, Zeglin et al. 2007, Treseder 2008). Nitrogen enrichment could either enhance microbial growth by improving N availability in litter and increasing C availability by increasing aboveground primary production (LeBaur and Treseder 2008), or diminish microbial growth by decreasing soil pH, causing direct toxicity to microbes or promoting production of recalcitrant litter (Soderstrom et al. 1983, Treseder 2008). Additionally, N enrichment has been known to alter the composition of plant communities, typically by decreasing diversity by promoting nitrophilous

species and excluding plants less tolerant to high N conditions (Tilman 1991, Tilman 1997). Similar effects can be observed for microbial communities, where increased N availability tends to increase the relative abundance of putatively copiotrophic microbial groups and decrease the relative abundance of oligotrophic groups (Johnson et al. 2003, Ramirez et al. 2012, Coolon et al. 2013, Leff et al. 2015). Because fire and N deposition are two phenomena of concern to tallgrass prairie managers, it's important to understand the interaction of these processes and their feedback to microbial dynamics. For example, N deposition has been shown to increase ANPP, which increases fire fuel loads and therefore fire intensity. Fire intensity can affect direct microbial mortality from soil heating, or the quantity of soil nutrients volatilized from the soil. Furthermore, fire may be a process to remediate the undesirable effects of N deposition in a management scenario, as fire is known to volatilize N. Overall, it is unclear how ecological disturbance interacts with global change phenomena, such as atmospheric N deposition, and how these interactions feedback to microbial communities and ecosystem processes.

In addition to nutrient availability from fire or N deposition being an influential stand-alone driver of microbial diversity and function, nutrient availability and fire/fertilization events also vary throughout a growing season and over winter due to changes in air temperature, moisture, plant growth and litter inputs, and plant-microbe competition (Hodge et al. 2000, Schimel and Bennett 2004, Schmidt et al. 2007). Therefore, one may expect to observe temporal variation in microbial community composition because changes in microbial community composition are often correlated with changes in the soil environment. Soil microbes are sensitive to environmental change and can respond to these changes rapidly because of their small size, fast generation time, and large surface area to volume ratio (Schmidt et al. 2007, Shade et al. 2012). Microbial communities have the potential to turnover on short (hours to days)

and long (seasonal or annual) time scales and display drastic responses to changes in soil physical and chemical properties, such as soil moisture, temperature and nutrient availability (Garcia and Rice 1993, Schmidt et al. 2007). For example, microbial ammonia oxidizing bacteria in wetland ecosystems have been shown to be diurnally regulated in response to changes in oxic and anoxic conditions that are driven by plant activity (Nikolausz et al. 2008). Studies in alpine ecosystems have indicated that microbial communities also display distinct seasonal patterns driven by physiological responses to snow accumulation and melting (Schmidt et al. 2007). Additionally, microbial communities have the potential to change in response to long-term management. In a long-term field manipulation, Coolon et al. (2013) showed divergence in microbial community composition from decades of N fertilizer inputs. While microbial communities have been shown to turnover and respond to environmental change on multiple time scales, it is currently unclear how management in remaining tallgrass prairie fragments impacts microbial community turnover on multiple temporal scales.

Objectives and Justification

The objective of this thesis research was to address how microbial community turnover is impacted by land management practices at multiple time scales, with two specific objectives: 1) characterize event-based, seasonal, and long-term responses of bacterial community and microbial population size turnover to 30 years of fire and fertilization treatments, and 2) relate changes in fungal and plant community composition to fire/fertilization management on short and long time scales. We conducted this research in the Flint Hills ecoregion, which holds the majority of the remaining tallgrass prairie due to the shallow, rocky, soils that are unfavorable for cultivated agriculture. Instead, grasslands in this region have been used for rangeland or preserved for research and recreation. Soil microbes are well-known to regulate critical

ecosystem processes, but there is no consensus on how these changes in microbial community composition may impact ecosystem function or if soil microbes are functionally redundant. One matter that hinders microbial ecologists from understanding functional changes in response to disturbance or global change is the knowledge of specific ecosystem responses of microbial taxa to environmental disturbances. Therefore, a comprehensive understanding of microbial compositional responses to global change phenomena, such alterations of biogeochemical cycles or climate change, can assist in understanding changes in ecosystem function and assist in making informed management decisions in threatened ecosystems. Recent efforts have focused on elucidating responses of soil microbial communities to ecosystem change and management practices in the tallgrass prairie. Understanding microbial responses to environmental drivers can help ecologists and conservationists best manage the remaining tallgrass prairie ecosystem fragments. Ecologists are now able to access an abundance of ecologically relevant microbial diversity and function information through low-cost sequencing of small subunit ribosomal genes, such as assigning microbial life history traits (copiotrophic/oligotrophic), nutrient acquisition strategies (saprotrophic/mycorrhizal/pathogenic), quantifying specific microbial populations (bacteria/fungi), and assigning taxonomic identities to microorganisms (Fierer et al. 2007, Pace 1997). For threatened ecosystems such as tallgrass prairie, connecting seasonal responses of microbial and ecosystem function in the face of global changes can help ecologists and land managers understand the implications of management decisions.

Correlating ecosystem functional responses to changes in community composition will continue to be a challenge for microbial ecologists, and one way to better understand this relationship is through long-term experimental manipulations. In tallgrass prairie, experimental manipulations of N additions and fire regimes can aid in understanding phylogenetic responses

to environmental change. By sampling experimental management manipulations at various temporal scales, one can elucidate microbial turnover phylogenetic, life history, and functional turnover in response to environmental and temporal change. The Belowground Plot Experiment (BGPE) at Konza Prairie Biological Station is one such field manipulation.

This study analyzed bacterial and fungal community and population turnover in a subset of the BPGE plots that have been annually burned or unburned, and N fertilized or unfertilized since 1986. This work both builds upon previous studies focusing on belowground responses to long-term land management, and provides critical information on belowground communities for future studies at the BGPE. We sampled soils monthly from November 2014 to December 2015 to add temporal resolution and a greater understanding of over-winter dynamics to our current knowledge of microbial community turnover in response to fire and fertilization.

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Chapter 2 - Long-term management of N availability alters grassland soil bacterial community composition, but not seasonality of microbial population sizes

Abstract

Nitrogen (N) availability is a driver of soil microbial diversity and function, and is affected by prescribed burning (N removal through volatilization) and fertilization (N addition). Because soil microbes control critical feedbacks to ecosystem function, it is important to understand the dynamics and responses of microbial populations under conditions of contrasting N availability. This study took place at a long-term field manipulation in which native tallgrass prairie was annually burned or left unburned, and annually fertilized or left unfertilized, in a factorial design, since 1986. Composite surface soil samples (0-15 cm) were collected monthly (November 2014 - December 2015) from replicate plots to measure event-based (post-fire, post-fertilization), seasonal and 29-year turnover of soil microbial communities. Bacterial 16S rRNA gene and fungal ITS population sizes were estimated by qPCR, and bacterial community composition (BCC) was measured using Illumina MiSeq sequencing of 16S rRNA genes. We expected seasonal and event-based change in all parameters, and that total microbial population sizes and diversity would be lower in soils with higher N availability, due to greater competitive dominance of nitrophilic taxa. Bacterial and fungal population sizes varied significantly by sampling month, not with long-term treatment, in that bacterial populations were at least 10x greater in summer (June-August). In contrast, BCC did significantly vary by season, but was strongly impacted by both long-term fire and fertilization treatments. Additionally, we observed increases and decreases in "copiotrophic" and "oligotrophic" taxa in response to long-term N

fertilization, which were significantly stronger and more predictable in unburned soils, but no changes in richness or evenness. These results reveal that while long-term grassland management changes BCC beyond typical levels of seasonal variability, total bacterial populations change coherently month-to-month, potentially due to plant activity supplying similar levels of labile carbon during summer. Furthermore, ambient soil N availability, controlled by fire in tallgrass prairie, can influence the sensitivity of bacterial communities to N fertilization.

Introduction

As multiple global environmental factors continue to change, it is essential to understand how biotic communities respond to environmental variability, particularly in threatened ecosystems such as the tallgrass prairie (Seastedt et al. 2008, Jackson and Blois 2015). Tallgrass prairie ecosystems compose <5% of their original range due to conversions to agriculture, urbanization, and woody encroachment (Riggs et al. 2015, Veach 2015). Ecologists and land managers recognize the need to manage the remaining tallgrass prairie to ensure habitat conservation for species that rely on this ecosystem, as well as for the maintenance of regionally and globally important ecosystem functions. Soil microbes mediate valuable grassland functions, including decomposition, soil fertility, and carbon storage, and understanding the environmental controls over microbial communities will help inform future land management decisions that maintain these critical ecosystem services (Torsvik and Ovreas 2002, van der Heijden 2008, van der Putten et al. 2013).

Microbes are often sensitive to environmental change due to their relatively short generation time, small size, and large surface area to volume ratio (Schmidt et al. 2007, Shade et al. 2012, Zeglin 2015), and within the heterogeneous soil habitat, soil microbial communities are taxonomically and functionally diverse. Therefore, environmental drivers of changes in soil

microbial dynamics have the potential occur on multiple time scales with differential effects on specific populations with different niche preferences (Bardgett et al. 2005, Fierer et al. 2010, Shade et al. 2013). It is well-known that tallgrass prairie ecosystems have undergone considerable long-term changes due to anthropogenic activities such as fire suppression, which has led to woody encroachment (Ratajczak et al. 2012) and accumulations of soil organic carbon (C) and nitrogen (N) (Turner et al. 1997, Dooley and Treseder 2011), and increases in biologically available soil N from atmospheric deposition, caused primarily by increased use of fossil fuels and N fertilizers (Galloway et al. 2004, Farrer et al. 2013). However, less is known about whether microbial turnover on shorter time scales is reflective of cumulative long-term ecosystem change, and an understanding of the typical range of microbial cell and community composition variability in the short term is needed to provide context to understand the novelty of the impacts of long-term change on microbial communities (Shade et al. 2013).

In addition to long-term changes in tallgrass prairie ecosystems, these regions have distinct seasonal changes in climate and plant phenology, ranging from hot, dry plant growing seasons to colder and wetter dormant periods (Knapp 1998), which could also drive microbial community or population dynamics. In winter, freeze-thaw of surface soils might impose physiological limitations on cell survival to the summer season and affect the decomposition of soil organic matter (SOM), which could have variable feedbacks on N availability during the growing season (Bardgett et al. 2005, Schimel et al. 2007). Seasonal patterns of soil microbial turnover have been well described in alpine and arctic ecosystems, where saprophytic fungi and slow-growing, oligotrophic, bacteria with heightened depolymerization strategies dominate in winter, whereas mycorrhizal fungi and fast-growing, copiotrophic, bacteria dominate in summer (Jaeger et al. 1999, Schmidt et al. 2004, Schimel and Mikan 2005, Schmidt et al. 2007). In

tallgrass prairies, less is known about seasonal turnover, and far less is known about how seasonal turnover is impacted by long-term environmental changes in soil nutrient availability from fire or fertilization.

In a managed tallgrass prairie, drivers of microbial dynamics could also occur on relatively short time-scales. For example, event-based pulses of rhizodeposited soil carbon (C) from root growth, spikes of N availability from direct fertilization events, or mobilization of nutrients following rainfall, can stimulate microbial activity in the short-term, sometimes in association with community composition change (Woods et al. 1987, Fauci and Dick 1994, Stark et al. 2008, Kuzyakov and Blagodatskaya 2015, Armstrong et al. 2016). In addition to greater root production following spring burning (Johnson and Matchett 2001), initial responses to fire can include higher microbial activity via increased soil temperature in the weeks following combustion of surface litter (Hulbert 1988, Ojima et al. 1997, Treseder et al. 2004). However, microbial responses to events that modify nutrient availability may be ephemeral, or different from, longer-term responses (Stark et al. 2004, Kuzyakov et al. 2000, Bardgett et al. 2003, Ramirez et al. 2010). Altogether, there is currently a lack of understanding of how short, seasonal, and long-term management and change interact to alter soil microbial communities and populations, and how these interacting time-scales may impact the tallgrass prairie ecosystem.

The objective of this study was to assess microbial community and population dynamics, at event-based, seasonal and decadal resolutions, in response to two management practices that drive N availability in tallgrass prairie: prescribed annual fire and N fertilization. Fire volatilizes organic N in plant litter, maintaining an N-limited situation in which native prairie plants with low N demand are competitively dominant (Seasted et al. 1991, Tilman and Wedin 1991, Blair 1997, Yu et al. 2015), in contrast, the lack of fire allows available soil N to accumulate (Blair

1997). We sampled a 30-year field manipulation of annual burning and fertilization once per month for one year to address the following questions: 1) What is the response of the soil microbial community to event-based (fire, resource pulse addition) and seasonal environmental variation? 2) Does long-term management of N availability impact the seasonal turnover of soil microbial communities, or modify the microbial community beyond the seasonal range of variability?

We predicted that: 1) Microbial populations would display distinct responses to fire and fertilization pulses, at both initial event-based and long-term time scales, with spring burning increasing microbial population sizes by promoting greater plant belowground production (Johnson and Matchett 2001), and N pulses decreasing microbial population sizes through direct shifts in microbial community composition to favor a more copiotrophic community (copiotrophic hypothesis, Ramirez et al. 2012), including a reduction in fungal populations (Treseder 2008). Additionally, we expected to detect seasonal changes in population sizes, with higher microbial populations in the summer due to availability of labile C from plant rhizodeposition, and lower in the winter when heterotrophic microbes rely on decomposition of soil organic matter for energy and C (Jaeger et al. 1999, Schmidt et al. 2007). We also predicted that: 2) Microbial community composition would differ between winter and summer (Schmidt et al. 2007), due to a higher relative abundance of populations that grow well on complex soil organic matter in the winter turning over different populations that grow faster on labile C in summer. Also, we expected that increased N availability, through either fertilization or lack of fire, will create an environment that favors a greater proportion of fast-growing copiotrophic taxa, as opposed to slower-growing oligotrophic taxa, and as evidenced by a greater community mean rRNA operon copy number (Roller et al. 2016).

Methods

Study Site and Experimental Design

This study was conducted at Konza Prairie Biological Station (KPBS). Konza Prairie Biological Station is located in the Flint Hills region of Kansas (39°05'N, 96°35'W) and is characterized by warm, dry summers and wet, cool winters, with MAP of 835 mm and MAT of 26.6°C. During the one-year sampling period, total monthly precipitation ranged from 6.2 mm in March 2015 to 147.3 mm during July 2015. Daily mean soil temperature ranged from 2.3° C in December 2015 to 23.5° C in July 2015, and daily mean air temperature ranged from 0.7° C in January 2015 to 37° C in July 2015. While mean temperatures were near or only slightly above average during the study period, the total annual precipitation of 1002.5 mm was 20% greater than average, reflecting a growing season with soil water content rarely much below field holding capacity (approximately 0.25 g·g^{-1}, Zeglin et al. 2013). Although specific micro-meteorological variables were not measured in each treatment, meteorological data for KPBS were collected for a site near the experimental plots. The vegetative cover of grasslands at KPBS is dominated by perennial C$_4$ grasses, such as *Andropogon gerardii*, *Sorghastrun nutans*, *Panicum virgatum*, and *Schizachyrium scoparium*, while unburned plots feature less grass cover and more woody plants such as *Juniperus virginiana, Cornus drummondii,* and *Rubus occidentalis* (Ratajczak et al. 2013).

The Belowground Plot Experiment (BGPE) was established in May 1986 at KPBS as part of the Konza Prairie Long-Term Ecological Research (LTER) program. The Belowground Plot Experiment is located on Irwin silty clay loam (fine, mixed, mesic, Pachic Arguistoll) (Garcia and Rice 1993, Wilson et al. 2009). This experiment was arranged in a split-strip block design, where whole-plot treatments are manipulated by fire. Then, a split-plot mowing treatment was

randomly assigned to half of the whole-plot. Within each treatment split, the plots were stripped

and randomly assigned a nutrient enrichment treatment (No fertilizer addition (control), N

fertilizer addition (10g $N \cdot m^{-2}$ as NH_4NO_3), phosphorus (P) addition, or N and P fertilizer

addition). For the purposes of this study, we sampled soils from just the control and the N

fertilizer addition plots under annually burned and unburned management history.

Sample Collection

Soils were collected once per month from November 2014 to December 2015, excluding

December 2014 and February 2015. Soils from all treatment plots were collected one week

following the annual burn treatment in April 2015, and one week following the annual

fertilization treatment in June 2015. Three random 2 cm diameter soil cores from the top 15 cm

of mineral soil were collected in each subplot and mixed to create a composite sample. Soils

were collected using aseptic techniques, placed on ice in the field and immediately carried back

to the laboratory and frozen at -20° C until further analysis. For each of these 192 samples, soil

gravimetric water content (GWC) was measured as mass lost from soil after drying at 105°C

overnight. Soil organic matter (SOM) and pH were estimated for all samples collected during

just one month (June 2016). SOM was measured by loss-on-ignition (LOI), and pH was

measured in 1:1 slurry of deionized water.

DNA Extraction and Polymerase Chain Reaction (PCR)

Total genomic DNA (gDNA) was extracted from approximately 0.5 g of homogenized

soil per sample using physical lysis, cetyltrimethylammonium Bromide (CTAB) and phenol:

chloroform extraction and overnight precipitation in PEG 6000 (DeAngelis et al. 2010). From

these gDNA extracts, the 16S rRNA gene was targeted for Illumina bacterial sequencing using

universal bacterial primers (515F/806R) following the Earth MicroBiome Project protocols

(Caporaso et al., 2012), with two exceptions: PCR was run for 25 cycles instead of 35, and 0.04% Bovine Serum Albumin (BSA) was added to each reaction. Gel electrophoresis was used to confirm amplification of each reaction. Triplicate technical replicates were run for each barcoded sample, amplicon amounts normalized and combined into one library and cleaned using a QIAquick Gel Extraction Kit. These samples were sequenced with a 2 x 150 paired-end read Ilumina MiSeq run (Caporaso et al. 2012, Zeglin et al. 2016).

