# SOIL MICROBIAL COMMUNITY CARBON AND NITROGEN DYNAMICS WITH ALTERED PRECIPITATION REGIMES AND SUBSTRATE AVAILABILITY

By

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## ABSTRACT

Understanding the nature and extent of the feedback between soil microorganisms and ecosystem processes is of great concern as we are faced with multiple elements of global environmental change. In this dissertation, I explore how anthropogenically induced environmental changes affect soil microorganisms' resource use, and how, in turn, changes in microbial resource use alters ecosystem processes. These explorations were conducted in grassland systems, which contain 12% of global soil carbon (C) stocks and can serve as large C sources or sinks depending on environmental conditions such as nitrogen (N) availability and precipitation regimes.

Nitrogen availability in grasslands can control plant primary productivity as well as rates soil organic matter decomposition and the fate of soil organic C. In grassland systems undergoing N addition through fertilization, resource inputs to soils increase in both quality and quantity. In Chapter 1, I investigate the fate of enhanced biomass inputs due to N addition by determining the direct and indirect effects of N addition on the activity of the soil microbial decomposers. Through measurement of extracellular enzyme activities and isotopic analysis of the microbial biomass relative to substrate sources, I found enhanced mineralization of newly incorporated soil organic C with N addition. This increase in soil C break down was associated indirectly with N addition through increases in plant litter quality and not directly with increased soil N availability. These results suggest that increased biomass input resulting from N addition does not necessarily result in increased soil C accrual.

Climate change in the Great Plains region will likely cause increases in drought severity and precipitation event size with little change in annual precipitation totals. Precipitation events, particularly those following periods of drought, can create large flushes of resources for microbial communities, but these same pulses also can cause high levels of physiological stress and disturbance. When faced with increased soil moisture stress and re-wetting disturbance, microorganisms must accumulate and release protective osmolytes. The acquisition and release of protective osmolytes, apparently of sufficient magnitude to influence ecosystem level N and C fluxes, makes understanding the mechanisms behind these fluxes critical for predicting not only microbial community responses to global change, but ecosystem responses as well. In Chapter 2, 3 and 4, I use soils from four locations across the Great Plains precipitation gradient in a combination of laboratory and *in situ* soil incubations to explore the effects of soil moisture stress on flows of C and N though the microbial biomass.

In Chapter 2, I focus on links between soil moisture stress and resource use efficiency by manipulating the frequency and magnitude of soil wetting and drying cycles in laboratory soil incubations. As soil moisture stress was increased with longer drought intervals and larger water pulse events, I saw a decline in C use efficiency and a 360 – 4800% increase in net N mineralization in soils from four sites along the Great Plains precipitation gradient. In Chapter 3, I employed the use of stable isotopes at the end of a similar incubation, to trace the C and N during a soil wetting-drying cycle. In this study I found that increased levels of soil moisture stress shifted microbial preference from N-rich protective osmolytes to N-free osmolytes. I also found that soils from the mesic end of the precipitation gradient were more sensitive to changes in soil moisture stress than soils from the semi-arid end of the gradient and that nitrification appeared to be less sensitive than denitrification, leading to increased soil $NO_3^-$ concentrations and a decoupling in the N cycle.

Finally, in Chapter 4, I reciprocally transferred soils between four study sites along the precipitation gradient and allowed them to incubate *in situ* for 1.5 and 2.5 y. After collection I assessed nitrification and denitrification potentials and the abundance of functional genes associated with these processes. I compared effects of both the initial community composition and the change in environment on the process rates. I found that as soil moisture stress increased across the precipitation gradient, nitrification potential decreased and nitrification functional gene abundance increased. Depending on soil origin, denitrifiers were either sensitive, resistant or functionally redundant after 1.5 y of altered precipitation regimes. In contrast, after 2.5 y denitrifiers in soils of all origins exhibited declines in process rates and functional gene abundance with increased soil moisture stress.

Overall, I found that microbial communities are sensitive to environmental change, and as these communities shift in structure and function C and N cycling in these grasslands is altered. In particular, the perturbations explored in this dissertation, N addition and climate change, may induce increased rates of C release and N loss from these grassland soils.

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# GENERAL INTRODUCTION

Microbial communities mediate processes that determine flows of carbon (C) and nitrogen (N) through ecosystems. Without an understanding of how microbial communities are structured and what factors most influence changes in these structures and ultimately community function, we cannot fully understand the impacts of anthropogenic disturbances such as global climate change on ecosystems. Most biogeochemical or ecosystem models used to predict ecosystem functioning incorporate microbially mediated processes in an opaque 'black box', relying on the assumption that these processes are not affected by the composition of the community (Schimel, 2001). Several studies however, have shown that community composition can influence ecosystem processes (Allison and Martiny, 2008; Balser and Firestone, 2005; Fierer et al., 2003; Nogales et al., 2002; Cavigelli and Robertson, 2001).

A review of microbial community response to disturbance suggests that the composition or structure of microbial communities is, in many cases, not resistant to environmental changes that cause disturbance or increases in physiological stress (Allison and Martiny, 2008; Schimel et al., 2007). When stress or disturbance does cause a shift in microbial community structure, there are three possible outcomes. First, the post disturbance community may be resilient, exhibiting no changes in structure or function, or changes for a limited time before returning to the pre-disturbance state. Second, the community may be functionally redundant so that changes in structure are not reflected in function. Finally, community structure and function may be permanently altered, or exhibit resilience only after relatively long time periods (Allison and Martiny, 2008). It appears that in many cases microbial communities are sensitive to environmental change and as these communities change, ecosystem process rates may also be altered.

In this dissertation I explore how environmental change can alter microbial community resource use and thus fluxes of C and N through the grassland systems of the Great Plains. I first examine the direct and indirect effects of N addition on microbial decomposers and soil organic C mineralization in an experimental grassland subjected to common land management practices such as fertilization and haying (Chapter1). Second, I explore C and N resource use under differing levels of wetting-drying stress in soils collected across the Great Plains precipitation gradient (Chapters 2 and 3). Finally, I utilize reciprocal transplants of soil cores across the same precipitation gradient to explore the connections between increasing environmental stress and microbial community composition and resulting N cycling process rates (Chapter 4).