The number of bacterial 16S rRNA gene copies and fungal ITS copies was estimated in all gDNA extracts using a Quantitative Polymerase Chain Reaction (qPCR) on a Bio-Rad CFX CONNECT system with Bio-Rad SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA). For 16S and ITS assays respectively, 1–10 ng template soil gDNA was used at 10 μL assay volume, 0.02% and 0.04% final BSA concentration, 100 nM and 500 nM final primer concentrations, using primer sequences and thermal cycler programs following Fierer et al. (2005). Standard curves for 16S assays were prepared using *E. coli* ATCC 25922 at $5x10^0$ - $5x10^{-6}$ ng ul^{-1} DNA concentrations (efficiency = 85%-114%, R^2 = 0.979 - 0.991). ITS standard curves were prepared using *Candidia albicans* SC5314 at $5x10^0$ - $5x10^{-6}$ ng ul^{-1} concentrations (efficiency = 81%-95%; R^2 =.988-.998). All assays included three technical replicates of gDNA per sample, as well as no-template controls and melting curves to confirm there was no amplification of non-target genes. Bacterial 16S rRNA gene and fungal ITS copy number was compared among field treatments on a g^{-1} dry soil basis, which was derived by normalizing the qPCR results by soil gDNA yield.

Bioinformatics and Statistical Analyses

The QIIME software package (Caporaso et al. 2010) was used to process raw Illumina sequence data. Sequences were quality filtered, joined and demultiplexed, and assigned to

operational taxonomic units (OTUs, representing 97% DNA sequence similarity) that were picked using the open-reference workflow. Taxonomy was assigned using the RDP classifier, OTU sequences were aligned to the GreenGenes v. 13.8 16S rRNA gene reference database, and non-aligned OTUs, chimeric sequences (identified using CHIMERASLAYER) and singletons and doubletons were removed prior to further analysis. The resulting dataset included 10,719,215 reads and 59,641 OTUs, and the dataset was trimmed to include an equal number of sequences per sample. This final rarefied dataset included 172 samples with 19,000 reads per sample, for a total 3,287,000 reads and 49,968 OTUs. The same steps were used to estimate average mean weighted 16S rRNA gene copy number per sample as a proxy for bacterial life history strategies, with the exception that OTUs were picked using the closed reference workflow and OTUs were aligned to the GreenGenes v. 13.5 reference database, then the closed reference OTU table was imported into the web-based Galaxy version of PICRUSt to derive weighted mean 16S rRNA gene copy number (Nemergut et al. 2015). Weighted copy numbers were calculated by multiplying copy number by relative abundance of each OTU, then summing these values within a sample. Sequences were uploaded to GenBank with accession number XXXXX.

For all soil microbiological response variables, the individual and interactive effects of burn history, fertilizer additions and sample date were evaluated with a repeated-measures Analysis of Variance (RM-ANOVA) test using mixed-effect models with block nested within replicate as a random variable, in the "lme" package in R Studio. All data were checked for normality and log-transformed when appropriate to meet assumptions of normality before statistical analysis (16S rRNA gene and ITS copy numbers, 16S:ITS gene copy ratios, Simpson evenness, dominance, and taxon relative abundance). A Bray-Curtis dissimilarity matrix from QIIME was used to create a nonmetric multidimensional scaling (NMDS) ordination using the

metaMDS function in the vegan package of R Studio, and the permuational analysis of variance (PERMANOVA) was determined by the "adonis" function of the vegan package in R Studio (Oksanen et al 2010). The strength of correlation between NMDS axes and taxon relative abundances was evaluated using Pearson's R coefficient and associated p-values (R Commander) (R Development Core Team 2011).

Prokaryotic taxon responses to N additions were calculated by $\log_{10}$-transforming the ratio of the average relative abundance in N-amended soils to the average relative abundance in non-amended soils for both burned and unburned treatments. For this analysis, high-order taxa were selected that were either predicted to be copiotrophic or oligotrophic, as indicated from previous studies (Ramirez et al. 2012, Leff et al. 2015). For each response, paired t-tests specified responses that were significantly greater or less than zero, and one-way ANOVA indicated significant differences between burned and unburned treatments for each taxon.

Results

Soil GWC ranged from 0.093 to 0.587 $g{\cdot}g^{-1}$ and showed an interactive effect of sampling date and burning treatment (Month*Burn: F = 4.54, P < 0.001): Soils in burned plots had greater GWC than unburned plots in November 2014, September 2015 and November 2015 (Figure S2.1). Soil organic matter (SOM) ranged from 0.05 to 0.11 $g{\cdot}g^{-1}$, and pH ranged from 5.50 to 6.80, but did not vary significantly between treatments (Table 2.1).

Bacterial 16S rRNA gene and fungal ITS copy numbers ranged from 5.69×10^6 to $5.86 \times 10^{10}\,g^{-1}$ dry soil and 2.8×10^6 to $5.50 \times 10^9\,g^{-1}$ dry soil for 16S rRNA gene and ITS copy number, respectively, and the ratio of ITS copies to 16S gene copies (F/B) ranged from 0.0016 to 4.1. These proxies for bacterial and fungal abundance, (or at a coarse taxonomic level, and henceforth, "population sizes") did not differ between the long-term management treatments, but

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all displayed seasonal variation (Figure 2.1). Bacterial 16S rRNA gene copy number was significantly greater during the summer (June - August) than in other months, showing an order of magnitude increase between May and June 2015, and an order of magnitude or greater decrease between August and September 2015 (Figure 2.1a). Fungal ITS copy number also decreased in the fall, being significantly lower in October 2015 than other sampling dates, but was otherwise relatively constant (Figure 2.1b). Variability in F/B was primarily driven by the strong seasonal patterns in bacterial 16S gene abundance, and correspondingly tended to be lower in summer, and was significantly higher in September 2015 than in other months (Figure 2.1c). Bacterial 16S rRNA gene copy numbers were correlated with monthly changes in soil and air temperature (t=7.66; p<0.0001), but not changes in soil water or precipitation, whereas fungal ITS copy numbers were correlated with monthly mean precipitation (t=2.33, p=0.0208) and GWC (t=3.68, p=0.0003), but not soil or air temperature. Despite the differences in total ribosomal gene abundance, there were no significant effects of management treatment or month on bacterial 16S rRNA weighted mean copy number (Table 2.1), thus no indication of changes in the dominant growth rate strategy within the prokaryotic community.

Bacterial 16S rRNA gene richness ranged from 2632 to 4863 observed OTUs per sample, evenness ranged from 0.0069 to 0.0562, and Shannon's diversity ranged from 8.35 to 10.40. While richness and diversity varied primarily by month, evenness data reflected a management treatment effect (Table 2.1, Figure S2.2). OTU richness was significantly lowest in all treatments in March 2015, and in the unburned fertilized treatment, richness remained significantly lower through April 2015 (Figure S2.2a). The temporal variation in bacterial diversity was primarily driven by low diversity in the unburned fertilized plots in April 2015 and an increase in diversity in all soils in August 2015 (Figure S2.2b). Bacterial 16S rRNA gene evenness did not vary

seasonally, instead it was higher on average in the burned, unfertilized plots than the unburned, unfertilized plots (Table 2.1, Figure S2.2c).

The best NMDS ordination model of prokaryotic community distance among all samples had 2 axes and a stress of 0.127 (Figure 2.2). Soil microbial communities from fertilized plots had significantly higher NMDS Axis 1 scores than from unfertilized plots, but there were no significant differences between burned and unburned treatments, while NMDS Axis 2 scores showed an interaction between burning and N additions, with bacterial communities from fertilized unburned soils grouping separately from the other treatments (Table 2.1). PERMANOVA results confirmed that a significant amount (11%) of variability in community composition was explained by the direct and interactive effects of long-term burning and N additions (Table 2.3), while notably, there was no significant seasonal variability in community composition (Figure S2.3a, b).

NMDS Axis 1 scores were most strongly correlated with differences in the relative abundance of sequences affiliated with (sub-)Phyla Verrucomicrobia, Acidobacteria, Nitrospirae, and δ-Proteobacteria, and NMDS Axis 2 scores were best correlated with differences in α-Proteobacteria, Chloroflexi, γ-Proteobacteria, Crenarchaeota, and Planctomycetes relative sequence abundance (Table S2.1). Notably, genus DA101 of phylum Verrucomicrobia, class Spartobacteria, composed 20% of the dataset on average, in parallel with other findings from tallgrass prairie soils that report high abundances of DA101 (Fierer et al. 2013). Relative abundance of sequences affiliated with "Other Verrucomicrobia" (excluding genus DA101) varied significantly with N addition, and were typically higher in the unfertilized treatments (Figure S2.4f), while DA101 relative abundance was highest in burned, fertilized soils (Figure S2.4b). Other major (sub-)Phyla relative abundances that varied significantly with nitrogen

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addition treatment included α-Proteobacteria, δ-Proteobacteria, Gemmatimonadetes, Verrucomicrobia (excluding genus DA101), and Crenarchaeota (Figure S2.4). While no significant treatment differences were observed for β-Proteobacteria, a dominant β-Proteobacterial order, Burkholderiales, did vary significantly with fertilization treatment (Figure S2.4l). Only γ-Proteobacteria varied significantly with burn treatment, and both γ-Proteobacteria and genus DA101 showed an interactive response to nitrogen additions and burn treatment (Figure S2.4).

While there was less overall seasonal variation in prokaryotic community composition, some (sub-)Phyla relative abundances did vary significantly with sample time. Planctomycetes relative abundance generally increased from May through December 2015. δ-Proteobacteria and Nitrospirae relative abundance was lower in spring (March/April), Nitrospirae and δ-Proteobacteria were more abundant in July, δ-Proteobacteria were most abundant in October, and Gemmatimonadetes were most abundant in April. While Crenarchaeota varied significantly by month, no post-hoc differences were observed. While subphylum β-Proteobacteria did not vary as a whole by month (F= 1.863, P= 0.0518), the relative abundance of Order Burkholderiales was significantly temporally variable, with a significant month*burn interaction in April, one week post-fire (Figure S2.4l).

Taxon response ratios to N additions indicated significant positive ratios for all putative copiotrophic (sub-)Phyla, except Actinobacteria and γ-Proteobacteria, which had negative and no response, respectively, in burned soils (Figure 2.3). All copiotrophic taxa had significantly stronger responses to N additions in unburned than burned soils (t-test, P < 0.05). Of the putative oligotrophic taxa, only δ-Proteobacteria and Planctomycetes had consistently negative responses to N additions in both burned and unburned soils, and only Verrucomicrobia and δ-

Proteobacteria significantly differed between burned and unburned soils, in that Verrucomicrobia had a significant positive response to N additions in burned soils (Figure 2.3).

Discussion

It is well known that microbial turnover can occur on both relatively long and short time scales (Thormann et al. 2003, Schmidt et al. 2007, Dornelas et al. 2013, Shade et al. 2013). During this study, we found that microbial communities have the potential to change on event-based, seasonal, and historical time-scales, although the magnitude and response at each temporal scale varies depending on populations of interest or the metric used to describe changes in microbial turnover. In our study system, native tallgrass prairie, long-term changes in disturbance regime and N addition had a greater propensity to change soil bacterial community composition than seasonal variability. Seasonality had a greater impact on bacterial population size, and event-driven responses to fire and N addition were subtle and not necessarily predictive of the long-term soil microbial community turnover.

Because microbial communities have the potential to respond quickly to fertilization pulses (Woods et al. 1987, Stark et al. 2007, Kuzyakov and Xu 2013) and to the direct or indirect effects of burning events (Dooley and Treseder 2011), we expected to observe pulses in microbial population sizes or changes in the relative abundance of certain taxa immediately following burning in April and fertilization June, respectively. Surprisingly, responses to these events at both the population and community levels were minimal, except for two responsive populations that displayed transient dynamics in response to management events (Figure S4). Order Burkholderiales relative abundance increased in only burned treatments immediately following the burn, indicating sensitivity to prescribed burning. The dominant fire-responsive Burkholderiales OTUs in our dataset had a taxonomic affiliation at the genus level with the soil-

associated, cultured representative of this order *Janthinobacterium lividum* (genus

Janthinobacterium changed in burned soils between March and April from $0.099 \pm 0.02\%$ to 1.05

$\pm 0.2\%$ relative abundance) (Pantanella et al. 2007). We speculate that this transient response

could be associated with indirect effects of fire, such as higher temperature or ultraviolet

radiation on the bare soil, or changes in phosphorous and/or cation availability, rather than to the

direct heat of the fire event. Only Crenarchaeota appeared to respond to the fertilization event,

particularly in the unburned, N amended soils (changed from $0.015 \pm 0.002\%$ to $0.024 \pm 0.004\%$

between May and June). Crenarchaeota are generally more abundant in fertilized than

unfertilized soils (Leff et al. 2015), and are a widespread and often dominant ammonia oxidizer

group (Leininger et al. 2006, Prosser and Nicol 2008, Taylor et al. 2012), signifying that their

response to the fertilizer pulse may be associated with transformations of available ammonium to

nitrate. The Crenarchaeota were higher in relative abundance in long-term fertilized soils on

average, in addition to being sensitive to the fertilization event. In contrast, the Burkholderiales

order was responsive to the fire event but not indicative of a long-term change in burning regime.

This is most likely due to the nature of the environmental changes that each factor affects at

different time scales: N retained in the soil post-fertilization can remain available throughout the

year, but increased light following fire is a transient phenomenon, as plant growth closes the

canopy in a matter of weeks.

There were many more changes in soil microbial community composition in response to

long-term management regime than to episodic management events. Overall, the dampened

short-term responsiveness of bacterial community and microbial population dynamics may be

explained by the lack of direct heat penetration, and therefore mortality, from grassland fire, or

the persistence of elevated available soil N associated with long-term fertilizer additions.

Because some microorganisms are known to have generation times shorter or longer (ranging from hours to days to many weeks) than our one-week post-event sampling window, there may have been dynamics of certain bacterial taxa that were not detected. Direct fertilization may have more influence on soil nutrient status and microbial sensitivity on daily time scales, whereas fire may have more indirect impacts over multi-week time scales (post-fire soil exposure increasing soil temperature, water loss, or increased light).

Although we did not observe evidence for pulses of growth or death in total soil bacteria or fungi following fire or fertilization events, bacterial population size dynamics were notably variable by season (Figure 2.1). Our estimates of bacterial and fungal population size are in a consistent range as compared to other studies addressing growing-season microbial population sizes (e.g. Lauber et al. 2008, Fierer et al. 2009, Barnard et al. 2013). However, we know of no studies that estimated bacterial and fungal population sizes during the non-growing season using qPCR, and few that have examined F:B biomass in winter using other methods (Bardgett et al. 1997, Schadt et al. 2003). Based on the patterns in our data, we suggest that high F:B ratios may be typical of local conditions that do not favor bacterial growth, such as lack of active rhizodeposition. Among different biomes, high F:B ratios are generally found in forest soils, under conditions including higher ectomycorrhizal colonization of bulk soils and a greater presumed microbial dependence on less labile organic substrates due to lower soil C:N ratios (Lauber et al. 2008, Fierer et al. 2009, Strickland and Rousk 2010). In this way, the high F:B ratio in our study soils during the non-growing season supports our prediction that bulk soil microbial community seasonal turnover would reflect greater reliance on soil organic matter during this time. However, more studies investigating over-winter microbial activity and

diversity are needed to better understand the dynamics of soil microbial substrate use preferences.

The mechanism for the sharp increase in prokaryotic population size in summer may be plant rhizodeposition providing labile C resources (Kuzyakov and Xu 2013), as bacterial population size increases were correlated with changes in monthly mean air temperature, and thus roughly correlated with the timing of onset of the growing season (Garcia and Rice 1994, Dell et al. 2005) and decreases roughly correlated with the timing of plant resources shifting in major allocation to aboveground reproductive structures, followed by senescence. If each soil bacterial cell contains 100 fg C (Whitman et al. 1998) and 2.325 copies of the 16S rRNA gene (Table 2.1), the average summer increase in 16S rRNA gene copies of 1.46×10^{10} is roughly equivalent to 626 μg C g^{-1} dry soil, which would comprise a substantial proportion (50-100%) of the estimated total soil microbial biomass C in these soils (Ajwa et al. 1999, Zeglin et al. 2013). There is a considerable amount of uncertainty associated with this estimate, both in the amount of cellular C associated with each 16S rRNA gene based on within and among-taxon variability in cell size, morphology and intra- and extracellular C storage (Paul 2014), and in the amount of cellular C extracted from the soil during chloroform incubation, fumigation and extraction assays (Jenkinson et al. 2004). Still, this observation suggests that bacterial growth and death associated with availability and cessation of plant rhizodeposits over the growing season can have a large impact on the soil C cycle.

An alternative explanation of the seasonal variation in microbial population sizes could be climatic variability or soil properties, because the tallgrass prairie has distinct seasonal changes in temperature and water availability (Knapp et al. 1997) and soil factors are well-known to drive microbial community processes (Fierer et al. 2007, Regan et al. 2017). Soil GWC

was assessed for individual treatments; unfortunately, soil temperature was not, however local monthly air and soil temperature was used as a proxy for monthly changes in temperature. Neither soil water nor precipitation was correlated with 16S rRNA gene copy numbers: soil GWC varied relatively little, particularly over the growing season, during this wetter-than-average year (Figure S2.1), in contrast to the sharper shifts in 16S rRNA gene abundance in June-August. However, ITS copy numbers were correlated with annual shifts in precipitation and GWC, perhaps highlighting differences in the spatial distribution of bacteria and fungi in the soil habitat. A reason that fungi may be sensitive to changes in soil water is their location on surfaces of soil aggregates, whereas smaller bacterial cells are more likely found in smaller, more consistently wet soil pore spaces (Frey et al. 1999, Strickland and Rousk 2010). On the other hand, fungi are generally expected to be resistant to low soil water (Schimel et al. 2007), so indirect or other mechanisms may be at play. We were also surprised that the long-term fertilization and fire treatments did not affect estimated population sizes of soil bacteria or fungi. This result is in contrast to many studies in which increased available soil N has reduced microbial biomass (Treseder 2008). Since QPCR population estimates are related to the amount of DNA, or number of genomes, in microbial cells, it is possible that N-driven biomass reductions detected with PLFA or chloroform fumigation methods may be due more to changes in cell size than cell number. Taken together, these results suggest that the availability of labile C resources could be a stronger driver of bacterial growth, and thus soil F:B cell ratio, than the monthly changes in air and soil temperature and soil water content or the differences in plant community composition and soil N availability between long-term land management treatments.

We also expected to observe seasonal shifts in prokaryotic community composition, particularly in relation to the striking seasonal fluctuations in bacterial population sizes.

However, prokaryotic community composition was not significantly temporally variable (Figure S2.3), and the only temporal pattern in diversity was an increase in bacterial richness between March and September (Figure S2.2). While this change in richness could be related to increased growth of taxa that were rare before the growing season, the pattern was quite subtle, and occurred with different timing, relative to the growing-season 16S rRNA gene copy number increase, suggesting that all detected co-occurring bacterial populations increased and decreased in size nearly synchronously in June and September. While these results do not fit our original predictions regarding temporal turnover, they do corroborate patterns of temporal variability in bacterial community turnover in other managed grassland ecosystems. In cropped soils, where the growing season was marked by seeding and harvesting corn, the bacterial community showed a high degree of monthly turnover relative to a temporally stable bacterial community in adjacent successional grassland soil (Lauber et al. 2013). This suggests that in addition to rhizodeposition influences on bacterial growth, the year-round presence of plant cover could dampen variation in bacterial community turnover due to some alleviation of microbial C limitation via leaching and depolymerization of labile C from fresh litter. Also, compared to the predictable seasonal patterns in temperature and/or soil water, as well as soil microbial community composition, in alpine or Mediterranean ecosystems (Schmidt et al. 2007, Cruz-Martinez et al. 2009), our study site experiences less predictable drought/storm cycles during the summer and freeze/thaw cycles during the winter, with no stable snowpack. In this temperate grassland, bacterial communities may not show significant seasonal turnover because selective pressures from freezing and thawing are akin to pressures from drought, resulting in similar constraints on community composition through the growing season and over-winter (Schimel et al. 2007).

Another, non-exclusive, explanation for the strong seasonal variation in bacterial population size coupled with a lack of seasonal community turnover may be a shift of all populations comprising the bacterial community to a "dormant" state during the non-summer months (Lennon and Jones 2011). We do not have data to assess this hypothesis, which might be addressed using microbial activity or rRNA analyses. We do note that our DNA-based results are unlikely to be strongly influenced by the presence of microbial extracellular DNA, which could bias measurements of microbial diversity, because previous evidence suggests that extracellular DNA is relatively insignificant in this soil type (<1% prokaryotic DNA, <8% fungal DNA), due to low soil sorption capacity for these molecules (Ciarini et al. 2016). In addition, the sharp decrease in 16S rRNA gene copies between August and September is evidence for degradation of prokaryotic DNA molecules in the soil. Most microbial taxa in these soils appear to be resistant to the challenges presented by seasonally dynamic environmental conditions, which include substrate limitation, variable temperatures, drying/rewetting and freezing/thawing.

Instead of event-based or seasonal variability, soil microbial communities differed most strongly with historical long-term management treatment (Figure 2.2). Specifically, N fertilization explained the most variability in prokaryotic community composition, and there were important interacting effects of fire history and fertilization. Individual high-level taxonomic responses to long-term N manipulations supported predictions based on previous work from other studies (Ramirez et al. 2012, Leff et al. 2015), in that putatively copiotrophic taxa (Actinobacteria, Crenarchaeota, α-Proteobacteria and γ-Proteobacteria) generally had positive responses to N additions and were more abundant in unburned soils where N is more available, and putatively oligotrophic taxa (Acidobacteria, δ-proteobacteria, Planctomycetes and Verrucomicrobia) generally had negative responses to N additions and were more abundant in

burned soils where N is less available (Figure 2.3). Overall, our data support the copiotrophic hypothesis, which states that community shifts associated with N additions are a function of increased competitive dominance of fast-growing taxa (Ramirez et al. 2012). Further, our results suggest that while the phylum-level affiliation of N-responsive taxa may be consistent, the magnitude of response, which is highly variable among systems (Leff et al. 2015), may be related to the baseline level of soil N availability. In annually burned soils, only half of the phyla responded significantly to long-term N-addition as predicted, and two phyla significantly responded contrary to predictions (Actinobacteria were less abundant in fertilized soils and Verrucomicrobia were more abundant in fertilized soils); while in unburned soils, all phyla responded as predicted, most (75%) in a significant manner, and the expected changes in relative abundance were generally greater in magnitude in unburned soils versus burned soils. In grasslands, the lack of fire increases the amount of organic N returned to the soil, and subsequently N availability is higher due to greater mineralization of plant litter N (Blair 1997, Dell and Rice 2004, Hobbie 2015). Our data suggest that the sensitivity of soil microbial responses to N fertilization may be greater in soils with already-elevated N availability due to changes in land management.

Overall, these results highlight the need to consider the interactions between the factors controlling soil N and C availability, particularly over the decadal time scales at which the soil fertility outcomes of land management decisions accumulate, to better understand and predict microbial responses to environmental change. The temporal resolution of any study affects data interpretation, since microbial communities and populations have the propensity to respond on multiple time scales. The timing of changes in soil microbial populations and communities is critical to plant nutrition and nutrient cycling in ecosystems, because microbial turnover releases

nutrients that support plant growth, and soil microbial communities are responsible for retention of nutrients during the non-growing season (Schimel and Bennet 2004, Schmidt et al. 2007, Shade et al. 2013,). Our data suggest that there are strong plant phenological effects on soil microbial populations, and that microbial community sensitivity to global change, such as N deposition, can be mediated by management, such as disturbance from fire. Because increased biologically available N from deposition events may have undesirable effects on plant or soil microbial diversity, decrease root and microbial biomass, or alter plant-soil feedbacks, fire can be considered a potential mechanism for mitigating these changes (Bardgett et al.1999, Baer et al. 2003, Johnson and Matchett 2001, Coolon et al. 2013, van der Putten et al. 2013). This study aids in elucidating microbial responses to global change in grasslands by adding temporal resolution to the current knowledge of microbial community turnover.

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Tables and Figures

Table 2.1: Soil environmental variables.

Burn Fertilizer B N	SOM -	GWC -	pH -
BC Burn -N	0.067 (0.006)	0.270 0.009	6.44 0.15
BN Burn +N	0.065 (<0.001)	0.267 0.010	6.35 0.15
UBC Unburn -N	0.067 (0.005)	0.305 0.039	6.31 0.18
UBN Unburn +N	0.0862 (0.008)	0.288 0.012	6.00 0.28

Means (SE) of measured environmental variables. Results of RM-ANOVA, with notation for all significant ($p<0.05$) effects burn treatment (B), and fertilization treatment (N). Abbreviations: SOM, soil organic matter (mass loss on ignition, $g\ g^{-1}$), GWC, Gravimetric water content ($g\ g^{-1}$).

Table 2.2: Prokaryotic community and microbial population metrics associated with long-term fire and fertilization treatments.

Month M / Burn B / Fertilizer N	Observed OTUs	Shannon Diversity	Evenness	NMDS1	NMDS2	16S Weighted Mean Copy #	log 16S Copy #	log ITS Copy #	F:B
	M, MxB	M	BxN	N	M, BxN	-	M	M	M
BC Burn -N	3610 (29)	9.35 (0.03)	0.019[b] (0.001)	0.11[b] (0.02)	-0.06[a] (0.01)	2.33 (0.004)	8.92 (0.06)	8.19 (0.07)	0.55 (0.12)
BN Burn +N	3682 (28)	9.29 (0.03)	0.015[ab] (<.001)	-0.11[a] (0.02)	-0.12[a] (0.02)	2.32 (0.005)	9.00 (0.06)	8.23 (0.07)	0.56 (0.13)
UBC Unburn -N	3589 (23)	9.31 (0.03)	0.017[a] (<0.001)	0.12[b] (0.02)	0.02[b] (0.02)	2.32 (0.006)	9.02 (0.05)	8.17 (0.05)	0.58 (0.17)
UBN Unburn +N	3482 (34)	9.31 (0.03	0.021[ab] (0.001)	-0.11[a] (0.04)	0.15[c] (0.03)	2.33 (0.006)	8.82 (0.06)	8.11 (0.05)	0.50 (0.10)

Means (SE) of measured variables for microbial community composition and qPCR analyses.
Results of RM-ANOVA, with all significant (p<0.05) effects of sample date (M), burn treatment (B), and fertilization treatment (N) noted for each variable. Subscript letters indicate significant treatment differences by Bonnferoni post-hoc tests. Observed OTUs varied significantly (p<0.05) by burn treatment, but no post hoc differences were observed.

Table 2.3: Correlations between16S rRNA gene OTU relative abundance of dominant taxa (p value and Pearson's R) with nonmetric multidimensional scaling axes one and two.

Taxa correlation with 16S NMDS Axes	NMDS 1 (P, Pearson's R)	NMDS 2 (P, Pearson's R)
Other Verrucomicrobia	**<.0001, 0.68**	1.0000, 0.12
∂-Proteobacteria	**<.0001 0.55**	**0.0009, 0.34**
Acidobacteria	**<.0001, 0.48**	**0.0425, 0.27**
Nitrospirae	**<.0001, 0.45**	1.0000, 0.16
Bacteroidetes	**0.0031, 0.36**	1.0000, 0.004
Chloroflexi	**0.0115, 0.29**	**<.0001, 0.42**
α-Proteobacteria	**0.0002, -0.36**	**<.0001, -0.63**
DA101	**<.0001, -0.41**	**<.0001, 0.39**
γ-Proteobacteria	1.0000, -0.11	**<.0001, -0.43**
Crenarchaeota	0.3616, 0.22	**<.0001, -0.42**
Planctomycetes	1.0000, 0.16	**<.0001, 0.41**
ß-Proteobacteria	1.0000, 0.08	**0.0382, 0.27**
Gemmatimonadetes	1.0000, -0.15	**0.0392, -0.27**
Actinobacteria	0.5592 -0.21	0.0001, -0.36
Other	1.0000, 0.06	1.0000, 0.12
Other Proteobacteria	1.0000, -0.04	1.0000, 0.03
Firmicutes	0.9782, 0.19	1.0000, -0.01

Bold values indicate a significant correlation (p<0.05).

Table 2.4: Permutational Analysis of Variance (PERMANOVA) results for 16S rRNA gene community composition data.

	Sum of Squares	F	R^2	P
Month	1.1168	1.1164	0.06692	0.099
Nadd	**0.8138**	**8.9481**	**0.04876**	**0.001**
Burn	**0.6367**	**7.0010**	**0.03815**	**0.001**
Month*Nadd	0.7996	0.7993	0.04791	0.998
Month*Burn	0.8867	0.8864	0.05313	0.916
Nadd*Burn	**0.3935**	**4.3270**	**0.02358**	**0.001**
Month*Nadd*Burn	0.7654	0.7650	0.04586	0.999
Residuals	0.09095	-	0.67570	-

Bolded factors explained significant ($P < 0.05$) levels of variability.