## CHAPTER 1: Indirect effects of nitrogen amendments on organic substrate quality increase enzymatic activity driving decomposition in a mesic grassland

*Abstract*

The fate of soil organic carbon (SOC) is determined, in part, by complex interactions between the quality of plant litter inputs, nutrient availability and the microbial communities that control decomposition rates. This study explores these interactions in a mesic grassland where C and nitrogen (N) availability and plant litter quality have been manipulated using both fertilization and haying for 7 years. We measured a suite of soil parameters including inorganic N, extractable organic C and N (EOC and EON), soil moisture, extracellular enzyme activity (EEA) and the isotopic composition of C and N in the microbial biomass and substrate sources. We use these data to determine how the activity of microbial decomposers was influenced by varying levels of substrate C and N quality and quantity and to explore potential mechanisms explaining the fate enhanced plant biomass inputs with fertilization. Oxidative EEA targeting relatively recalcitrant C pools was not affected by fertilization. EEA linked to the breakdown of relatively labile C rich substrates exhibited no relationship with inorganic N availability but was significantly greater with fertilization and associated increases in substrate quality. These increases in EEA were not related to increase in microbial biomass C. The ratio of hydrolytic C:N acquisition enzymes and $\delta^{13}C$ and $\delta^{15}N$ values of microbial biomass relative to bulk soil C and N, or EOC and EON suggest that microbial communities in fertilized plots were relatively C limited, a feature likely driving enhanced microbial efforts to acquire C from labile sources. These data suggest that in mesic grasslands, enhancements in biomass inputs and quality with fertilization can prompt increase in EEA within the mineral soil profile with no significant increases in microbial biomass. Our work helps elucidate the microbially mediated fate of

enhanced biomass inputs that are greater in magnitude than the associated increases in mineral soil organic matter.

## INTRODUCTION

The link between rising atmospheric $CO_2$ concentrations and global climate change makes it increasingly important that we determine the fate of organic carbon (C) in terrestrial ecosystems. Soils contain the largest near-surface reservoir of terrestrial C, and the factors controlling the release and storage of this C are tightly linked with nitrogen (N) availability (Conant et al., 2005; Neff et al., 2002; Asner et al.,1997). For example, N additions influence microbially mediated processes such as decomposition that govern the fate of C inputs to soil profiles (Knorr et al., 2005; Waldrop et al., 2004; Hobbie 2000). Nitrogen availability may influence decomposition rates through the direct influence of inorganic N availability on microbial function, or indirectly through changes in the quality of organic substrates (Hobbie, 2005).

Microbial resource availability can influence organic matter in multiple ways. For example, experiments that explore land management practices in North American grasslands, like fertilization and haying, have shown that the resulting alterations in nutrient and substrate availability can have significant influence on mineral SOC pool sizes and dynamics. Such studies are important, because grasslands contain approximately 12% of Earth's SOC pool (Schlesinger 1997). Nitrogen addition in grasslands can increase SOC concentrations (Malhi et al.,1997; Conant et al., 2001; Billings et al., 2006), but the stability of this additional SOC and the extent to which it is incorporated into long-lived soil organic matter (SOM) pools varies greatly. In one study of a mesic grassland system undergoing N additions for five years, SOC concentrations

increased significantly, but this additional SOC resided primarily in the most labile SOM fraction (Billings et al., 2006). Another study of the same duration reported increased organic matter content in stabilized fractions with N addition, but only when coupled with plant litter with high lignin content (Dijkstra et al., 2004). Resource removal from grassland soils can also influence soil C and N cycles; in mesic grassland systems, aboveground biomass removal through haying can decrease both SOC and soil N content (Franzluebbers and Stuedemann, 2005).

Grassland studies exploring the effects of N addition on SOM decomposition by measuring soil extracellular enzyme activity (EEA) report contrasting results. One study of N addition in a semi-arid grassland revealed a correlation between inorganic N availability and increases in multiple EEAs associated with C acquisition (Stursova et al., 2006), while a study of three different grasslands, widely variable in edaphic properties, concluded with the observation that EEA in grassland soils may be "insensitive to N amendment" (Zeglin et al., 2007). It thus remains unclear how the importance of inorganic N availability relative to organic substrate quality and, important for grasslands experiencing haying, quantity drives microbial C vs. N limitation and rates of SOC decomposition.

We explored drivers of SOC cycling in a relatively mesic grassland in eastern Kansas, USA that has experienced manipulations of N and C availability through fertilization and haying over multiple years (Billings et al., 2006; Billings and Gaydess, 2008; Tiemann and Billings, 2008; Foster et al., 2009). At this site, fertilization has resulted in significant increases in labile SOC stocks, though the magnitude of these increases is less than the increases in plant biomass inputs (litterfall and root) with fertilization (Billings et al., 2006). We use this site to explore this discrepancy by studying how SOM decomposition rates relate to changes in both the quantity and quality of C and N substrates available to soil microbial communities. Understanding drivers

of SOC dynamics, and ultimately SOC retention or release as $CO_2$, is particularly critical in mesic grasslands, given their relatively high concentrations of SOC compared to more xeric systems (McCulley et al., 2005). To determine how heterotrophic soil microbial activity at this site may change with nitrogen addition or plant biomass removal via haying, we measured EEA associated with the decomposition of labile and recalcitrant pools of SOM multiple times during a growing season. We also measured isotopic composition ($\delta^{13}C$ and $\delta^{15}N$) of the litter, the bulk soil, extractable organic C and N (EOC and EON), and the microbial biomass at the end of the growing season as a means of further assessing microbial substrate use. We measured these indices of microbial activity in conjunction with microbial biomass C and N, inorganic N and EOC. We use these data to assess patterns of microbial resource use with long-term alterations in C and N substrate form and availability.

## METHODS

### *Study Site*

Our study site is located at the University of Kansas Field Station in northeastern Kansas, USA (KUFS; 39˚03′ N, 95˚12′ W). Average annual rainfall is 971 mm and the average annual temperature is 13.5°C (High Plains Regional Climate Center). During the course of this study, the site received 549 mm rainfall, while average air temperature was 19.6 and average soil temperature was 19.8°C. The experimental grassland plots were managed as a hayfield until 1984. In 2000, 32 experimental plots (10x10 $m^2$) were established (Billings et al., 2006; Tiemann and Billings, 2008; Foster et al., 2009). There are 8 plots of each of 4 treatments, fertilized (F), fertilized and hayed (FH), hayed (H), and untreated early succession grassland (E). A ‘N-P-K’ fertilizer mix (N:P:K; 29:3:4) commonly used in the region was applied to the F and FH plots in

early April of each year from 2000 to 2006 and on April 17, 2007, at a rate of approximately 15 g inorganic N $m^{-2}$ with a $\delta^{15}N$ of -0.2 ± 0.1‰ (Billings et al., 2006). Haying was performed from 2000-2006 in July or early August, but not at all in 2007 due to unusually hot and dry conditions. Haying removed all plant biomass from just above ground level; total aboveground biomass removed from the hayed plots annually was approximately 550 kg $km^{-2}$ (Foster et al., 2009). The dominant grassland plant species include the perennial grasses *Bromus inermis*, *Poa pratensis* and *Festuca arundinacea*, and native grasses *Andropogon virginicus* and *Andropogon gerardi* (Foster and Dickson, 2004). Soils are fine, smectitic, mesic Argiudolls formed from glacial deposits of loess over till, and are naturally fertile with high water holding capacity (NRCS, USDA). Fertilization over the first 6 y at this site resulted in significantly higher bulk SOC and organic N concentrations in the top 15 cm of the soil profile and higher plant biomass (litterfall and root) inputs compared to control plots with no significant effects of haying observed (Billings et al., 2006).