Figure 2.1: Quantitative Polymerase Chain Reaction (qPCR) data for (a) log 16S rRNA gene copy numbers g⁻¹ dry soil, (b) log ITS copy number g⁻¹ dry soil and (c) fungal:bacterial (F/B) ratios for all soils collected between Nov 2014 – Dec 2015 (BC = Burned control, BN = Burned fertilized, UBC = Unburned control, UBN = Unburned fertilized). Repeated measures ANOVA post-hoc results (bold letters) indicate significant (p<0.05) changes by month; there were no significant treatment differences. Bars indicate standard error of the mean.
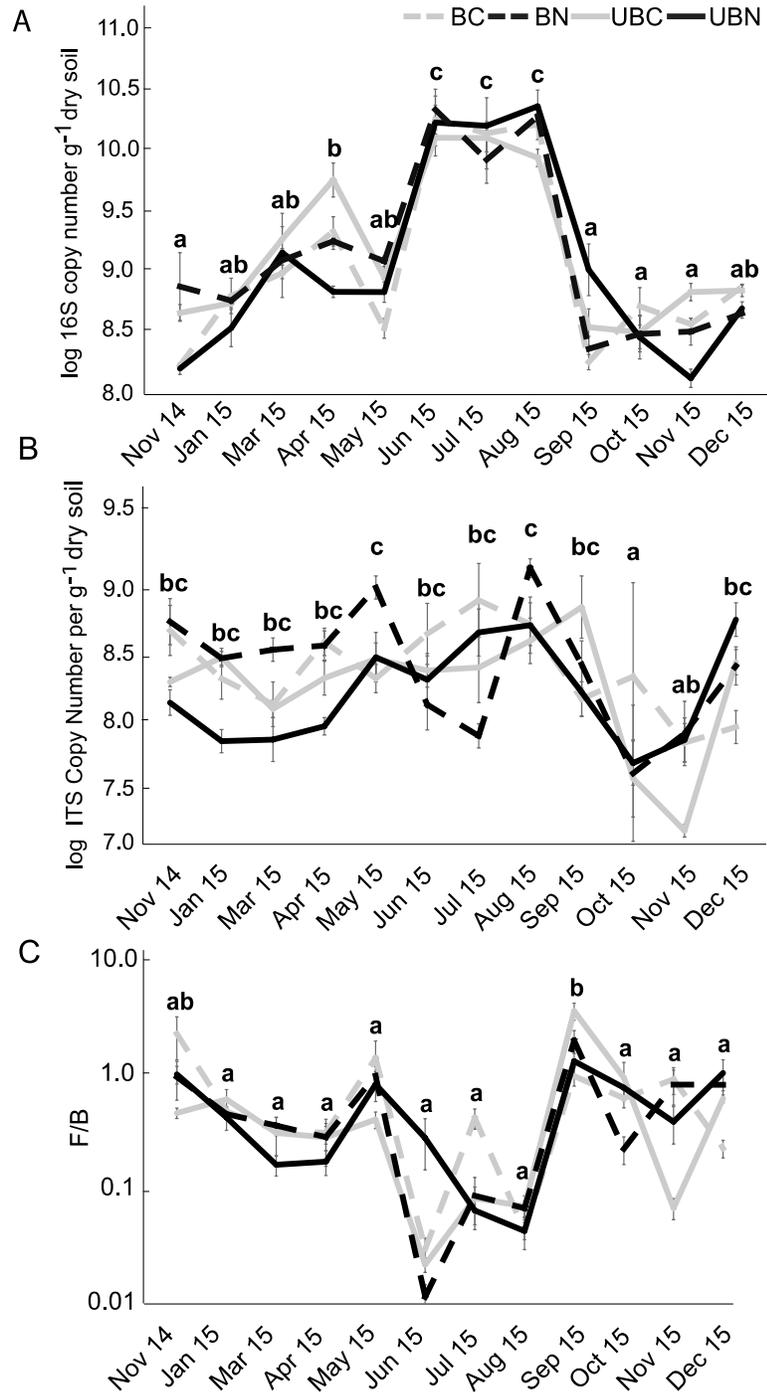
Figure 2.2: Nonmetric multidimensional scaling (NMDS) ordination model axes one and two of Bray-Curtis dissimilarity in soil 16S rRNA gene OTU composition, comparing four treatments (BC = Burned control, BN = Burned fertilized, UBC = Unburned control, UBN = Unburned fertilized) and including all samples collected between Nov. 2014 – Dec. 2015. Each line endpoint represents where a sample falls in the ordination space, and the connecting centroid box notes the long-term treatment associated with the sample.
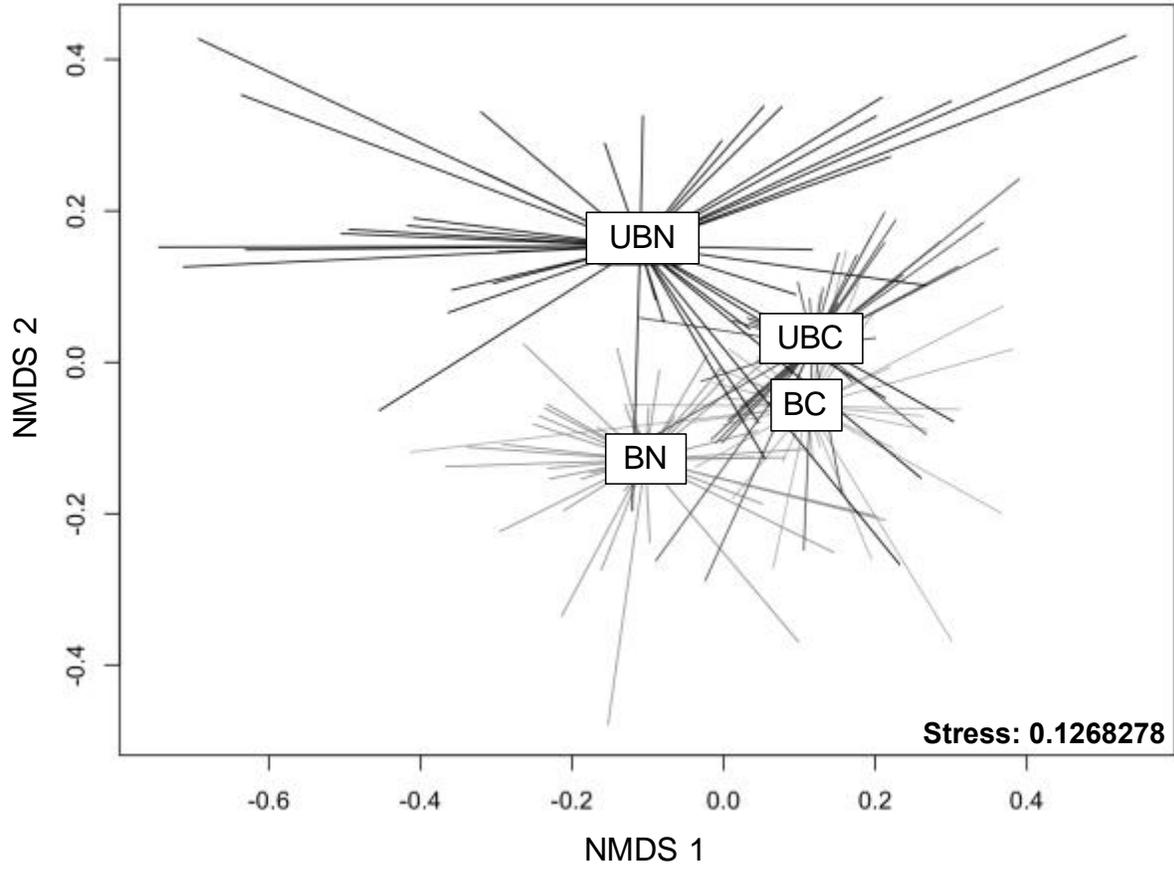
Figure 2.3: Log response ratio (response to long-term N additions, averaging across all time points sampled) of dominant prokaryotic taxa in burned (light grey) and unburned (dark grey) soils. Taxa are sorted into those predicted to be copiotrophic (left of vertical dashed line) and oligotrophic (right of vertical dashed line) based on previous studies (Ramirez et al. 2012; Leff et al. 2015). Boxes represent the quartile values for each taxon, and dots indicate outliers. Asterisks represent values significantly (p<0.05) greater or less than zero, and a bar above a taxon response represents a significant difference between burned and unburned treatments.
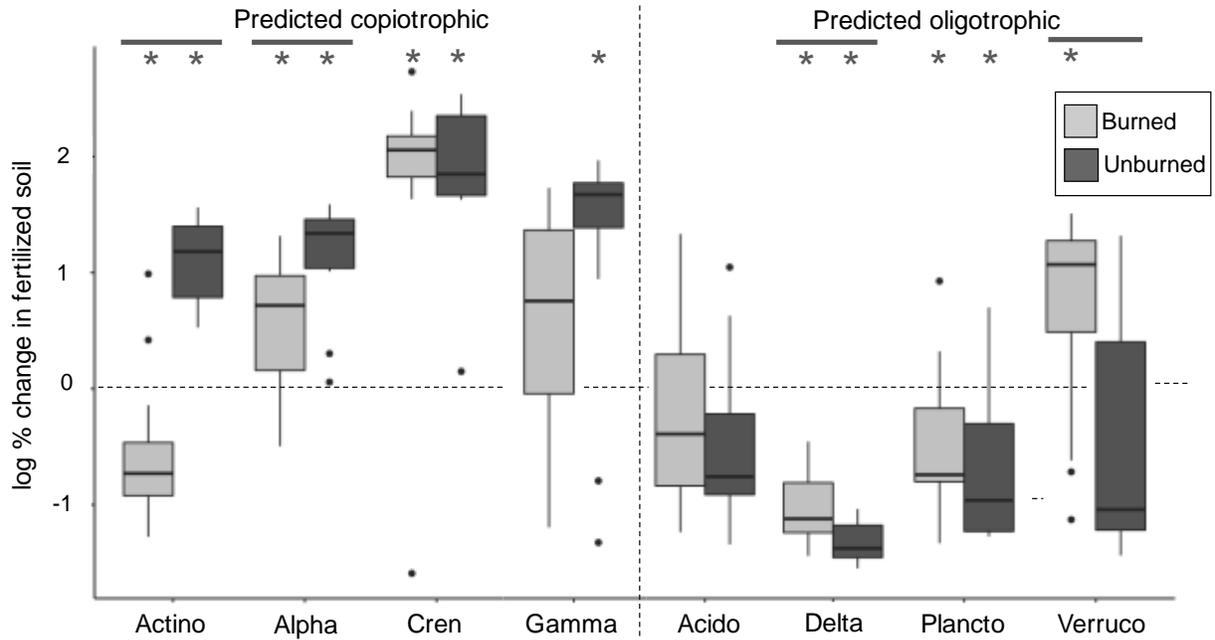
Figure S 2.1: Average soil gravimetric water content (GWC) measured in each treatment (BC = Burned control, BN = Burned fertilized, UBC = Unburned control, UBN = Unburned fertilized). Letters denote significant differences between months (Bonnferoni post-hoc, p < 0.05) and asterisks indicate significant treatment differences (RM-ANOVA indicated a significant (p<0.05) interactive effect of sample date and burn treatment (F=4.541 p=<.0001) on soil GWC). Bars indicate standard error of the mean.
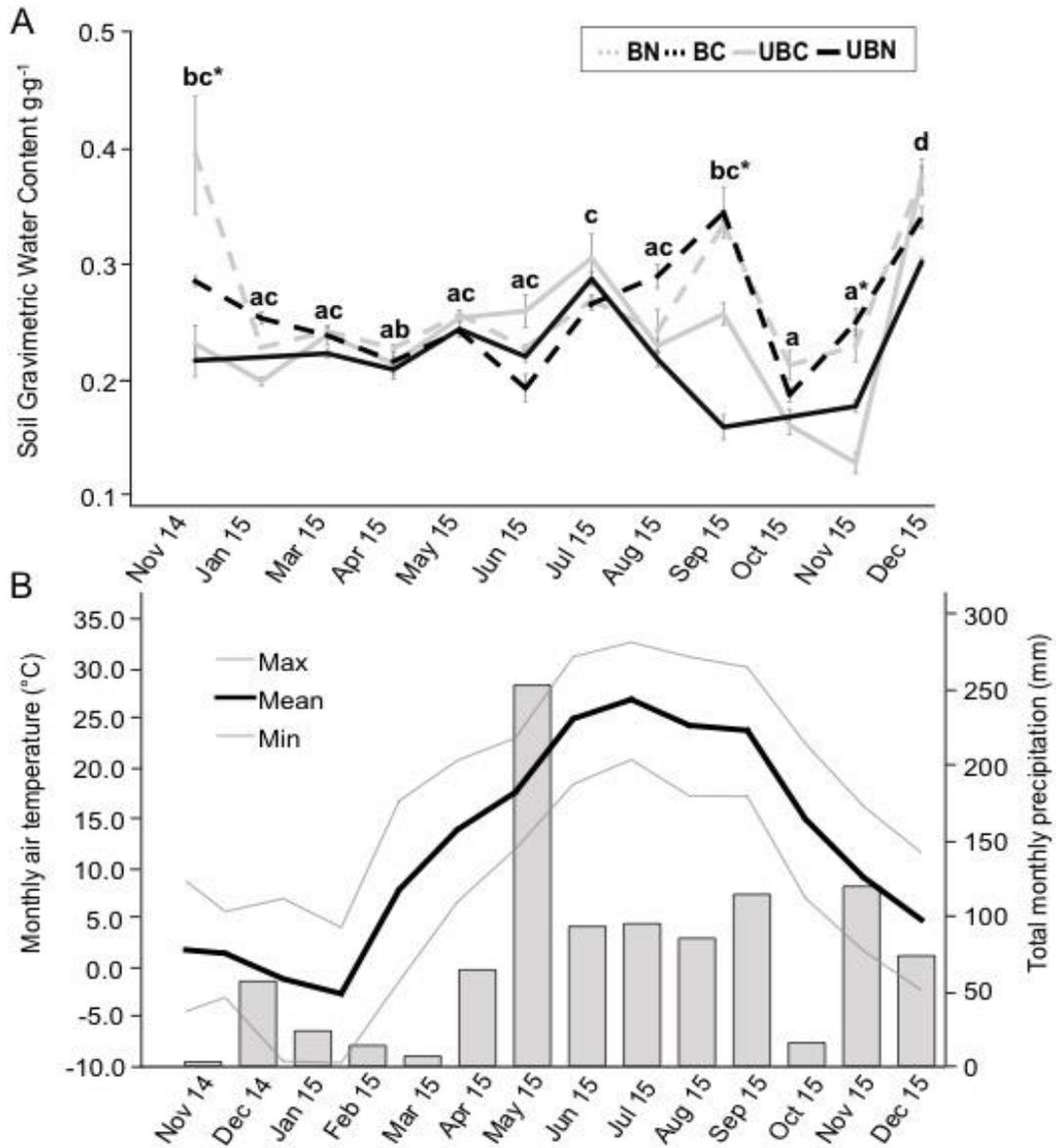
Figure S 2.2: 16S rRNA gene (a) taxonomic richness (observed OTUs), (b) Shannon diversity, and (c) Simpson's evenness in all four treatments (BC = Burned control, BN = Burned fertilized, UBC = Unburned control, UBN = Unburned fertilized) from samples collected between Nov. 2014 – Dec. 2015. Bonferonni post-hoc differences (P < 0.05) are indicated by subscript letters (month) and asterisks in panel C indicate differences between burned and unburned soils. Bars indicate standard error of the mean.
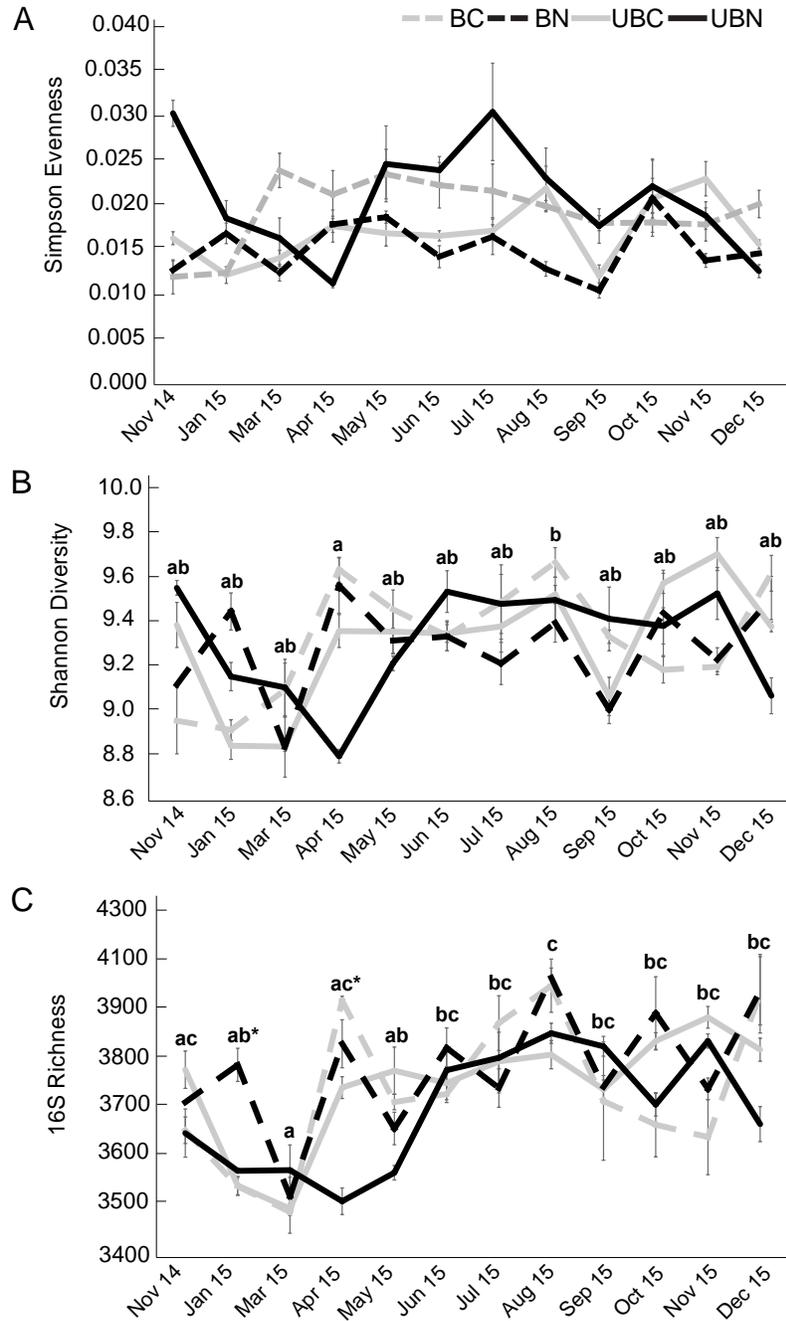
Figure S 2.3: Nonmetric multidimensional scaling axis (a) one and (b) two in each treatment (BC = Burned control, BN = Burned fertilized, UBC = Unburned control, UBN = Unburned fertilized) from Nov 2014 – Dec 2015. Repeated measures ANOVA indicated significant (p < 0.05) changes with (a) fertilization treatment ([F, P]: 8.35, 0.0136) in Axis 1, and (b) an interaction of burn and fertilizer treatment ([F, P] 1.99, 0.0352) in Axis 2, as well as an interaction of sample date and burn treatment ([F, P] 11.30, 0.0152) with no post hoc differences. Bars indicate standard error of the mean.
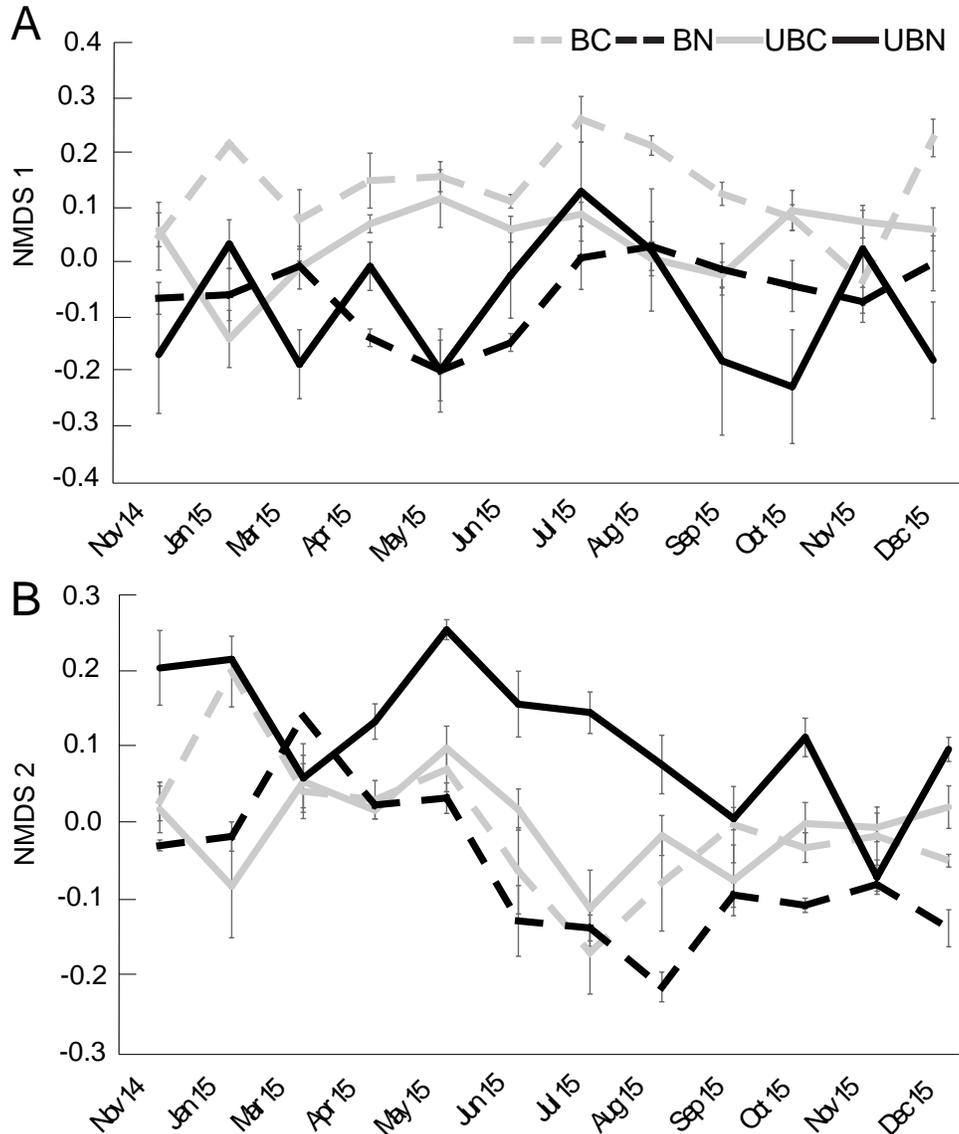
Figure S 2.4: Treatment and temporal changes in taxon relative abundances (BC = Burned control, BN = Burned fertilized, UBC = Unburned control, UBN = Unburned fertilized) in soil sampled between Nov 2014 – Dec 2015 for (a) genus DA101, (b) sub-Phylum δ-Proteobacteria, (c) sub-phylum α-Proteobacteria, (d) Phylum Planctomycetes, (e) Phylum Verrucomicrobia excluding genus DA101, (f) Phylum Gemmatimonadetes, (g) Phylum Nitrospirae, (h) Phylum Crenarchaeota, and (i) Order Burkholderiales (Betaproteobacteria). Significant (p<0.05) sources of variation (burn treatment, fertilizer treatment, and sample date), F, and p-values are indicated in bold on each chart. Subscript letters and asterisks indicate significant differences by Bonnferoni post-hoc tests. Bars indicate standard error of the mean.
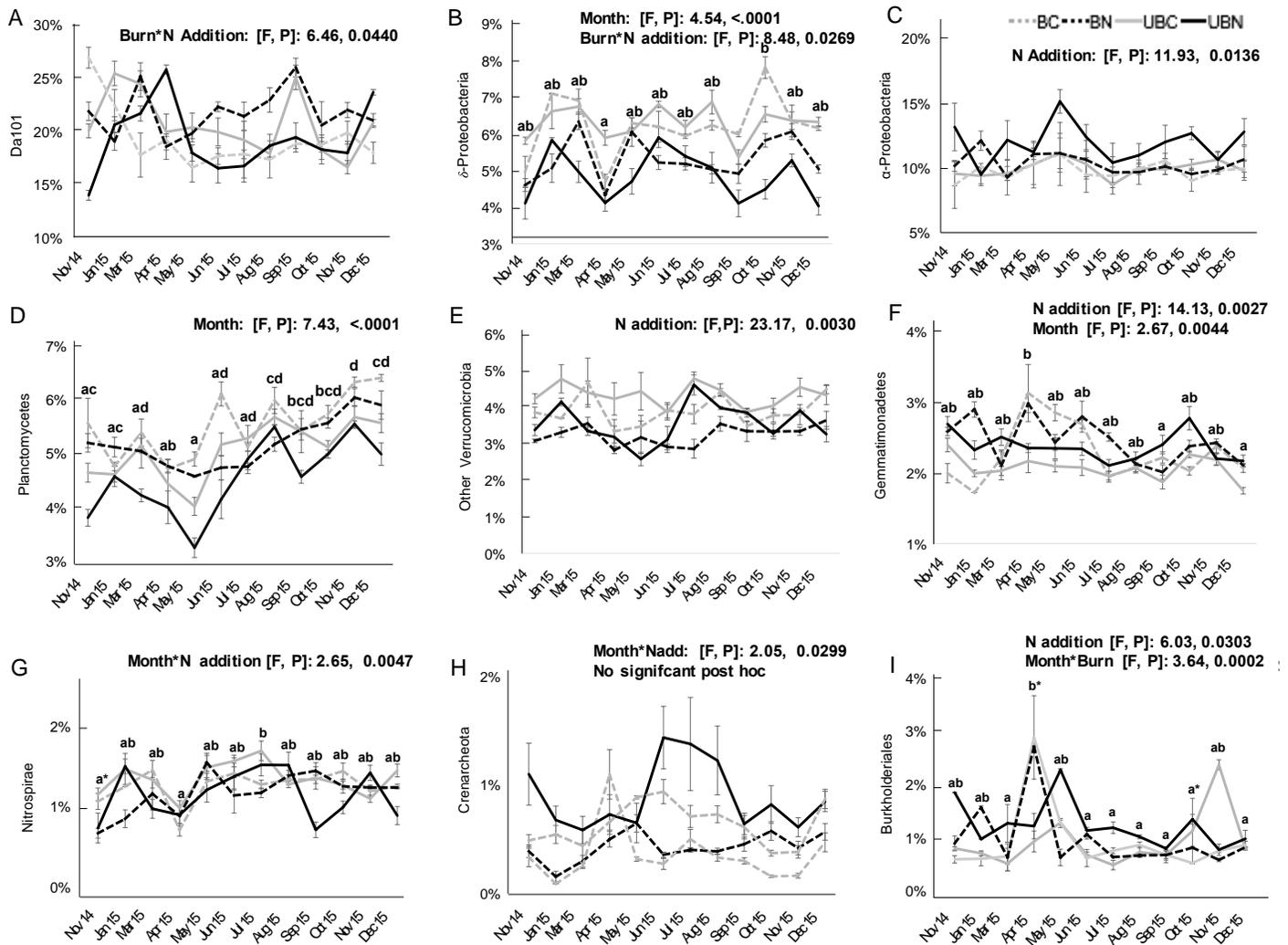
Chapter 3 - Soil fungal response to long-term grassland management changes is mediated by both plant and environmental heterogeneity