*Measurements Over the Growing Season*

At five time points, approximately 6 weeks apart during the 2007 growing season (April 4, May 16, July 2, August 14, and September 24, 2007), we collected six soil cores (5 cm deep, 2 cm diameter) from each of the 32 plots. Soils were placed in a cooler and returned to the University of Kansas where they were stored at 4°C until processed. Roots >1mm were removed and the soils from each plot homogenized. Approximately 5 g of soil from each plot were weighed and dried at 60°C for >48 h to determine gravimetric soil moisture. We froze a 10 g sub-sample of soil from each plot at -65°C for determination of EEA, inorganic N, DOC, and microbial biomass C and N (Lee et al., 2007).

*Inorganic N*

We extracted inorganic N from 2.5 g frozen soil from each plot with 12.5 ml 0.5M $K_2SO_4$. Concentration of $NH_4^+$-N in these extracts was determined colorimetrically using a diffusion block (Doyle et al., 2004) and $NO_3^-$-N concentrations were determined using cadmium reduction on a Lachat Quik-Chem 8000 FIA (Lachat Instruments, Milwaukee, WI).

*Soil and Litter C and N Analyses*

Soils for C and N and isotopic analyses were collected on October 17, 2007 and processed in the same manner as presented above, except that all visible roots were picked and then 5 g samples were dried and ground to a fine powder. Litter was collected on September 26 and 29, 2007 as part of a total standing biomass collection. In each plot total above-ground biomass was removed in two 2 $m^2$ strips using clippers and then sorted into litter and living components. Samples of litter from each of the two strips per plot were homogenized into one sample per plot, then ground on a Wiley mill fitted with a 1 mm mesh screen. Litter cellulose and lignin concentrations were determined by acid detergent digestion in an Ankom fiber analyzer and sulfuric acid digestion respectively per manufacturer instructions (ANKOM Technology, Macedon, NY, USA). Soil samples and litter were analyzed for [C], [N], $\delta^{13}C$, and $\delta^{15}N$ on a ThermoFinnigan MAT 253 Continuous Flow System interfaced with a Costech 4010 elemental analyzer EA at the University of Kansas Keck Paleoenvironmental and Environmental Stable Isotope Laboratory (KPESIL). Precision for this instrument is better than ± 0.42‰ for $\delta^{15}N$, and better than ± 0.22‰ for $\delta^{13}C$.

*Microbial Biomass and Soil EOC and EON*

We determined microbial biomass C and N (MBC and MBN) using fumigation-extraction (Brookes et al., 1985; Doyle et al., 2004). We exposed 2.5 g of soil from each plot to chloroform for 24 h. After venting, these soils were extracted with 12.5 ml of 0.5 M $K_2SO_4$ and filtered through 20-25 µm pore size filter paper (Whatman #4) in order to capture all EOC and EON that is readily available for breakdown by EEA. Fumigated and un-fumigated extracts were subjected to persulfate digestion (Doyle et al., 2004). We used a NaOH concentration of 0.5 M to increase the final digest pH, to ensure retention of dissolved inorganic C in solution until analysis. The concentration of dissolved inorganic C in the digested extracts was determined using a diffusion block (Doyle et al., 2004) on a Lachat auto-analyzer. Nitrate concentrations in the extracts were determined via cadmium reduction. Microbial biomass C and N were calculated as EOC or total dissolved N in fumigated soils minus EOC or total dissolved N in un-fumigated soils, divided by an efficiency factor of 0.45 (Jenkinson et al., 2004). Glycine and nicotinamide standards were included in each analysis to check for digestion efficiency and potential losses of dissolved inorganic C as $CO_2$ from the digested extracts. We used measurements in the un-fumigated extracts to determine soil EOC and to calculate soil EON as total dissolved N minus inorganic N. We corrected soil EOC and EON for digestion efficiency when necessary using the above standards.

*Extracellular Enzyme Assays*

We analyzed the activity of eight enzymes, β-1,4-glucosidase (BG), α-1-4-glucosidase (AG), cellobiohydrolase (CBH), β-1-4-N-acetylglucosaminidase (NAG), β-1-4-xylosidase (BXYL), leucine amino peptidase (LAP), phenol oxidase and peroxidase. These enzymes are

representative of a wide range of substrate utilization and were assayed for each of the 5 sampling dates. Throughout this study, we consider polymeric substrates such as cellulose, hemicellulose, starch, chitin and peptides as relatively labile, and lignin and humic substrates relatively recalcitrant due to their amorphous arrangement, aromatic ring structures (Sinsabaugh et al., 2002; Fog, 1988). The activities of BG, AG, CBH, NAG, BXYL, PHOS and LAP were determined using corresponding substrates fluorescently labeled with methylumbelliferone (MUB) or methyl coumarin (MC) as per Saiya-Cork et al. (2002). Soils were homogenized with 50 mM NaAcetate buffer at pH 5.5, which is the average pH for the four treatments. After incubating for approximately 18 h, we added 10 µl 0.5M NaOH to each well and then measured fluorescent values on a SpectraMax Gemini XS Fluorescence Platereader (Molecular Devices, Menlo Park, CA, USA) with 365 nm excitation and 460 nm emission filters.

Determination of peroxidase and phenol oxidase activity was performed using 3,4-dihydroxy-L-phenylalanine (L-DOPA) as the substrate in 96-well microplates (Saiya-Cork et al., 2002). We added 25 mM L-DOPA to two of three columns used per soil sample, and the third column received buffer. Columns of buffer-only and buffer plus L-DOPA were used as a negative control. To measure peroxidase activity we also added 10 µl of a 0.3% hydrogen peroxide solution to each well. Plates were read on a SpectraMax 340PC 384 Absorbence Platereader (Molecular Devices, Menlo Park, CA, USA) at 460 nm.

*Microbial Biomass and Dissolved Organic $\delta^{13}C$ and $\delta^{15}N$ Analyses*

To obtain the $\delta^{13}C$ and $\delta^{15}N$ values of MBC, MBN, EOC and DON, fumigated and un-fumigated extracts from September 24, 2007 were dried at 60°C and then ground to a fine powder using a mortar and pestle (Dijkstra et al., 2006). Dried extracts were then weighed out

into silver capsules for analysis at KPESIL as described for soils and litter above. Using microbial biomass C and N and EOC and TDN measurements obtained through colorimetric analyses as described above, we calculated $MB\delta^{13}C$ and $MB\delta^{15}N$ following Dijkstra et al. (2008) as:

$MB\delta^{13}C = (EO\delta^{13}C_{fum} * EOC_{fum} - EO\delta^{13}C_{unfum} * EOC_{unfum}) / (EOC_{fum} - EOC_{unfum})$ and

$MB\delta^{15}N = (TD\delta^{15}N_{fum} * TDN_{fum} - TD\delta^{15}N_{unfum} * TDN_{unfum}) / (TDN_{fum} - TDN_{unfum})$.

Fertilizer application has significantly increased the abundance of C3 relative to C4 species (Foster et al., 2009) and has lowered the $\delta^{15}N$ of plant available N, so that the isotopic composition of litterfall in fertilized plots exhibits significantly lower $\delta^{13}C$ and $\delta^{15}N$ values than in unfertilized plots (Tiemann and Billings, 2008). Therefore, MB $\delta^{13}C$ and $\delta^{15}N$ are presented relative to EOC and bulk soil $\delta^{13}C$, or EON and bulk soil $\delta^{15}N$, using:

$\Delta^{13}C\text{-}MB_{EOC} = \delta^{13}C_{MB} - \delta^{13}C_{EOC}$

$\Delta^{13}C\text{-}MB_{soil} = \delta^{13}C_{MB} - \delta^{13}C_{soil}$ and analogous calculations for MB $\delta^{15}N$.

*Statistical Analyses*

To determine the effects of treatment, date, and their interaction on EEA, microbial biomass C and N, inorganic N, EOC, and EON, we perform repeated measures ANOVA using PROC MIXED in SAS (SAS v8.2, SAS Institute, Cary, NC, USA). Covariance structure was modeled using the spatial powers law. We generated least-squares means tables for all pairwise comparisons with Tukey-Kramer adjusted p-values in order to minimize the experimentwise error rate. Using SAS's PROC GLM, we performed two-way ANOVA to determine the effects of fertilization, haying and their interaction on $\Delta^{13}C\text{-}MB_{EOC}$, $\Delta^{13}C\text{-}MB_{soil}$, $\Delta^{15}N\text{-}MB_{EON}$, $\Delta^{15}N\text{-}MB_{soil}$, soil C, soil N, soil C:N ratio, cellulose, lignin, lignin:N ratio, litter C, litter N, litter C:N

ratios, and the $\delta^{13}C$ and $\delta^{15}N$ of bulk soil. We again used LS means tables for pairwise comparisons with Tukey-Kramer adjusted p-values. Correlation analyses were performed in PROC CORR and normality tests and calculation of measures of skewness and kurtosis were performed in PROC CAPABILITY. Results of correlation analyses are presented as Pearson correlation coefficients. Activities BG, AG, CBH and BXYL did not vary by date so we averaged these measures across the growing season for correlation analyses with $\Delta^{13}C$-MB and $\Delta^{15}N$-MB or measures of litter quality, soil C:N, litter C:N, and litter lignin:N ratios as measured at the end of the growing season (n = 32). To separate the fertilization induced effects of increased inorganic N availability versus increased litter quality and quantity on BG, AG, CBH and BXYL, we performed repeated measures ANCOVA in SAS PROC MIXED with inorganic N as covariate. Each model initially contained interaction terms between the covariate and fixed effects. When these interactions were non-significant we assumed homogeneity of the regression line slopes and re-ran the analyses without these interactions in the model. Data were transformed when necessary to achieve a normal distribution. Differences were considered significant at $\alpha$=0.05 unless otherwise noted, and all errors presented are one standard error of the mean.

## RESULTS

### *Measures of Substrate Quantity and Quality*

Measures of inorganic N were significantly influenced by a treatment*date interaction. Inorganic N availability in the fertilized plots ranged from 7.8 to 56.0 mg-N $kg^{-1}$ soil, was highest in the fertilized plots when first measured following fertilizer application, and declined

throughout the 2007 growing season (Figure 1a). In unfertilized plots, N availability ranged from 6.2 to 31.9 mg-N $kg^{-1}$ soil. Within the treatment*date interaction, inorganic N was not significantly different between treatments prior to spring fertilizer application. On the first sampling date post fertilization and on August 14 and September 24, 2007, fertilized plots had significantly higher inorganic N than unfertilized plots (Figure 1a). There were significant treatment and date effects on soil EOC (Figure 1b). Fertilized plots had significantly more EOC across the growing season that unfertilized plots ($0.18 \pm 0.01$ vs. $0.14 \pm 0.01$ g-C $kg^{-1}$ soil, Figure 1b).

Bulk soil total C was higher in fertilized than unfertilized plots (Table 1), and bulk soil N was also higher in fertilized plots compared to unfertilized plots (Table 1). Soil C:N ratios were significantly higher in the unfertilized compared to fertilized plots but were also affected by a significant fertilization*haying interaction such that H plots had higher soil C:N than E plots, though this effect was small (Table 1; E, $11.00 \pm 0.10$; H, $11.38 \pm 0.08$). The bulk soil $\delta^{13}C$ reflects differences in plant community composition driven by fertilization at this site (Foster et al., 2009). Soil $\delta^{13}C$ was significantly lower in the fertilized compared to unfertilized plots ($-23.8 \pm 0.1$ vs. $-22.8 \pm 0.3$ ‰) while the $\delta^{15}N$ of the bulk soil was not significantly affected by treatment.

Plant litter quality was significantly altered with fertilization but has not been significantly influenced by haying. Litter C and N concentration was significantly greater in the fertilized compared to unfertilized plots; litter C:N was significantly greater in the unfertilized plots than the fertilized plots, as were litter lignin:N ratios (Table 1).

*Microbial Biomass C and N*

Treatment did not affect MBC, which ranged from 0.36 to 2.53 g C $kg^{-1}$ soil, but there were significant differences between sampling dates (Figure 2a). Microbial biomass N also varied significantly by date and with treatment (Figure 2b) such that fertilized plots had greater MBN than unfertilized plots (85.6 ± 3.6 vs. 64.7 ± 1.9 mg N $kg^{-1}$ soil). The ratio of microbial biomass C:N was lower in F and FH plots (20.9 ± 1.2 and 21.0 ± 1.2) than in H and E plots (27.7 ± 1.6 and 24.7 ± 1.4). There were no significant treatment*date interactions.