Abstract

In tallgrass prairie, many soil fungi and plant communities are linked through direct interactions, and fungal community seasonal turnover may be related to plant composition and phenology, litter composition, or litter availability. Nitrogen (N) availability is an ecological driver of plant community composition and productivity in grasslands, where fire suppression has promoted growth of woody plants and N availability through litter accumulation, and greater anthropogenically driven N deposition (N addition) has increased aboveground production. Surface soils (0-15 cm) were sampled monthly for one year (November 2014 – December 2015) from a long-term field multifactorial experimental manipulation of fire management (burned/unburned) and N enrichment (fertilized/unfertilized) to evaluate fungal community dynamics. Treatment-level plant community composition and soil environmental factors were also measured at a single time point. Fungal taxa were parsed into ecologically meaningful functional guilds based on putative carbon acquisition strategy using the FUNGuild database. We predicted that fungal communities would change between growing and non-growing seasons, long-term management would alter functional guild relative abundance, and differences in plant and fungal communities would be correlated. Surprisingly, fungal community composition was seasonally stable, although obligate mycorrhizae increased during summer. In contrast, long-term management altered fungal communities and plant communities. During July when soil and vegetation variables were measured, burning, fertilization and their interaction explained 28%

and 50.9% of the variation in fungal and plant composition, respectively. Additionally, fungal and plant compositional differences, as well as fungal composition and environmental variation, were correlated across all long-term management treatments, unlike soil prokaryotic community composition. These results highlight potentially important changes in nutritional links between soil fungi and plant communities in response to long-term management and global change and their potential importance for tallgrass prairie management.

Introduction

Soil fungi are critical for grassland ecosystems because of their contributions to grassland soil fertility and plant nutrition (Reynolds et al. 2003, van der Heijden et al. 2008, Schnitzer et al. 2011, Hodge et al. 2013). Soil fungi are phylogenetically and functionally diverse in grasslands (Blackwell 2011, van der Putten et al. 2013), including regulating plant community structure and nutrition through symbiotic relationships (van der Heijden 1998, Hartnett and Wilson 1999), facilitating the release of plant available nutrients through decomposition (Deacon et al. 2006), modifying plant communities through plant-pathogen interactions and negative feedback loops (Bever et al. 2015), as well-as contributing to soil aggregate stability and carbon (C) retention (Six et al. 2006, Wilson et al. 2009).

While soil fungi may affect plant community composition, plants are also the source of fungal nutrition, either directly through symbiotic interactions or indirectly through litter that feeds fungal saprotrophs. Therefore, drivers of plant community structure, such as natural and anthropogenic disturbance regimes, can also structure fungal communities. One such driver in tallgrass prairie ecosystems is fire, which historically maintained herbaceous plant communities (Hulbert 1969) and contributed to low available soil N (Hobbs et al. 1991, Blair 1997). Recently,

anthropogenic suppression of fire across the Great Plains has caused encroachment of native woody shrubs and trees, such as *Cornus drummondii* (roughleaf dogwood), and *Juniperus virginiana* (eastern red cedar) (Ratajczak et al. 2013), and increased available soil N (Seastedt et al. 1991, Ojima et al. 1994). Woody invasions may modify fungal communities by altering litter quality or quantity (Johnson and Matchett 2001) and their associated saprophytic fungi, or altering plant composition and positively- or negatively-associated fungal symbionts (Egidi et al. 2016). While plant responses to fire suppression (Briggs et al. 2002, Ratajczak et al. 2013), and plant responses to changes in N availability (Clark and Tilman 2008) in grasslands, and microbial responses to fire in forested ecosystems (Horton et al. 1998, Hart et al. 2005, Dooley and Treseder 2012) are well-researched, much less is known about how these ecosystem drivers impact soil fungal communities in grasslands – particularly in multifactorial combinations.

While fire supression increases N availability due to decreased litter volatilization, N availability has also increased due to anthropogenic alterations of the N cycle (Vitousek et al. 1997, Galloway et al. 2004). The consequences of higher N availability in the tallgrass prairie are increased biologically available N, greater aboveground net primary production (ANPP) and lower belowground net primary production (BNPP) (Kitchen et al. 2009, Wilson et al. 2009), altered plant community structure and through decreased plant diversity (Collins et al. 1998, Bardgett et al. 1999), and changes in soil nutrient stoichiometry (Johnson et al. 2003). These changes have variable impacts on soil fungal communities, although meta-analyses and work in forest ecosystems have reported increased N availability to frequently cause declines in fungal biomass, inhibition of fungal growth and even direct toxicity, as well as lower fungal ligin-modifying enzyme activity (Fog 1988, Gallo et al. 2004, Treseder 2008, Edwards et al. 2011). While some studies have investigated arbuscular mycorrhizal (AM) fungal responses to

increased available soil N in grassland ecosystems (Jumpponen et al. 2005, Wilson et al. 2009, Johnson et al. 2015), it is unclear how the individual and interactive effects of historical fire suppression and N fertilization impact the composition of the entire bulk soil fungal community, including AM, pathogenic, and saprophytic fungi.

Within the fungal community, taxa can be broadly sorted into functional guilds, each interacting differently with the plant community and the soil environment (Garrett 1951, Nguyen et al. 2015, Cho et al. 2017). For example, fungi in Phylum Glomeromycota, the arbuscular mycorrhizal (AM) fungi, directly contribute to plant nutrition through their symbiotic relationships in which fungi alleviate plant phosphorus limitation through enhanced nutrient acquisition (Hartnett and Wilson 1999, Johnson et al. 2015). Additionally, AM fungi dependencies vary among hosts, as seen in tallgrass prairies, where AM promote competitive dominance of C_4 grasses (Wilson and Hartnett 1998). Saprophytic fungi are critical decomposers, producing extracellular enzymes that breakdown and mobilize plant available nutrients, including N and P, from plant litter and soil organic matter (Deacon et al. 2006, Purahong et al. 2016). Pathogenic fungi can affect plant community composition through negative interactions in the rhizosphere, suppressing growth of some plants and thus regulating grassland plant diversity (Reinhert 2012, Bever et al. 2015). Ectomycorrhizal (EcM) fungi are more abundant in forest ecosystems, due to their common symbiotic association with dominant tree species; however, likely due to plasticity in the symbiotic versus saprotrophic lifestyle of many EcM taxa as well as transient dispersal, they may also be found in grassland soils (Theit and Borener 2007). While EcM fungi tend to be found in the Basidiomycota phylum, functional guild association is more often conserved at lower taxonomic levels (Bruns et al. 1998, Horton

2002). Taken together, community composition and functional roles of soil fungi can have significant feedbacks on plant community structure and nutrient availability in grasslands.

Due to their known relationships with plants and the soil environment, soil fungi are potentially sensitive to global change phenomena, including changes in disturbance regimes and N availability, as well as to seasonal variability in photosynthate and litter availability, and to discrete environmental changes such as pulses of nutrient availability and fire (Bentivenga and Hetrick 1992, Allison et al. 2007, Collins et al. 2007, Fontaine et al. 2011, Klaus et al. 2016). Because AM fungi form obligate symbiotic relationships with plants and derive their C resources from the plant photosynthate, AM relative abundance within the bulk soil fungal community may increase during the growing season (Mandyam and Jumpponen 2008). Plant litter inputs, soil moisture, and soil temperature also fluctuate between the growing and non-growing seasons. As a result, the relative abundance of saprophytic fungi and decomposition activity may also fluctuate seasonally (Waldrop and Firestone 2008). While fire may cause fungal mortality due to heat or substrate removal, total microbial biomass is often enhanced by annual burning in grasslands (Dooley and Treseder 2013). As a result, more information on the mechanistic links between fire events and their long-term consequences is needed. In general, it is of interest to understand how seasonal or event-based fungal turnover interacts with long-term ecosystem changes, since understanding the impact of long-term management on seasonality of fungal communities can aid in understanding the availability of inocula for plant-beneficial fungal relationships, and consequent feedbacks to grassland plant and soil nutrition.

The objective of this study was to assess seasonal dynamics of soil fungal communities on multiple temporal scales, including fungal community responses to contrasting long-term land-management practices, seasonal turnover of fungi associated with plant communities that

have changes in response to those contrasting land-management histories, and fungal responses to fire or fertilization events, to address the following questions: 1) How does fire and fertilization history affect soil fungal communities? 2) Does management history alter seasonal and event-based fungal community turnover? 3) Are fungal responses related to changes in grassland plant communities? To address these questions, we analyzed fungal community composition monthly and surveyed plant community composition during the growing season in a long-term factorial grassland field manipulation where plots had been either annually burned or unburned and fertilized or unfertilized since the experiment's inception in 1986. We predicted that: 1) Disturbance history, specifically fire exclusion, would promote saprophytic fungi as a result of accumulation of litter (Boutton et al. 2006), whereas AM fungi would have greater relative abundance in fertilized soils due to greater N:P ratio, therefore an increased reliance of plants on AM partners (Johnson et al. 2003, Wilson et al. 2009). 2) On seasonal scales, shifts in plant phenology would promote pathogen and AM populations in summer, due to actively photosynthesizing plants as a primary C source, and saprophytic fungal abundance would be higher in winter due to reliance on litter or SOM for growth resources (Schmidt et al. 2007), and 3) plant community composition, as modified by disturbance and N fertilization, would be correlated with changes in fungal community structure (LeBlanc et al. 2014, Cassman et al 2016).

Methods

Study Site and Experimental Design

This research was conducted at Konza Prairie Biological Station (KPBS) (39°05'N, 96°35'W) at the Belowground Plot Experiment (BGPE), which includes a long-term field manipulation of N availability through a split-strip block experimental design. We sampled from

plots that had been fertilized (10g N·m^{-2} as NH$_4$NO$_3$) or unfertilized, and burned or unburned since 1986, to investigate soil fungal dynamics in differently-managed tallgrass prairie. The dominant vegetation at KPBS was perennial C$_4$ grasses, such as *Andropogon gerardii*, *Sorghastrun nutans*, *Panicum virgatum*, and *Schizachyrium scoparium*, although fire suppression promoted dominance by woody plants such as *Juniperus virginiana, Cornus drummondii,* and *Rubus occidentalis* (Ratajczak et al. 2013). This area experiences warm, dry summers and cool, wet winters: mean annual precipitation (MAP) is 835 mm and mean annual temperature (MAT) is 26.6°C. Specific micro-meteorological variables were not measured within each treatment, however meteorological data was collected at a location adjacent to the BGPE (cite the website for the met station?). During the one-year sampling period, total monthly precipitation ranged from 6.2 mm in March 2015 to 147.3 mm during July 2015. Daily mean soil temperature ranged from 2.3° C in December 2015 to 23.5° C in July 2015, and daily mean air temperature ranged from 0.7° C in January 2015 to 37° C in July 2015. Total annual precipitation during the study of 1002.5 mm was ~20% greater than average, reflecting a growing season with soil water content rarely below field holding capacity (approximately 0.25 g·g^{-1}, Zeglin et al. 2013), and mean temperatures were near or slightly above average through the study.

Surface soil (top 15 cm) cores from all treatment plots were collected once per month from November 2014 to December 2015, excluding December 2014 and February 2015. Soils from all treatment plots were collected one week following the annual burn treatment in April 2015, and one week following the annual fertilization treatment in June 2015. Soils were collected in were collected using aseptic techniques in 2 cm diameter cores from three random locations within each subplot and homogenized in the field to create a composite sample, placed

on ice in the field and immediately carried back to the laboratory and frozen at -20° C until further analysis.

Environmental and Soil Properties

For each sample (n = 192), soil gravimetric water content (GWC) was measured as mass loss from soil after drying at 105°C overnight. Soil organic matter (SOM), pH and mineralizeable C were measured from a one-month subset of soils from the same experiment (collected June 2016, n = 16). SOM was measured by loss-on-ignition (LOI) and pH was measured in 1:1 slurry of deionized water. Mineralizeable soil C (Cmin) was the integrated amount of soil respiration (μg C g^{-1} dry soil) over a 30-day laboratory incubation, with CO_2-C measured using a Picarro G2101-i Analyzer (Picarro Inc., Santa Clara, CA, US) (Baer and Blair 2008, Zeglin and Myrold 2013). Total available N (Navail.) over the growing season (June - Sept 2016) was indexed as the mass (μg bag^{-1}) of nitrate plus ammonium sorbed to resin bags (buried to 10 cm with four replicates averaged per plot to integrate sub-plot heterogeneity) (Baer and Blair 2008, Binkley 1984). Belowground net primary production (BNPP, g m^{-2}) was estimated as the mass of root ingrowth between June - Sept 2016 into 5-cm diameter, 30-cm depth, fine mesh bags (n = 16, average of 4 replicates per plot to integrate sub-plot heterogeneity) (Johnson and Matchett 2001).

DNA Extraction and Polymerase Chain Reaction (PCR)

Total genomic DNA (gDNA) was extracted from approximately 0.5 g of homogenized soil per sample using physical lysis, cetyltrimethylammonium Bromide (CTAB) and phenol: chloroform extraction and overnight precipitation in PEG 6000 (DeAngelis et al. 2010) Fungal community sequence libraries were constructed from these gDNA extracts, with the ribosomal Internal Transcribed Spacer (ITS2) as the target locus, using barcoded universal fungal primers

(fITS7/ITS4, White et al. 1990, Ihrmark et al. 2012). Each sample was PCR-amplified in triplicate 25 µl reactions with 10 ul GoTaq Flexi Buffer with MgCl$_2$, 5 ul of 0.04% Bovine Serum Albumin, 5 µl of uniquely barcoded primers 0.25 µl of HotStart GoTaq Polymerase, 2.5 µl of dNTP, between 1 – 100 ng/µl gDNA, and brought to volume with Nuclease-free PCR grade H$_2$O. Cycling conditions were 94˚ for 3 min. initial denaturation, followed by 35 cycles of 94˚ denaturation 10 sec., 54˚ annealing for 10 sec., and a final 1 min 72˚ extension step. Amplification success and amplicon length were confirmed with horizontal gel electrophoresis in a 2% agarose gel. Triplicate technical reactions were combined and cleaned using Axygen PCR Magnetic Bead Clean-up (Axygen Scientific, Inc., Union City, CA), following the manufacturer's protocol with 1:1 volume ratio of Axygen Magnetic beads for each reaction volume. The cleaned products were combined into one library with Illumina specific adapters. Indices were added using a NEBNextDNA MasterMix (Protocol E6040, New England Biolabs Inc., Ipswich, MA, USA) and sequenced using a MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA) with 600 cycles and sequenced on an Illumina MiSeq using a 2x300 cycle run at the Integrated Genomics Facility at Kansas State University (Manhattan, KS, USA).

Bioinformatics

The sequence data were processed using mothur (v. 1.35.1) informatics software (Schloss et al. 2009). The paired-end library contained 5,779,183 sequences after joining forward and reverse reads. The data were screened to remove sequences with ambiguous bases, sequences shorter than 250 base pairs, contiguous sequences with less than 100 base pair overlap, or homopolymers longer than 8 base pairs, which removed 65 sequences. Sequences were then cut to 232 bases, and near-identical sequences were preclustered to decrease sequencing bias and putative chimeras were identified and removed (UCHIME algorithm; Edgar et al. 2011) (Huse et

al. 2008). After these quality control steps, all data were rarified to an equal depth of 7,446 sequences. A pair-wise distance matrix was calculated, Operational Taxonomic Units (OTUs) were defined by clustering sequences to 97% sequence similarity, and taxonomic annotations were assigned using the UNITE database. The final dataset contained 4049 unique OTUs in 166 remaining samples that yielded adequate depth. Sequences were uploaded to GenBank with accession number XXXXX.

Functional Guild Assignment

Mothur-generated taxonomy was used to assign OTUs to functional groups, or guilds, using the UNITE-compatible FUNGuild (v1.0) database (Nguyen et al. 2015). This online database designates OTUs into ecologically relevant functional groups based on nutrient-acquisition strategies, e.g., pathogen, saprotroph, or symbiont (Nguyen et al. 2015). Because arbuscular mycorrhizal fungi are particularly important for plant nutrition in grasslands (Hartnett et al. 1999) and ectomycorrhizae form associations with many woody species (Nilsson and Wallander 2003), we parsed the coarse assignment of symbionts into arbuscular mycorrhizae (AM), ectomycorrhizae (EcM), and symbiont OTUs that did not fall into these two categories as "other symbionts". Of the 4,049 OTUs assigned by the UNITE database, 1,290 (32%) were assigned to a functional guild by FUNGuild using any available assignment. Fungal community composition data from Morrison et al. (2016) was compared with our data to evaluate functional and taxonomic differences between forested ecosystems, woody prairie, and tallgrass prairie.

Plant Community Composition and Production

Plant community composition data were collected in late July at two subplots within each of the 16 plots sampled. In each subplot, canopy cover was described in a surrounding 5-m^2 area and coverage of individual species was assessed by visual estimates based on a modified

Daubenmire scale. Estimated cover was then averaged for the two subplots. Additionally, foliage

biomass was collected and recorded in two separate 0.1 m^2 quadrats in each plot during peak

biomass (September) to measure live grass and forbs, the current and previous year dead

biomass, and woody biomass in each plot, and the total amount of new biomass was used to

estimate aboveground net primary production during the full growing season (ANPP, g m^{-2}).

Statistical Analysis

All variables were checked for normality and transformed when appropriate to meet

assumptions of normality (ITS taxon relative abundance and plant productivity values) before

statistical analysis. The individual and interactive effects of burning, fertilization and sample date

for fungal NMDS scores were evaluated with a repeated-measures two-way Analysis of Variance

(RM-ANOVA) test using mixed-effect models with replicate as a random variable, in the "lme"

package in R Studio. Similarly, effects of burning and fertilization, but not sample date, were

evaluated for all plant variables using mixed-effect models using the same R package. A Bray-

Curtis dissimilarity matrix was created using the "vegdist" function to create a nonmetric

multidimensional scaling ordination with the "metaMDS" function, and the "adonis" function

was used to determine the permuational analysis of variance (PERMANOVA) for both plant and

fungal communities (vegan package, R Studio) (Oksanen et al. 2010). Sub-plot heterogeneity

was evaluated by measuring and comparing ordination cloud size (function "betadisper", vegan

package) Correlation of plant and soil fungal community variability, and variability in

environmental factors (ANPP, BNPP, plant growth form coverage, SOM, pH, GWC, N

availability (Navail), mineralizable C (Cmin)), in July was determined using Mantel tests

(function "mantel," vegan package). Correlations between NMDS axes and fungal taxa relative

abundances were evaluated using Pearson's R coefficient and associated p-values using the

"cor.test" function in R Studio. The integrated N fertilization response ratios of each fungal guild and phylum were calculated by averaging the percent difference of the relative abundance in the N-fertilized plot from the relative abundance in the paired control plot for all sampling points. Paired t-tests were used to evaluate changes in fungal taxa relative abundances between treatments (R Commander) (R Development Core Team).

Results

Key soil variables, including SOM, GWC and pH, did not vary significantly between long-term burn disturbance or N fertilization treatments (Table 1, $P > 0.05$). Cmin and Navail did vary between long-term treatments, in that both Cmin and Navail were altered by chronic N additions, and Navail was affected by burn history. Cmin ranged from 7.00 to 8.29 µg C g^{-1} g^{-1} dry soil, and was higher in unfertilized plots. Navail ranged from 4.98 to 11.04 µg bag^{-1}, and was higher in both unburned and fertilized soils, and lower in both burned and unfertilized soils. There was no interactive effect between fire and N fertilization history on Navail ($P > 0.05$).

Fungal OTU richness ranged from 146 – 524 and varied significantly by sample month and fire history (RM two-way ANOVA: Burn [F, P] = 24.4, 0.0003; Month [F, P]: 4.43, < 0.0001). Across all treatments, fungal richness was lowest in March, increased in April and was higher for most of the remainder of the study duration, and fungal richness was lower on average throughout the year in annually burned soils (Table 3.2, Figure 3.1c). Shannon diversity was lowest in March but did not vary with long-term treatment history (Month [F, P]: 2.1452, 0.0230), and fungal evenness was not different between treatments or by sample date (Table 3.2).

The NMDS ordination of fungal community composition in all samples had an optimal two-axis solution and resulted in a stress value of 0.150. Results from PERMANOVA and two-way RM ANOVA on individual NMDS axes (Table 3.2) indicated that fungal community

composition was significantly affected by both direct and interactive effects of burning and fertilization (Figure 3.1a, b) (Table 3.2). NMDS axis one indicated a strong effect of fertilization ([F, P]: 26.87, 0.0002) and burn history ([F, P]: 19.95, 0.0008), where unburned, fertilized soils had the lowest NMDS scores and burned, unfertilized soils had the highest scores. NMDS axis two indicated an interaction of burn and fertilization history ([F, P]: 4.76, 0.0497) (Figure 3.1a & b). These results were confirmed by PERMANOVA, which showed fire history, N fertilization, and their interaction explained 5.1%, 4.7% and 1.7% of the total variation in the distance matrix, respectively (Table 3.3). Notably, neither NMDS axis changed by sample date. Because no significant seasonal turnover was observed in the fungal community, fungal communities in soils collected in July were used to construct a subset NMDS ordination better suited for comparison with plant community and environmental data (Figure 3.2a). This model also had an optimal two-axis solution with a stress value of 0.094. July ITS PERMANOVA results and two-way RM ANOVA on individual NMDS axes also indicated that burning history and N additions significantly explained variation in the July community subset, where fertilization, burning and their interaction explained 11.6%, 9.9%, and 6.5% of the variation in the July ITS community, respectively (Table 3.3). Additionally, Navail, Cmin, and % grass were significantly correlated with the July NMDS ordination (Table 3.3, Figure 3.2a).

Ascomycota dominated this dataset (76.14%), followed by Basidiomycota (21.50%), then basal lineages assigned to Zygomycota (0.8%) and Glomeromycota (0.2%). Of the 4049 total OTUs, 0.20% were AMF, 2.32% were EcM, 6.09% were pathogens, 19.50% were saprotrophs, 3.90% were an assortment of other symbionts (including lichenized fungi, endophytes, and other mycorrhizae), and 68% of the community was unassigned, on average. Of the assigned taxa, saprotrophs, pathogens, and other symbionts belonged primarily to Phylum

Ascomycota (70.7%; 55.8%; and 71.4% of the assigned taxa in each group, respectively), and EcM belonged primarily to Basidiomycota (58.8%). Within the fungal guilds, two-way RM-ANOVA indicated that AM ([F, P] 58.85, 0.0003), ECM ([F, P]: 8.27, 0.0282), pathogens ([F, P]: 16.26, 0.0069), and saprotrophs ([F, P]: 41.59, 0.0007) significantly differed between fertilized and unfertilized soils (Figure 3.3). AM ([F, P]: 1.99, 0.0369 and saprotrophs ([F, P]: 1.90, 0.0475) significantly differed among sample months, although no post hoc differences were significant. Arbuscular mycorrhizae relative abundance was 11.63% higher in July and August than other sampling months in burned, fertilized soils, but this pattern was not statistically significant. Unassigned taxa and other symbionts were not significantly affected by treatment or sampling month (p > 0.05).

All assigned fungal guilds, except for ECM, had positive responses to N additions, and none of the assigned fungal guild responses differed between burned and unburned soils. Unassigned taxa in unburned soils had a negative response to N additions (Figure 3.4a). There were overall fewer significant responses in coarse fungal classification between burned and unburned treatments (Figure 3.4a): Of the guild responses, pathogen and saprotroph abundance was significantly higher in unburned soils, and no Phylum was consistently indicative of fire history. Treatment interactions were observed as EcM-assigned sequences being most abundant in the burned, unfertilized treatment, and Ascomycota and Zygomycota being more abundant, and Basidiomycota less abundant, in fertilized and unburned soils; but there were no differences between burned and unburned responses to N additions across any assigned fungal guilds (one-way ANOVA, p>0.05).

Plant community composition was significantly affected by the direct and interactive effects of burning and fertilization, and the PERMANOVA results indicated that fire history, N

fertilization and their interaction explained 24.7%, 15.6% and 10.5%, respectively, of the

variation in plant community (Table 3.2). The best NMDS ordination for plant community

composition had an optimal two axis solution and resulted in a stress value of 0.077 (Figure

3.1b), and two-way RM-ANOVA of individual NMDS axis scores also showed that long-term

management significantly altered plant community composition, with burning treatment

explaining variability on NMDS axis one, and N additions explaining variability on NMDS axis

two (Table 3.1). The relative abundance of woody plants, live grass, and ANPP were correlated

with the plant community ordination model, whereas other measured environmental variables

were not (Table 3.3, Figure 3.2b).

Plant communities in burned, fertilized sub-plots had the lowest heterogeneity (Figure

3.2b), as these communities were strongly dominated by *P. virgatum* (95.25% ± 1.6%). In

contrast, burned, unfertilized plant communities were dominated by *A. gerardii* (big bluestem)

(49.37% ± 9.0%), *P. virgatum* (23.87% ± 9.8%), and *S. scoparium* (little bluestem) (7.12% ±

4.19%). Unburned, fertilized plant community composition had the highest spatial sub-plot

heterogeneity among field replicates, with the four individual sub-plots dominated by *P.*

virgatum, Sporobolus compositus, Symphoricarpos orbiculatus and *C. drummondii,* and *Rubus*

occidentalis. There were no observed differences in homogeneity in the unfertilized treatments,

although the communities differed significantly due to shifts from herbaceous to woody

vegetation (from 0.00% ± 0.00% in burned to 67.01% ± 17.96% in unburned) with long-term fire

suppression.

Plant species richness ranged from 4 to 21 species per 5 m^2, evenness ranged from 0.03 to

0.44, and Shannon diversity ranged from 0.15 to 1.97. Plant richness was higher in unfertilized

plots, plant evenness and diversity were lowest in annually burned and fertilized plots, and total

ANPP was two to three times higher in the fertilized than the unfertilized treatment (Table 3.1).

Fungal (July 2015), plant, and environmental distance matrices were all correlated

(Mantel tests, $P < 0.001$, Table 3.3). For comparison, the correlation between the plant, fungal

and environmental matrices and the prokaryotic 16S rRNA gene community distance matrix

from the same July 2015 samples (Carson and Zeglin, Chapter 3.2) was also evaluated. Bacterial

and fungal communities were correlated with one another ($P = 0.002$), but soil bacterial

communities were not correlated with either plant or environmental matrices.

Discussion

Here, we show empirical evidence that plant and soil fungal communities respond

somewhat coherently to long-term fire and N fertilization, and that the long-term differences in

land management alter the relative abundance of putative fungal functional groups. Although we

initially hypothesized that fungal communities would be seasonally variable due to differences in

the source and availability of plant inputs between months, we found that that neither fungal

communities as a whole nor the functional guilds changed seasonally. The correlation between

plant, soil, and fungal responses to long-term experimental manipulation of N availability points

to the importance, coupling, and complexity of aboveground-belowground interactions in

understanding ecosystem responses to global change.

While historical disturbance and management altered plant and fungal communities, we

observed surprisingly little seasonal turnover in the fungal community. We initially expected that

changes in plant phenology would affect soil fungi, in that photosynthate resources over summer

would increase the relative dominance of AM fungi and pathogens, while reliance on litter

resources would promote the dominance of saprotrophs fungi in winter. Both saprotrophic and

AM guilds significantly varied by month, although this effect was relatively weak for both groups (Figure 3.3). Further, neither within the whole community nor the observed fungal guilds, no significant seasonality was detected. This may reflect detection of dormant propagules of obligate plant-associated AM or pathogens, as AM and pathogens may be unable to receive photosynthates or other nutrients during non-growing seasons (Tommerup 1983, Lennon and Jones 2011). Also, some putative pathogens may form relationships with animals or other soil organisms, or may not be obligately pathogenic, so may persist through winter due to either alternative saprophytic states or reliance on other hosts for nutrition (Bever et al. 2015). In turn, saprophytic fungi may persist through both winter and summer, relying on relatively constant C availability from fine root turnover and soil organic matter (Schmidt et al. 2007). In addition to nutrient acquisition strategies, fungi may also be less sensitive to seasonal change due to their growth forms and rigid cell walls, providing possible protection from seasonal environmental stressors (Schimel et al. 2007, Strickland and Rousk 2010). While AM fungi as a whole did not differ across months, there was a notable increase in AM relative abundance in the burned fertilized soils over summer. These seasonal associations between AM and host plants can create positive-feedback loops year-to-year by offering competitive advantages to some hosts (Bever et al. 2012, van der Putten et al. 2013).

In contrast to long-term effects of fire and fertilization, we found no support for fungal responses to N additions or fire on short, event-based time scales. Fungal communities respond to fire in forest systems, where heat penetrates deeper in the soil profile (Dooley and Treseder 2012, Reazin et al. 2016, Muñoz-Rojas et al. 2016). In grasslands, soil temperatures reach only a fraction of the temperatures observed in forests, and much of the impact of fire is rather observed through indirect effects such as increased UV exposure, soil drying, and changes in nutrient

availability due to above-ground litter removal or volatilization, rather than direct mortality during the fire event (Ojima et al. 1994, Neary et al. 1999, Dooley and Treseder 2012). The lack of responsiveness to N addition pulses may be attributed to long-term community adaptation to high N conditions, as we observed significant differences in long-term, but not short-term, responses to N. We observed an increase in fungal richness after the fire event (between March and April), but this response was consistent among all treatments and therefore cannot be attributed to the burning event.

While fungal responses to short-term and seasonal change were minimal, long-term N-additions led to distinct fungal communities. In grasslands, N additions can have variable effects on the soil environment through changes in nutrient stoichiometry, and therefore variable feedbacks to biotic communities. In the Flint Hills ecoregion where soils have relatively low amounts of biologically available P, increasing available soil N tends to favor high AM colonization, and mycelial foraging responses to N addition are generally positive due to increased plant dependence on AM partners for P acquisition (Johnson et al. 2003, Avolio et al. 2014, Johnson et al. 2015). Our results support these observations, most notably in the burned, fertilized treatment where AM relative abundance in the bulk soil increased sharply over the summer due to an increase in the AM order Archaeosporales. This group differed significantly in relative abundance between N-amended and non-amended soils in July ([F, P]: 12.12, 0.0045), and on average, comprised 19.1%-59.7% of the Glomeromycota community in burned, fertilized plots, 17.5%-39.4% of the Glomeromycota community in the unburned, fertilized soils, and was absent in the unfertilized soils. AM fungi commonly associate with warm-season grasses in tallgrass prairies, and the burned fertilized soils support a dominantly warm-season grass plant community composed of 95% *P. virgatum* (Wilson and Hartnett 1998). Because we sampled the

bulk soils rather than root tissue, we cannot ascertain specific plant-AM symbioses, and this pattern may be attributable to other factors in part because *P. virgatum* is known to have relatively low mycorrhizal dependency compared to other warm-season grasses (Eom et al. 2011).

In addition to nutrient stoichiometry, N additions can alter soil nutrient distribution homogeneity, which has positive feedbacks to plant compositional homogeneity (Baer et al. 2004, Maestre et al. 2006, Ke and Miki 2015). Soil nutrient and plant distributional homogeneity may also concentrate negative plant-soil-feedbacks through increased prevalence of fungal pathogens (Maron et al. 2011, Schnitzer et al. 2011, van der Putten et al. 2013). Our data support this hypothesis, as fungal pathogen relative abundance was highest in N added soils. In general, pathogens can be more prevalent in soils with higher and more stable nutrient availability, which allows for the accumulation and maintenance of specialized pathogens (Wardle et al. 2004). This is widely accepted in agroecosystems where plant pathogens are of key concern for crop yield (Altieri 1999). Fungal pathogens are recognized as a critical force for maintaining plant species diversity in grasslands, which has important feedbacks for other biotic communities at multiple trophic levels (Reynolds et al. 2003, van der Heijden et al. 2008, Bever et al. 2015), but it may be that positive interactions from mycorrhizal colonization may play a more important role in structuring plant communities than negative interactions with fungal pathogens in our *Panicum*-monodominant burned, fertilized soils. Also, enriched fungal pathogens in the unburned, N-amended plots may represent propagule banks from buildup of litter. Further work is necessary to understand whether the higher putative fungal pathogen abundance is mechanistically related, or whether plant responses to N addition are concomitant but independent of fungal responses.

Annual burning removes aboveground plant material (Blair 1997). As a result, we expected the accumulation of litter in unburned plots would promote a higher relative abundance of saprophytic fungi; however, while saprotrophs tended to be more abundant in unburned soils (Figure 3.4b), this pattern was weak in the context of overall variability (Figure 3.3d). This result is analogous to previous work from this tallgrass prairie site, in which total decomposition rates were not altered by burning legacies, despite differences in total soil C and C:N (Reed et al. 2009). Despite the lack of clear responses to fire suppression at the coarse levels of functional guild or taxonomic phylum to long-term burning treatment, fire history explained a higher amount of variation in the overall relative abundance of fungal OTUs than N fertilization history or the fire by N interaction. Because OTUs from the Ascomycota are predominant within our dataset, the response to the lack of fire is due primarily to a turnover of OTUs at lower taxonomic levels within this Phylum. Since most Ascomycota were classified as saprotrophs (and inversely, most saprotrophs were Ascomycetes), the functional implications of this shift are not clear, but this does point to the importance of considering lower-taxonomic level responses to an altered environment.

Instead of showing a strong fire response, putative saprotrophs were significantly more abundant in soils that experienced long-term N amendments. This was not related to an increase in belowground litter inputs (Table 3.1), but could plausibly be related to greater aboveground inputs, since even in burned treatments the 2-3x higher level of ANPP with fertilization (Collins et al. 1998, Wilson et al. 2009) causes increased fresh litter cover between the end of the growing season and late spring when the organic matter is burned off, and significant infiltration of litter-derived OM into the soil occurs (Cotrufo et al. 2015). The positive responses of fungal saprotrophs to N additions may also suggest that the saprophytic community invests less in

decomposition of complex litter resources due to the greater availability of simple N sources, and invests more energy into growth (Morrison et al. 2016, van Diepen et al. 2017). While not a significant treatment response, we found the highest SOM contents in the experimental plots with the highest N availability (Table 3.1), suggesting that the complex interactions between plant and soil microbial responses to higher N (Wilson et al. 2009, Riggs et al. 2015) may begin to affect soil carbon stocks after three decades of fertilization.