*Extracellular Enzyme Activity*

Extracellular enzyme activities related to labile C substrate release (BG, AG, CBH, and BXYL) were an average of 1.24 times higher in the F and FH plots, compared to the unfertilized plots across the growing season ($P < 0.0001$; Figure 3a-d), with no significant differences between sampling dates. When inorganic N availability was included as a covariate, these same enzyme activities were still significantly greater in fertilized compared to unfertilized plots (BG, $P < 0.0001$; AG, $P < 0.0001$; CBH, $P < 0.0001$; BXYL, $P = 0.05$). These activities averaged across the growing season were significantly correlated with measures of substrate quality, litter C:N and lignin:N ratios (n = 32; Table 2). There was an autocorrelation between inorganic N and these EEAs so we analyzed these data by treatment and found no significant correlations between inorganic N and BG, AG, CBH or BXYL for any treatment. We calculated a ratio of C:N acquisition EEA by summing BG, AG, CBH, and BXYL activities and dividing by the sum of LAP and NAG activity; fertilized plots exhibited a higher ratio of C:N acquisition across all sampling dates (2.52 ± 0.05 vs. 2.30 ± 0.04).

Oxidative enzyme activity, important for the breakdown of relatively recalcitrant substrates, was not affected by treatment but varied significantly with date (Fig 4a,b). Phenol

oxidase activity across the growing season and across treatments was weakly correlated with EOC (r = 0.18, $P = 0.02$) but not with any other of our measures of substrate quantity or quality, and we found no significant correlations between peroxidase activities and measures of substrate quantity or quality.

Activities of N acquisition enzymes, LAP and NAG, were not significantly different between treatments, but varied with sampling date (Figure 5a,b). LAP activity was negatively correlated with EOC (r = -0.28, $P = 0.0004$) while NAG exhibited a weak positive correlation with inorganic N (r = 0.15, $P = 0.06$). LAP activity averaged across the growing season was negatively correlated with multiple measures of substrate quality, including litter lignin:N (r = -0.53, $P = 0.002$) and soil C:N (r = -0.44, $P = 0.01$), and weakly correlated with litter C:N (r = -0.32, $P = 0.07$). There were no significant correlations between NAG and measures of substrate quality.

*$\Delta MB^{13}C$ and $\Delta MB^{15}N$*

There were significant effects of fertilization on the magnitude of the difference between $\delta^{13}C$ and $\delta^{15}N$ of multiple substrates and microbial biomass (Fig. 6). $\Delta^{13}C\text{-}MB_{EOC}$ and $\Delta^{13}C\text{-}MB_{soil}$ were lower in fertilized ($0.3 \pm 0.1$ and $-0.7 \pm 0.1$‰) compared to unfertilized ($1.5 \pm 0.2$ and $0.6 \pm 0.2$‰) plots. Both $\Delta^{13}C\text{-}MB_{EOC}$ and $\Delta^{13}C\text{-}MB_{soil}$ were significantly and negatively correlated with inorganic N, EOC, BG, AG, CBH and LAP and positively correlated with litter lignin:N ratios (Table 3). $\Delta^{15}N\text{-}MB_{EON}$ and the $\Delta^{15}N\text{-}MB_{soil}$ exhibited patterns that generally contrasted with those of $\Delta^{13}C\text{-}MB_{EOC}$ and $\Delta^{13}C\text{-}MB_{soil}$. $\Delta^{15}N\text{-}MB_{EON}$ and the $\Delta^{15}N\text{-}MB_{soil}$ were significantly higher in fertilized ($1.7 \pm 0.6$ and $0.0 \pm 0.5$‰) compared to unfertilized plots ($0.1 \pm 0.5$ and $-1.3 \pm 0.2$‰), and were positively correlated with inorganic N, EOC, BG, AG, CBH, and NAG and negatively correlated with litter lignin:N ratios (Table 3). There were no

significant correlations between measures of microbial biomass $^{13}C$ or $^{15}N$ enrichment and oxidative EEA.

## DISCUSSION

This study helps elucidate the microbially mediated fate of enhanced plant biomass inputs with fertilization in these mineral soil profiles. The data suggest that the more indirect effects of N addition – increases in substrate quality – were more closely associated with EEA than increases in inorganic N availability. Though we observed general trends of depressed EEA with haying in the fertilized plots (F vs. FH; Fig. 3), differences in enzyme activities between hayed and non-hayed plots were not significant. In addition, the isotopic composition of both microbial biomass C and N relative to the isotopic composition of EOC and EON and bulk soil C and N was significantly influenced by fertilization. We observed that the level of microbial biomass $^{13}C$ and $^{15}N$ enrichment relative to available substrates was significantly related to the rates of labile C acquisition enzyme activity. As discussed below, these results have important implications for our understanding of soil C and microbial community dynamics.

### *Potential Microbial C Acquisition with Increasing N Availability and Substrate Quality*

Though EEA was not significantly correlated with inorganic N concentrations in any of the treatments, we observed consistent, increased activity of multiple enzymes associated with labile C acquisition (BG, AG, CBH, BXYL) in fertilized grassland plots relative to E and H plots, where substrate quantity and quality (as measured by commonly used metrics, C:N and lignin:N ratios) were lower. These results are consistent with several other grassland studies that report increased activity of one or several enzymes that break down relatively labile C substrates

with N addition (Ajwa et al., 1999, BG; Stursova et al., 2005, BG and BXYL; Zeglin et al., 2007, CBH). There were no treatment effects associated with oxidative EEA, similar to other grassland studies (Stursova et al., 2005; Zeglin et al., 2007). Activity of these enzymes was significantly higher with fertilization even with inorganic N included as a covariate in ANCOVA analyses. In conjunction with no increase in MBC with fertilization, these results suggest that the indirect effects of higher litter quality with fertilization are more important drivers of SOM decomposition in these soils than enhancement of inorganic N availability.

The conclusion that indirect effects of increased substrate quality with fertilization appear to be more important determinants of EEA than inorganic N availability is a conclusion consistent with results from Hobbie (2005), as well as studies in other systems reporting that plant tissue quality can be an important determinant of SOM decomposition rates and EEA responses to N additions (Sinsabaugh et al., 2005; Gallo et al., 2005; Dijkstra et al., 2004). In forest studies, where litter quality varies due to variation in the dominant vegetation type, increases in both labile and recalcitrant C acquisition EEA with N addition can occur where litter is of higher quality relative to other litter types in these same studies (Sinsabaugh et al., 2002; Carreiro et al., 2000). In areas with relatively low quality litter in these same studies, labile C acquisition EEA can increase with N additions to a lesser degree or not at all, and recalcitrant C acquisition EEA tends to be depressed, emphasizing the role of litter quality in determining EEA response to inorganic N inputs (Sinsabaugh et al., 2002; Carreiro et al., 2000). We saw no evidence of the depressive effects of N addition on oxidative enzyme activity in this study. This observation coupled with the significant increases seen in labile C acquisition EEA, in spite of no increase in microbial biomass C in the fertilized plots, suggests that fertilization has promoted C

acquisition efforts per unit microbial biomass, and that these grasslands may be more susceptible than some forests to soil C losses with increased N availability.