Previous studies from forest ecosystems have suggested that N loading reduces decomposition, and promotes soil C accumulation, via loss of the EcM association of less N-limited trees with laccase-producing Basidiomycota. Our data do not support this mechanism being important in fertilized grassland soils, even in the unburned plots that show a significant amount of woody plant invasion. Temperate deciduous forest ecosystems have a soil C:N ratio of approximately 18.7, and temperate grasslands have a C:N ratio of approximately 13.3 (Wardle et al. 2004, DeDeyn et al. 2008, Fierer et al. 2009, Xu et al. 2013). Grassland fire suppression, and woody plant invasion, can substantially increase soil C and N inputs (McKinley and Blair 2008), with impacts that can include increases in soil C:N towards a ratio more typical of forested ecosystems (Kitchen et al. 2009, Carson and Blair 2013, unpublished data). In forests, however, higher soil C:N is generally associated with and even perpetuated by greater EcM fungal biomass, which contributes higher C:N necromass to soil OM pools (Strickland and Rousk 2010). Our study soils are dominated by non-EcM Ascomycetes, not EcM Basidiomycetes, with a surprising trend toward lower EcM/Basidiomycete relative abundance in soils with more woody plant cover (Figure 3.4b). The higher abundance of EcM in burned grassland soils in this study may indicate that we detected dormant propagules, since no plants in the annually burned plots form relationships with EcM (Eissenstat et al. 2015). Further, although woody plants are more

often associated with EcM symbiosis rather than AM symbiosis, all forbs and woody plants in our study plots form relationships with AM symbionts (with the exceptions of *Alliaria petiolata* (garlic mustard) and *Amarantus rudis* (common waterhemp), which are non-mycorrhizal) (Pendleton and Smith 1983, Medve 1984, Roberts and Anderson 2001, Kremer 2014, Eissenstat et al. 2015). Consequently, we highlight that a loss of fire may not alter grassland soil fungal communities or function to mimic that of forested ecosystems, due to the low abundance of Basidiomycota in the ecosystem, even though fire suppression alters the plant community to support more woody vegetation (Fig. 3.4a).

In addition to possible biogeochemical implications of grassland-characteristic plant-fungal interactions, the lack of required EcM symbiosis is a mechanism that may promote woody plant species establishment under fire suppression in tallgrass prairies, because many woody invaders are able to take advantage of the available AM symbionts (Williams et al. 2013) (Fig. 3.4b). Woody encroachment is of substantial concern, because this phenomenon is a widespread threat to the conservation and sustainable management of grasslands and rangelands (Ratajczak et al. 2012). Previous work has shown that simply reintroducing fire to an historically unburned grassland does not remediate long-term woody encroachment due to competitive advantages associated with woody plants including modified distribution of fuel loads, shading, clonal growth, and greater root access to deep water (Neary et al. 1999, Ratajczak et al. 2011, Nippert et al. 2013, Ratajczak et al. 2014). Further, our fungal composition data underscore that biological belowground legacies (i.e. AM fungal inocula) could support the establishment of specific invasive woody plants (i.e. those with AM rather than EcM associations). While the implications of links between woody invasion and fungal symbiont prevalence for management are not well

understood from our data alone, we argue that a greater understanding of these links will help in addressing management and remediation for woody encroachment in grasslands.

Within-treatment spatial heterogeneity in fungal community composition was less variable in comparison to plant community composition (Figure 3.2). In burned plots, fertilization caused major differences in warm-season plant cover due to a switch in dominance from *A. gerardii.* to *P. virgatum*, resulting in high homogeneity. This shift in dominance due to fertilization has been observed previously and can be attributed to *A. gerardii*'s competitive advantage for N acquisition in low N systems, whereas alleviation of N limitation has shown to increase the competitive dominance of *P. virgatum* (Wedin and Tilman 1993, Baer et al. 2016, Koerner et al. 2016). On the other hand, fertilized, unburned plots had the highest plant community sub-plot heterogeneity, with different dominants in each plot, possibly indicating priority effects (Fukami 2015, Adler et al. 2017). In contrast, fungal community sub-plot heterogeneity was different among all treatment replicates, despite turnover in OTU composition. This may be somewhat attributable to the difference in scale of measurement and growth from of plants versus fungi, as multiple cores from across each plot were composited for the measurement of fungal composition, while 5-m^2 plant composition plots established pre-woody invasion are less well suited to detection of large tree cover. Still, these considerations reflect information on within sub-plot heterogeneity, not among sub-plot heterogeneity, so it was still surprising that fungal sub-plot heterogeneity did not reflect the coarse scale plant community sub-plot heterogeneity that is a clear hallmark of the unburned, fertilized treatment. More likely, the bulk soil fungal communities represent the potential inocula for plant interactions, rather than the specific active interactions that would be better evaluated via direct measurement of root colonization by fungi.

Still, our data showed that plant communities, soil environmental variation, and fungal communities were all correlated (Table 3.3). It is widely known that AM and some pathogenic fungi form obligate relationships with plants (Smith et al. 1998, van der Heijden et al. 2008, van der Putten et al. 2013); however, AM and pathogens only consisted only a small (0.2% and 7% on average, respectively) proportion of the community. Therefore, our observed relationship between plant and fungal communities cannot be exclusively attributed to changes in root-associated symbiotic relationships. Rather, the range of nutritional preferences and affinities among free-living fungi may help drive these plant-fungal community associations. Evidence for the specialization of decomposer communities for localized litter types has accumulated, and a variety of supporting, non-exclusive, mechanisms exist to explain these observations (Austin et al. 2014). In our study system, litter quantity and quality is affected by both disturbance regime change and fertilization: litter C:N is lower with lack of fire as well as fertilization (Hobbie 1992, Kitchen et al. 2009), litter inputs are higher with fertilization as noted earlier, and plant community differences creates a different integrated matrix of litter biochemical composition (Freshshet et al. 2012), all of which may affect saprophytic fungal community composition. Soil environmental variables also reflect changes in organic matter quality and quantity, and the correlation among plant, soil and fungal responses to long-term grassland management changes reflect the complexity of feedbacks among these factors (Hobbie 2015), as well as the possibility that taxon-specific feedbacks may exist.

Furthermore, while bulk soil bacterial and fungal communities were correlated, bacterial community variation was not significantly related to plant composition or environmental distance matrices. In general, free-living decomposer fungi have several competitive advantages over decomposer bacteria for nutrient acquisition and survival in the soil habitat.

Morphologically, filamentous fungi are competitively advantageous because hyphal growth allows for penetration of complex substrates, where even hyphal actinomycete bacteria have been shown to be outcompeted for nutrient sources when compared to saprophytic fungi (deBoer et al. 2005). Additionally, saprophytic fungi have several physiological advantages over bacteria, namely their ability to access complex C sources, such as lignocellulose (de Boer et al. 2008, Schneider et al. 2012). These competitive advantages indicate that fungi are more likely to be specialists for litter decomposition and primary decomposers of plant litter, in addition to forming intimate symbiotic relationships. There is also evidence to suggest that bacterial communities may rely on fungal exudates as a source of nutrition (Linderman 1999, Garbaye 1991, Boer et al. 2005). In addition, previous work has suggested that AM communities modify rhizosphere bacterial communities, but rhizosphere bacteria do not modify mycorrhizal communities (Marschner et al. 2001). These lines of evidence provide mechanisms for our observation of links between fungal and bacterial communities, but not bacterial and plant communities or the soil environment.

In tallgrass prairies, plant and fungal communities have pivotal roles for ecosystem functions and are intimately linked through their nutritional requirements. In this study, we found that change in nutrient availability through fire and N enrichment alter plant and fungal communities somewhat coherently, and that fungal communities have the most distinct responses to ecosystem change on long-term scales, rather than short-term, in response to fire and N additions. Because soil fungi vary widely in their nutritional strategies within coarse taxonomic assignments, we used a novel fungal functional assignment database which allowed for the assignment of ecologically meaningful functional guilds to some of the fungal community sampled. While all the assigned functional groups responded positively to N additions,

unassigned taxa, which compose the majority of the fungal community, displayed a negative

response to N additions. These results suggest that the unknown portion of the fungal community

may be more sensitive to environmental change, and presents a demand for future research into

the functional ecology of these more cryptic fungal taxa to fully understand responses of fungal

biodiversity, potential feedbacks to plant and soil nutrition, and implications for management and

conservation in the face of global change.

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Tables and Figures

Table 3.1: Environmental and soil variables.

Burn B	Fertilizer N	SOM	GWC	pH	Cmin	Navail	BNPP	% Grass	% Wood	% Forb	ANPP
		-	-	-	N	B, N	-	B	B	-	N
BC Burn -N		0.067 (0.006)	0.270 0.009	6.44 0.15	7.94 (0.15)	5.15 (0.07)	132.78 17.19	96.66 (1.45)	0.00 (0.00)	3.34 (1.45)	501.2 (64.1)
BN Burn +N		0.065 (<0.001)	0.267 0.010	6.35 0.15	7.36 (0.06)	8.54 (0.26)	112.53 34.29	99.27 (0.46)	0.00 (0.00)	0.73 (0.46)	1492.2 (289.3)
UBC Unburn -N		0.067 (0.005)	0.305 0.039	6.31 0.18	7.63 (0.17)	6.75 (0.35)	164.97 28.32	33.40 (17.99)	66.01 (17.69)	0.59 (0.59)	543.1 (158.3)
UBN Unburn +N		0.0862 (0.008)	0.288 0.012	6.00 0.28	7.23 (0.20)	10.60 (0.37)	130.45 26.09	40.82 (23.10	48.06 (27.79)	11.13 (10.85)	1173.9 (484.5)

Means (SE) of measured environmental variables. Results of RM-ANOVA, with notation for all significant ($p<0.05$) effects burn treatment (B), and fertilization treatment (N). Abbreviations: SOM, soil organic matter (mass loss on ignition, g g^{-1}), GWC, Gravimetric water content (g g^{-1}), Cmin, 30-d mineralizeable carbon (ln μg C g^{-1} dry soil), Navail, mineralized N (ln μg bag^{-1}), BNPP, belowground net primary production (g m^{-2}), ANPP, aboveground net primary production (g m^{-2}).

Table 3.2: Fungal and plant community metrics.

	Fungal Community					Plant Community				
	S	J'	H'	NMDS 1	NMDS 2	S	J'	H'	NMDS 1	NMDS 2
Month Burn Fertilizer M B N	M, B	-	M	B, N	B	N	BxN	BxN	B	N
BC Burn -N	296 (16)	0.62 (0.01)	3.50 (0.11)	0.171 (0.01)	0.008 (0.01)	15 (2)	0.33 [b] (0.02)	1.52 [b] (0.08)	-0.25 (0.02)	-0.18 (0.06)
BN Burn +N	330 (12)	0.65 (0.01)	3.79 (0.09)	0.033 (0.02)	-0.111 (0.02)	5 (1)	0.05 [a] (0.01)	0.23 [a] (0.06)	-0.29 (0.11)	0.17 (0.01)
UBC Unburn -N	363 (14)	0.64 (0.01)	3.80 (0.08)	-0.004 (0.02)	0.084 (0.02)	15 (3)	0.30 [b] (0.06)	1.40 [b] (0.26)	0.36 (0.15)	-0.06 (0.03)
UBN Unburn +N	373 (11)	0.67 (0.01)	3.95 (0.07)	-0.191 (0.02)	0.021 (0.01)	11 (2)	0.24 [ab] (0.06)	1.15 [b] (0.27)	0.19 (0.18)	0.07 (0.10)

Means (SE) of measured variables for fungal and plant community composition. Results of RM-ANOVA, with all significant (p<0.05) effects of sample date (M), burn treatment (B), and fertilization treatment (N) noted for the fungal community, and burn and nitrogen effects are noted for the plant community. S = Species richness; J' = Simpson's evenness; H' = Shannon diversity, ANPP = Aboveground net primary production ($g \cdot m^{2\,-1}$). Superscript letters indicate significant treatment differences by Bonnferoni post-hoc tests.

Table 3.3: Permutational Analysis of Variance (PERMANOVA) results for all fungal community composition, July fungal community composition, and plant community composition data.

	Fungi (All Months)				Fungi (July)				Plant (July)			
	Sum of Squares	F	R^2	Pr(>F)	Sum of Squares	F	R^2	Pr(>F)	Sum of Squares	F	R^2	Pr(>F)
Nadd	**2.601**	**8.49**	**0.047**	**0.001**	**0.592**	**1.93**	**0.116**	**0.001**	**0.797**	**3.79**	**0.156**	**0.010**
Burn	**2.843**	**9.26**	**0.051**	**0.001**	**0.511**	**1.67**	**0.099**	**0.007**	**1.266**	**6.02**	**0.248**	**0.001**
Month	3.503	1.04	0.063	0.301	-	-	-	-	-	-	-	-
Burn*Nadd	**0.925**	**3.01**	**0.017**	**0.001**	0.332	1.08	0.065	0.310	**0.537**	**2.56**	**0.105**	**0.045**
Nadd*Month	2.976	0.88	0.054	0.973	-	-	-	-	-	-	-	-
Burn*Month	3.414	1.01	0.061	0.417	-	-	-	-	-	-	-	-
Nadd*Burn*Month	2.759	0.82	0.049	1.000	-	-	-		-	-	-	-
Residuals	0.307	-	0.656	-	3.683		0.307		2.523	-	0.493	-

Bolded values represent factors that explained significant (P < 0.05) levels of variability.

Table 3.4: Environmental variable correlations with July fungal and plant ordination axes.

	Fungal Ordination				Plant Ordination			
	NMDS 1	NMDS 2	R^2	P	NMDS 1	NMDS 2	R^2	P
GWC	0.02	-0.99	0.06	0.659	0.09	0.99	0.12	0.448
SOC	-0.99	0.05	0.34	0.054	0.56	-0.83	0.20	0.221
pH	0.16	0.99	0.33	0.084	0.23	0.97	0.03	0.828
Navail	**-0.99**	**0.02**	**0.63**	**0.001**	0.04	-0.99	0.17	0.316
Cmin	**0.99**	**-0.62**	**0.52**	**0.001**	-0.89	0.45	0.20	0.19
BNPP	-0.48	0.88	-0.04	0.787	-0.96	0.29	0.26	0.129
ANPP	-0.97	0.25	0.18	0.270	**0.02**	**-0.99**	**0.35**	**0.039**
% Grass	**0.86**	**0.51**	**0.38**	**0.040**	**-0.96**	**0.26**	**0.87**	**0.001**
% Woody	-0.83	-0.56	0.33	0.080	**0.99**	**-0.10**	**0.91**	**0.001**
% Forbs	-0.65	0.75	0.02	0.861	-0.37	-0.93	0.11	0.605

Bolded values represent significant (P < 0.05) correlations with NMDS axes. Abbreviations:

GWC, Gravimetric water content, SOC, soil organic carbon (mass loss on ignition), Navail,

available N (ln µg bag^{-1}), Cmin, mineralizable carbon (ln µg C g^{-1} 30 day^{-1}), BNPP,

belowground net primary production (g·m$^{2-1}$), ANPP, aboveground net primary production

(g·m$^{2-1}$).

Table 3.5: Mantel test output indicating Pearson's R-squared and p-values for correlations of plant, fungal (July only) and environmental variables.

	R^2	P
ITS & Plant	**0.46**	**0.001**
ITS & Environment	**0.34**	**0.001**
Plant & Environment	**0.50**	**0.001**
ITS & 16S	**0.47**	**0.002**
16S & Plant	0.18	0.075
16S & Environment	0.08	0.171

July prokaryotic 16S rRNA gene community composition matrix from the previous chapter was also used for comparison. Bolded values indicate significant (p>0.05) correlations.

Figure 3.1: Nonmetric multidimensional scaling axis (a) one and (b) two, and fungal richness (c) in soils sampled from each treatment (BC = Burned control, BN = Burned fertilized, UBC = Unburned control, UBN = Unburned fertilized) between Nov 2014 – Dec 2015. RM-ANOVA indicated significant (p < 0.05) changes with (a) fertilization ([F, P] 26.87, 0.0002) and burn history ([F, P]19.95, 0.0008) in Axis 1, (b) an interaction of fire and fertilization ([F, P] 4.76, p=0.0497) in Axis 2, and richness changes by (c) sample date ([F, P] 4.428, <0.0001) and burn history ([F, P] 24.395, 0.0003). Bars indicate standard error of the mean.
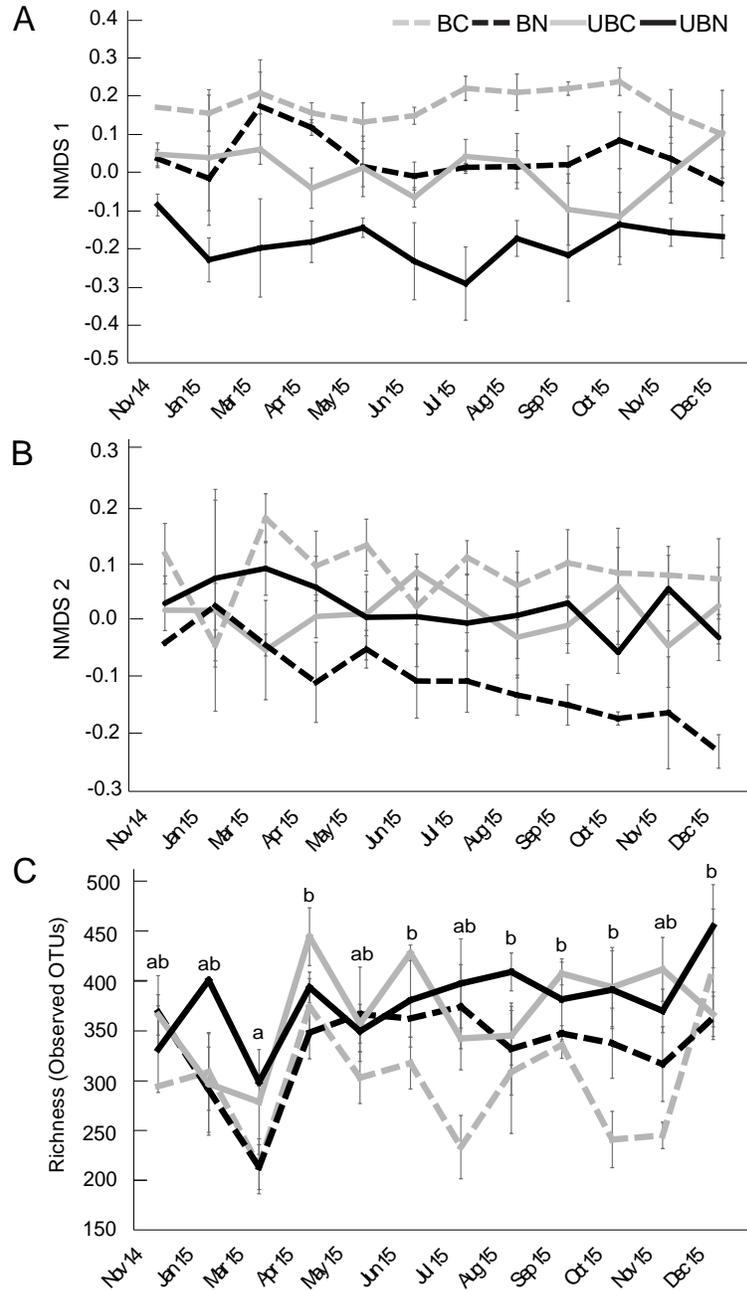
Figure 3.2: Nonmetric multidimensional scaling (NMDS) ordination model axes one and two of Bray-Curtis dissimilarity in (a) July soil fungal community composition and (b) plant community composition, comparing four treatments (BC = Burned control, BN = Burned fertilized, UBC = Unburned control, UBN = Unburned fertilized) from July samples, where each ellipse represents 95% confidence intervals for each treatment. Arrows represent significant ($p<0.05$) environmental correlations ((a)Navail available N, Cmin, availabile C). The size of each ellipse was compared using "betadisper", which indicated significant differences in plant, but not fungal, cloud size (Table inset B, observed and permuted p value above and below diagonal, respectively).