*Stable Isotopes of C and N as Indicators of Microbial Activity*

Isotopic enrichment of microbial biomass C relative to available substrates may offer further evidence that microorganisms in fertilized plots exploited the enhanced SOC availability associated with fertilization. Increases in microbial $^{13}C$ enrichment relative to two available substrate categories (EOC and bulk soil) have been observed and reported as a reflection of microbial fractionation during litter decomposition, but this $MB^{13}C$ enrichment has not been linked with C availability (Dijkstra et al., 2006; Coyle et al., 2009). We found that both $\Delta MB^{13}C_{EOC}$ and $\Delta MB^{13}C_{soil}$ measured in the current study were higher in unfertilized than fertilized plots. This is consistent with greater microbial use of fertilizer-enhanced SOC pools; Billings et al., (2006) found increased SOC with fertilization at this site residing in the most labile soil fraction, which was also the most $^{13}C$-deplete fraction. If microbes in the fertilized plots took advantage of this larger pool of relatively labile C, they would exhibit lower $\Delta MB^{13}C_{EOC}$ and $\Delta MB^{13}C_{soil}$ when compared to microbes in unfertilized plots, as we observed. Though not conclusive, these data are also consistent with enhanced microbial use of older C substrates in unfertilized compared to fertilized plots, given that older SOC pools typically exhibit relatively greater $^{13}C$ enrichment (Ehleringer et al., 2000; Billings, 2006; Coyle et al., 2009). In conjunction with the observed lower values of $\Delta MB^{13}C_{EOC}$ and $\Delta MB^{13}C_{soil}$ in fertilized plots, we also observed higher levels of labile C acquisition EEA, nutrient availability and litter quality. Together, these data indicate that the microorganisms in fertilized plots have likely

increased their acquisition of labile C and that these efforts target the larger labile soil C pool produced through fertilization during the past 7 years of treatment.

In contrast with $\Delta MB^{13}C_{EOC}$ and $\Delta MB^{13}C_{soil}$, $\Delta^{15}N\text{-}MB_{EON}$ and $\Delta^{15}N\text{-}MB_{soil}$ were significantly higher in fertilized than in unfertilized plots. These data suggest that microbes may have access to pools of $^{15}N$-enriched N in fertilized plots in spite of the relatively $^{15}N$-deplete fertilizer additions, and/or that fertilization may have influenced the relative degrees of microbial assimilation and dissimilation of N. Given the large effluxes of $N_2O$ recorded with fertilization at this and many other grassland sites (Tiemann and Billings, 2008; Jones et al., 2005; Mosier et al., 1998) and the $^{15}N$-depleted status of $N_2O$ relative to pools of inorganic N (Well et al., 2006), $^{15}N$-enrichment of microbially available N seems feasible, though it remains unclear why microbially available N would become $^{15}N$-enriched beyond the levels observed in unfertilized plots.

The relative availability of C and N, and the degree of microbial N dissimilation associated with relative substrate availability, provides another means of interpreting $\Delta^{15}N\text{-}MB_{DON}$ and $\Delta^{15}N\text{-}MB_{soil}$ data. These data are consistent with predictions of microbial biomass $\delta^{15}N$ relative to available substrates proposed by Dijkstra et al. (2008), who suggest that microbial biomass will become $^{15}N$-enriched under conditions of low C availability relative to N availability. Data in the current study are consistent with enhanced dissimilation of N by soil microbial communities in fertilized plots as they experience a relatively low C:N (Dijkstra et al., 2008) compared to communities in unfertilized plots. Such a phenomenon would result in greater $^{15}N$-enrichment of microbial biomass in fertilized plots, as $^{15}N$-depleted compounds are dissimilated. Extracellular enzyme data support the idea that microorganisms are relatively more C than N limited in the fertilized plots. The ratio of labile C:N acquisition EEA, greater in the

fertilized than unfertilized plots, implies greater microbial effort devoted to acquiring C than N in the microbial communities undergoing fertilization. Though not conclusive, these data provide independent confirmation of Dijkstra et al.'s (2008) model, and reinforce the idea that $\delta^{15}N$ of microbial biomass may serve as a valuable indicator of relative C vs. N limitation of soil microbial communities.

*Microbial Community Dynamics and the Fate of SOC*

Substrate quantity or quality had little measurable effect on microbial biomass C. Instead, MBC varied by sampling date, suggesting that the size of the microbial community across a growing season is more closely related to environmental factors than substrate availability or quality. Previous grassland studies with long-term N addition treatments have also reported temporal variability in microbial biomass C, but these same studies find contrasting effects of fertilization (Garcia and Rice, 1994; Lovell et al., 1995; Bardgett et al., 1999). Garcia and Rice (1994), working in a mesic tallgrass prairie similar to our study site, found no significant differences in microbial biomass C between fertilized and unfertilized soils over 2.5 y of measurements, while Lovell et al. (1995) and Bardgett et al. (1999), working in a New Zealand grassland, found decreased microbial biomass C in fertilized relative to unfertilized soils over a year of measurements. Other studies reporting increases in EEA associated with N addition have also observed no change in microbial biomass with fertilization (Sinsabaugh et al., 2004; Zeglin et al., 2007), similar to the current study. Our data, in conjunction with these studies, suggest that N addition may release microorganisms from N limitation for enzyme production, but may not necessarily lead to evident increases in growth yields.

If soil microorganisms invest more energy in producing enzymes that aid in the acquisition of C, why might that additional C not be found in the microbial biomass, as our data indicate? Carbon use efficiency of soil microorganisms may have decreased due to the enhanced C availability associated with fertilization, a result reminiscent of plant studies that find lower nutrient use efficiency with increased nutrient availability (Vitousek, 1982; Bridgham et al., 1995). However, given the added N availability this seems counterintuitive. Fertilization also could induce shifts in microbial community structure towards less efficient organisms. A shift in microbial community structure, favoring organisms with high growth rates, low growth yields, and constitutive production of enzymes with increases in SOC availability (Fierer et al., 2007) could explain increases in EEA without concurrent increases in microbial biomass C. An alternative explanation is that our measure of microbial biomass C may be unable to capture possible increases in microbial productivity in fertilized plots due to concurrent increases in microbial grazing (Frey et al., 2001). Though challenging to detect using the methods employed here, enhanced EEA with no increase in MBC is consistent with increased flows of organic matter through the microbial loop within the soil profile, which likely would induce increases in rates of microbial productivity unless C use efficiency has been altered.

Previous work at this site reports more than a doubling of organic matter inputs with fertilization (Billings et al., 2006; Foster et al., 2009). In contrast, mineral SOC with fertilization has 1.1 times the SOC of unfertilized plots (Table 1). We did not quantify EEA in litter layers in these plots, but fertilized plots' hydrolytic C EEA rates of 1.24 times those in unfertilized soils help to explain the fate of enhanced OM inputs at this site. We cannot directly link measurements of EEA in laboratory assays to C flows at the ecosystem scale, but examination of the relative changes in mineral soil EEA compared to alterations in OM inputs to soil profiles

can provide a greater understanding of the fate of SOC enhancements in mesic grasslands, and these ecosystems' subsequent potential to retain SOC.

*Conclusions*

Predicting how various forms of N influence organic matter decomposition has been a perplexing question, with conflicting answers, in ecosystem science for decades (Fog, 1988; Hobbie, 2005). In this mesic grassland, we observed positive relationships between increasing substrate quality and labile C acquisition EEA, but no relationship between substrate quality and recalcitrant C or N acquisition EEA. EEA did not vary with inorganic N availability for any enzyme assessed. These results reinforce that organic matter quality – even after it has become mineral-associated organic matter – is a critical determinant of microbial processes governing decomposition, and highlight the indirect effects of N additions on different groups of extracellular enzymes. At this site, increases in OM inputs to the soil profile with fertilization exceed increases in mineral SOC. The EEA data reported here help depict why this is so, and raise questions about the future of soil organic C stocks in this and other mesic grassland regions. Are the observed increases in EEA indicative of net C losses from these systems over longer time frames? To date, there is no clear link between measures of soil respiration and microbial biomass and measures of increasing EEA with N addition (Sinsabaugh et al., 2005, Waldrop et al., 2004) that are comparable in breadth and depth to our knowledge of the links between plant productivity and N availability (Chapin et al., 2002). To predict how alterations in EEA with varied quality of mineral SOM will influence C cycling on an ecosystem level, we need a better mechanistic understanding of the linkages between microbial community composition and function, and how to relate these measures to large-scale fluxes.

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**Table 1.** Soil and litter C and N concentrations and isotope values by experimental fertilization or haying treatments. Lower case letters indicate significant differences between treatments.

| | ***Fertilized (F and FH)*** | ***Un-fertilized (H and E)*** | ***Hayed (FH and H)*** | ***Non-hayed (F and E)*** |
|---|---|---|---|---|
| **Bulk soil C (mg $g^{-1}$)** | 21.2 ± 0.05[a] | 19.8 ± 0.04[b] | 20.7 ± 0.4 | 20.2 ± 0.6 |
| **Bulk soil N (mg $g^{-1}$)** | 2.0 ± 0.004[a] | 1.8 ± 0.003[b] | 1.9 ± 0.04 | 1.8 ± 0.04 |
| **Soil C:N** | 10.8 ± 0.07[b] | 11.2 ± 0.08[a] | 11.0 ± 0.1 | 10.0 ± 0.07 |
| **Bulk soil $\delta^{13}C$ (‰)** | -23.8 ± 0.1[b] | -22.3 ± 0.3[a] | -22.8 ± 0.3 | -23.2 ± 0.2 |
| **Bulk soil $\delta^{15}N$ (‰)** | 3.2 ± 0.1 | 3.2 ± 0.1 | 3.1 ± 0.1 | 3.3 ± 0.1 |
| **$\delta^{13}C$-EOC (‰)*** | -24.8 ± 0.06[b] | -23.2 ± 0.2[a] | -23.7 ± 0.3[a] | -24.3 ± 0.2[b] |
| **$\delta^{15}N$-TDN (‰)** | 1.5 ± 0.2 | 1.8 ± 0.3 | 1.8 ± 0.3 | 1.5 ± 0.1 |
| **$\delta^{13}C$-MBC (‰)*** | -24.5 ±0.08[b] | -21.7 ± 0.3[a] | -22.7 ± 0.5[a] | -23.5 ± 0.3[b] |
| **$\delta^{15}N$-MBN (‰)*** | 5.0 ± 1.6[a†] | 1.8 ± 0.3[b†] | 2.3 ± 0.4 | 4.6 ± 1.6 |
| **Litter C (mg $g^{-1}$)** | 416.4 ± 3.1[a] | 396.9 ± 3.4[b] | 404.0 ± 3.8 | 409.3 ± 4.2 |
| **Litter N (mg $g^{-1}$)** | 16.3 ± 0.6[a] | 8.1 ± 0.4[b] | 11.4 ± 1.0[b] | 13.0 ± 1.2[a] |
| **Litter C:N (mg $g^{-1}$)** | 26.0 ± 0.9[b] | 50.5 ± 2.3[a] | 40.2 ± 3.7 | 36.3 ± 3.5 |
| **Litter Lignin:N** | 7.6 ± 0.3[b] | 14.1 ± 0.5[a] | 11.2 ± 1.0 | 10.5 ± 0.8 |
| **Litter $\delta^{13}C$ (‰)** | -28.0 ± 0.1[b] | -20.2 ± 0.9[a] | -24.2 ± 1.1 | -24.0 ± 1.2 |
| **Litter $\delta^{15}N$ (‰)** | -1.6 ± 0.1[a] | -3.8 ± 0.2[b] | -2.5 ± 0.3 | -2.9 ± 0.3 |

* significant interaction between fertilization and haying
† $P = 0.06$

**Table 2.** Pearson correlation coefficients and *P*-values for relationships between hydrolytic extracellular enzyme activities and measures of substrate quality (n=32). See text for enzyme abbreviations.

| Enzyme | Litter C:N | Litter Lignin:N |
|---|---|---|
| β-1,4-glucosidase (BG) | -0.58<br>*0.0005* | -0.42<br>*0.02* |
| α-1-4-glucosidase (AG) | -0.74<br>*<0.0001* | -0.62<br>*0.0001* |
| cellobiohydrolase (CBH) | -0.70<br>*<0.0001* | -0.56<br>*0.0008* |
| β-1-4-xylosidase (BXYL) | -0.32<br>*0.07* | -0.19<br>*0.29* |

**Table 3.** Correlations of measures of substrate quantity, quality and microbial activity with the enrichment of microbial biomass $^{13}C$ and $^{15}N$ relative to EOC and EON or soil C and N. Pearson correlation coefficients followed by *P* values (n=32). Correlations with *P*-value < 0.05 are emboldened.