A

Stress: 0.094
No change in heterogeneity

% Grass
Navail
UBN
BN
BC
UBC
Cmin

NMDS 2

NMDS 1

B

Stress: 0.077

	BC	BN	UBC	UBN
BC	-	0.011	0.269	0.006
BN	0.011	-	0.048	0.001
UBC	0.277	0.043	-	0.496
UBN	0.003	0.000	0.522	-

BC
% Grass
UBC
UBN
% Woody
BN
ANPP

NMDS 2

NMDS 1

95
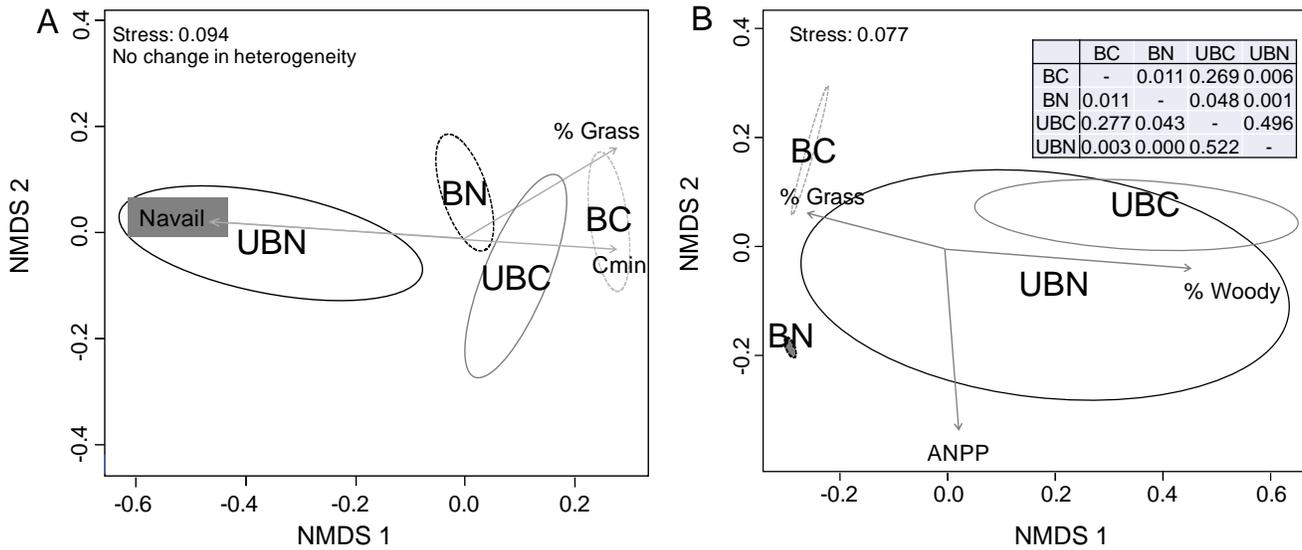
Figure 3.3: Treatment and temporal changes in FUNGuild-assigned functional group relative abundances (BC = Burned control, BN = Burned fertilized, UBC = Unburned control, UBN = Unburned fertilized) in soil sampled between Nov 2014 - Dec 2015 for (a) Arbuscular mycorrhizae (AMF), (b) ectomycorrhizae (EcM), (c) pathogens, and (d) saprotrophs. Repeated measures ANOVA indicated significant (p < 0.05) changes with fertilizer treatment for all groups ((a) [F, P]: 8.36, 0.0276; (b): [F, P]: 8.27, 0.0282; (c): [F, P]: 58.85, 0.0003; (d): [F, P]: 41.59, 0.0007), and a significant effect of (d) sample date ([F, P]: 1.90, 0.0475; No post-hoc differences). Bars indicate standard error of the mean.
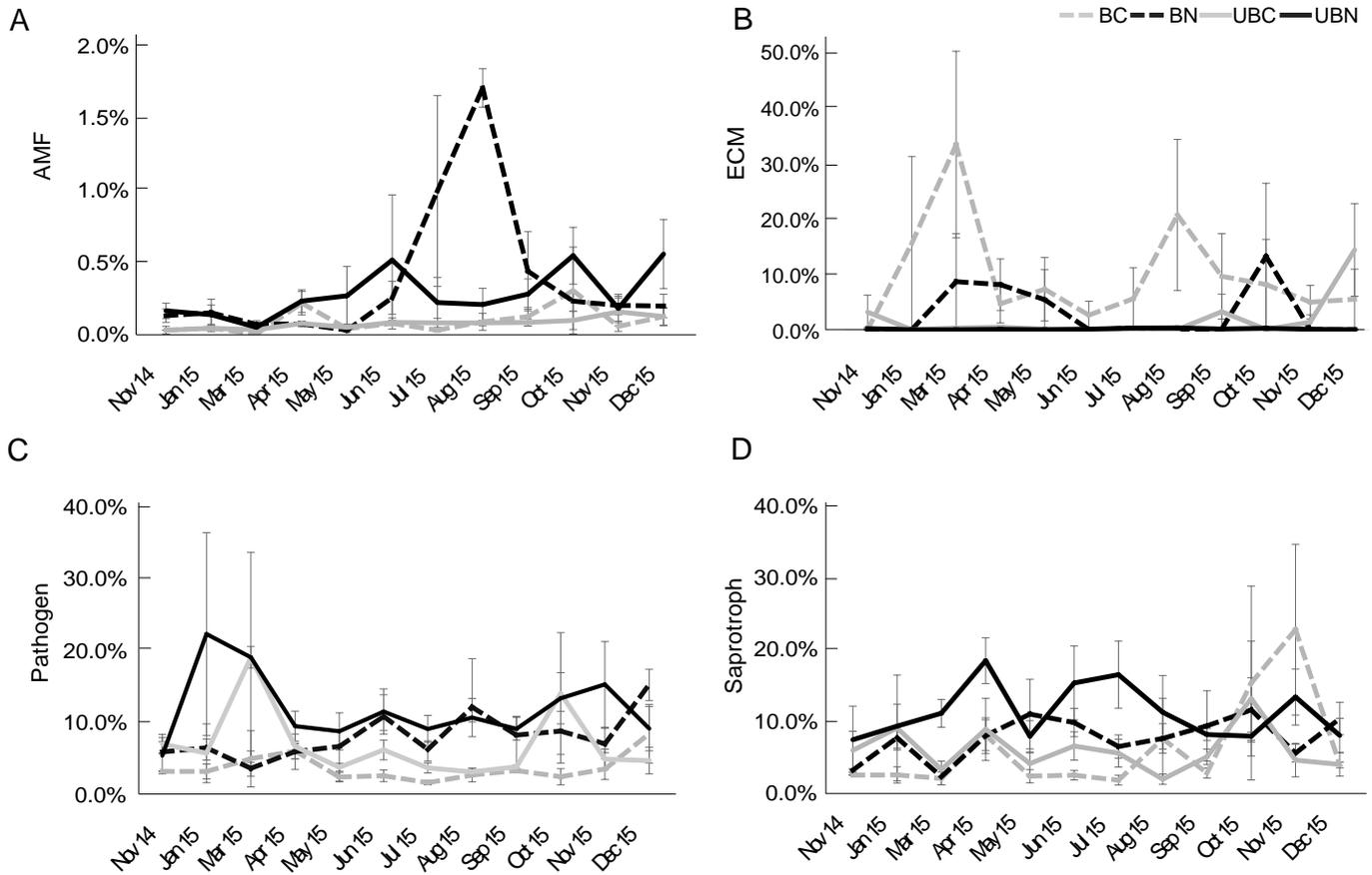
Figure 3.4: **Average relative abundance of dominant fungal (a) phyla and (b) functional groups from long-term field manipulations soils sampled between Nov 2014 - Dec 2015 (BC = Burned control, BN = Burned fertilized, UBC = Unburned control, UBN = Unburned fertilized; this study), and a fertilized coniferous forest (Morrison et al. 2016).**
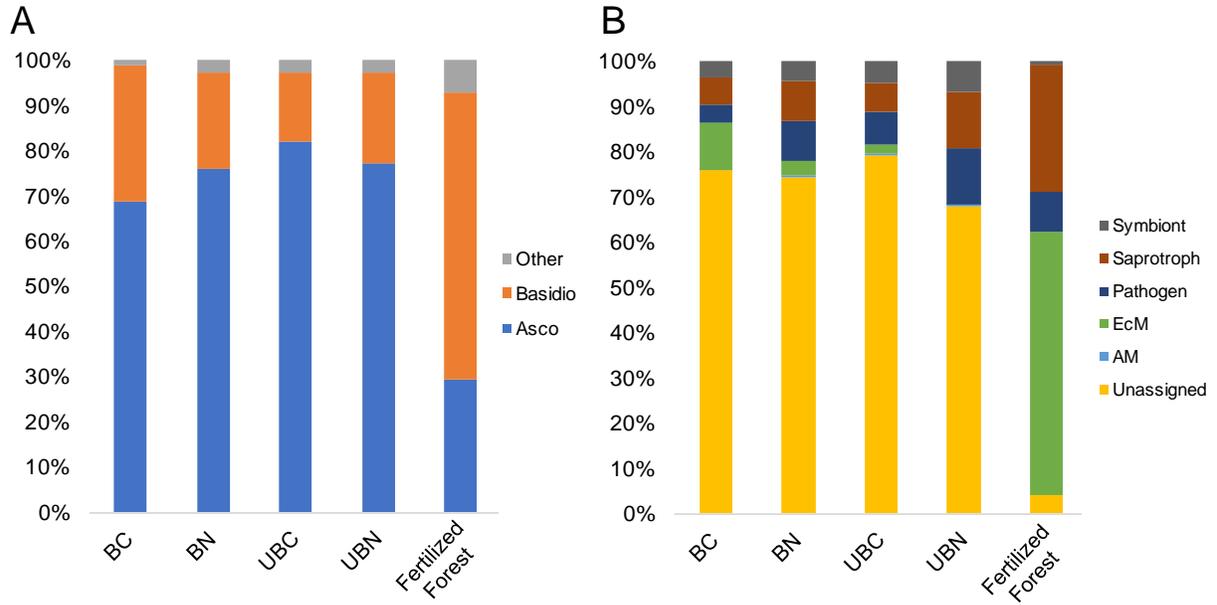
Chapter 4 - Conclusions

Soil microbial communities are highly diverse and are well-known for their critical roles in biogeochemical cycles, but the goal of relating microbial diversity to microbial ecology and ecosystem function remains a developing area of research (Horner-Devine et al. 2004, Philippot et al. 2013, Graham et al. 2016). Therefore, understanding specific responses of microbial communities to ecosystem drivers can aid in interpreting implications of management practices and global change phenomena. These consequences are potentially important for threatened ecosystems, such as tallgrass prairies. The <5% of remaining tallgrass prairie ecosystems are of critical conservation concern because tallgrass prairies sustain a significant portion of earth's biodiversity, have significant roles as C sequestration sinks, and face threats of land-use conversion through urbanization, agriculture, and woody encroachment (Samson and Knopf 1994, Knapp et al. 1998, Scurlock and Hall 1998, Briggs et al. 2006, Fierer et al. 2013). Therefore, it is critical to identify how drivers of ecosystem function are impacted by land management practices, anthropogenically-driven global change phenomena, and disturbance regimes, and what feedbacks these manipulated drivers have for biotic communities.

In tallgrass prairie ecosystems, N availability is both a stand-alone driver of ecosystem function and manipulated by prescribed burning and anthropogenic actions, where prescribed burning decreases N availability, and fossil fuels and fertilizer use increase N availability through long-term atmospheric deposition (Galloway et al. 2008, Coolon et al. 2013, Sutton and Bleaker et al. 2013). Grassland soil microbial communities are potentially sensitive to change in the soil habitat from ecosystem drivers on multiple time scales, ranging from days to decades (Schmidt et al. 2007, Lauber et al. 2013, Shade et al. 2013). Microbial turnover, in terms of

cellular or community turnover, can impact shifts in dominance of prokaryotes or fungi (Strickland and Rousk 2010), and functionality of groups within those broad domains (Fierer et al. 2007, Farrer et al. 2015), and therefore the transformations and retention of soil nutrients, plant composition, and litter quality (Garcia and Rice 1993, Ramirez et al. 2010, Riggs et al. 2015). In this thesis, we investigated microbial responses to fire and fertilization, which are drivers of N availability in tallgrass prairie, on event-based, seasonal, and long-term temporal scales, to add taxonomic and temporal resolution to the current understanding of mesic grassland soil ecology.

In chapter two, we investigated how these ecological drivers of N availability (chronic N additions and fire) affect sensitivities of microbial population sizes and bacterial community dynamics on short and long time scales. We found that bacterial and fungal population sizes are sensitive to seasonal change, where bacterial populations were the most responsive on seasonal scales most likely in response to plant rhizodeposits during the growing season. Fungal population sizes were more stable throughout winter and growing season months, although decreased during October and November 2015. These results may represent differences in fungal and bacterial reliance on active plant exudation, as rhizosphere-associated bacteria sharply increase in population size during summer, while fungi may have greater access to SOM stores during winter than bacteria. However, population sizes did not differ between long-term treatments for either bacterial or fungal populations. Opposite effects were observed for bacterial communities, as bacterial communities were seasonally stable, yet sensitive to historical changes in fire and fertilization management. Additionally, we observed no change in bacterial richness and evenness between historical management treatments. However, we did find support for the "copiotrohic hypothesis," which postulates that microbial communities change in response to

replacement of high substrate affinity oligotrophic taxa with low substrate affinity copiotrophic taxa (Ramirez et al. 2012, Leff et al. 2015). Further, our results reveal important interactions of fire and fertilization manipulations of N availability on the sensitivity of the bacterial community to ecosystem change, where the highest amount of copiotrophic taxa replacement occurred in unburned soils. These results indicate that baseline N availability modified by fire can act as a buffer to microbial community change under N enrichment in tallgrass prairies.

In our assessment of the bacterial community, we noted pronounced heterogeneity in the bacterial composition in the unburned, fertilized soils. While we originally hypothesized that this heterogeneity was attributed to seasonal dynamics in bacterial community composition, we did not find support for this hypothesis. As an alternative hypothesis, we predicted that this heterogeneity may be due to spatial, rather than temporal, heterogeneity among plots of similar treatments, as we had observed major differences in the plant communities among individual plots in the unburned, fertilized treatments. Because fungal communities have well-known direct nutritional links to plant communities (van der Heijden et al. 2008, Hodge and Fitter 2013), we investigated fungal community turnover between winter and during the growing season, how long-term changes N availability from fire and fertilization impacted grassland soil fungi and plant communities, and potential links between soil fungi and plant communities. Similar to bacteria, we found little evidence for fungal seasonal turnover, or change on short-time scales in response to fire or fertilization events, while long-term changes in N availability had the greatest impact on plant and fungal communities. Our results were supported by previous studies from KPBS, that found that N additions increase the relative abundance of obligate mutualistic arbuscular mycorrhizae (Wilson et al. 2009, Johnson et al. 2015), and this study also adds taxonomic and functional resolution to knowledge regarding fungal saprotrophs, pathogens, and

symbionts. Additionally, we found that changes in soil fungal communities, grassland plant communities, and changes in soil properties were correlated, soil fungal and bacterial communities were correlated, but changes in plant and bacterial communities were not correlated, highlighting possible differences in nutritional requirements within the soil microbial community. Soil fungi may be more tightly linked to plant nutrition through competitive advantage for litter resources due to specialized enzyme activities, morphology, and symbiotic relationships, while soil bacteria may be more linked to litter quality or fungal exudates (de Boer et al. 2005).

Taken together, these studies indicate that soil microbial communities and plant communities have most notable responses on long-time scales in response to long-term management of N availability through fire and chronic fertilization, However, since we also found that microbial population sizes change significantly between the growing season and non-growing seasons, this may represent a limitation to our methods which focused on measuring bulk soil community composition rather than focusing on microbial activity. Therefore, coupling our results with enzyme assays, measures of gene expression, or transcription through RNA-based analyses may detect greater seasonal shifts throughout the growing season. Overall, understanding seasonality in microbial systems can aid in understanding how management and global change affecting N availability impacts soil community activity, feedbacks to biogeochemical cycling, and ecosystem processes.

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