| | $\Delta^{13}C\text{-}MB_{EOC}$ | $\Delta^{13}C\text{-}MB_{soil}$ | $\Delta^{15}N\text{-}MB_{EON}$ | $\Delta^{15}N\text{-}MB_{soil}$ |
|---|---|---|---|---|
| Inorganic N | **-0.64** | **-0.62** | 0.32 | **0.40** |
| | *<0.0001* | *0.0002* | *0.08* | *0.03* |
| EOC | **-0.39** | **-0.44** | **0.47** | **0.57** |
| | *0.02* | *0.01* | *0.0064* | *0.0008* |
| Litter Lignin:N | **0.70** | **0.70** | **-0.40** | **-0.48** |
| | *<0.0001* | *<0.0001* | *0.02* | *0.0059* |
| Litter C:N | **0.70** | **0.60** | **-0.36** | **-0.43** |
| | *<0.0001* | *0.0003* | *0.05* | *0.02* |
| BG | **-0.41** | **-0.45** | **0.46** | **0.44** |
| | *0.02* | *0.0091* | *0.0088* | *0.01* |
| AG | **-0.60** | **-0.56** | **0.48** | **0.55** |
| | *0.0003* | *0.0009* | *0.0055* | *0.0014* |
| CBH | **-0.61** | **-0.55** | **0.49** | **0.53** |
| | *0.0002* | *0.0010* | *0.0042* | *0.0023* |
| BXYL | -0.15 | -0.30 | **0.38** | 0.34 |
| | *0.42* | *0.10* | *0.03* | *0.06* |
| LAP | **-0.48** | **-0.34** | -0.01 | 0.03 |
| | *0.0050* | *0.05* | *0.98* | *0.87* |
| NAG | -0.05 | -0.18 | **0.46** | **0.38** |
| | *0.77* | *0.32* | *0.0074* | *0.04* |
| Phenol Oxidase | -0.20 | -0.03 | -0.04 | 0.02 |
| | *0.28* | *0.88* | *0.84* | *0.94* |
| Peroxidase | -0.22 | -0.23 | -0.05 | -0.08 |
| | *0.23* | *0.21* | *0.77* | *0.68* |

**Figure 1.** Inorganic N (a) and extractable organic carbon (EOC; b) for fertilized (F), fertilized and hayed (FH), hayed (H) and early succession grassland (E) plots over the 2007 growing season. Inorganic N was significantly greater in F and FH plots on May 16, July 2, August 14 and September 24, 2007. EOC was significantly greater in F and FH plots across the entire growing season. Error bars represent one standard error of the mean (n = 8).

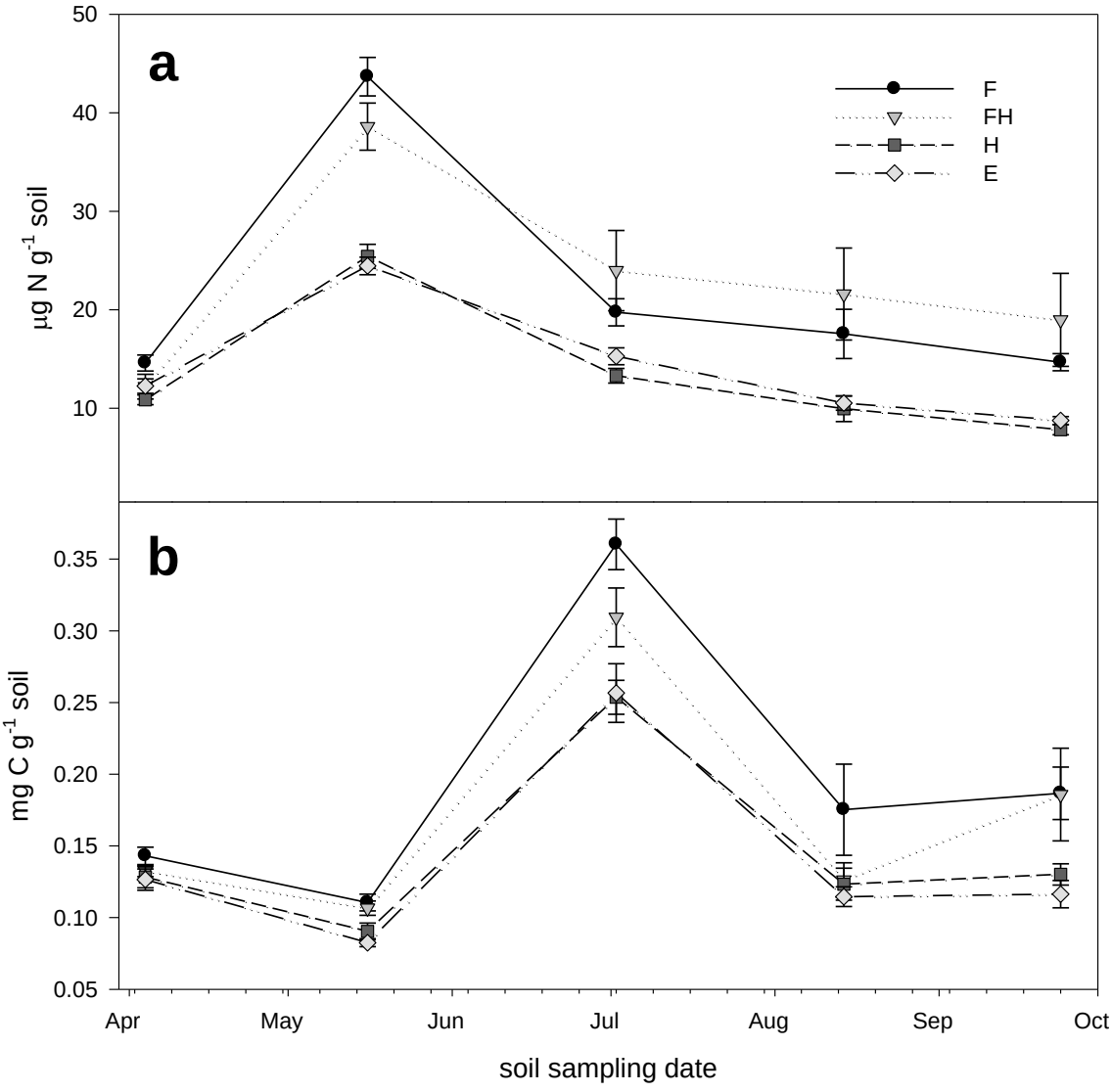


**Figure 2.** Microbial biomass C (a) and microbial biomass N (b) for fertilized (F), fertilized and hayed (FH), hayed (H) and early succession grassland (E) plots across the 2007 growing season. Microbial biomass C was not affected by treatment. Microbial biomass N was significantly higher in F and FH plots across the growing season. Error bars represent one standard error of the mean (n = 8).

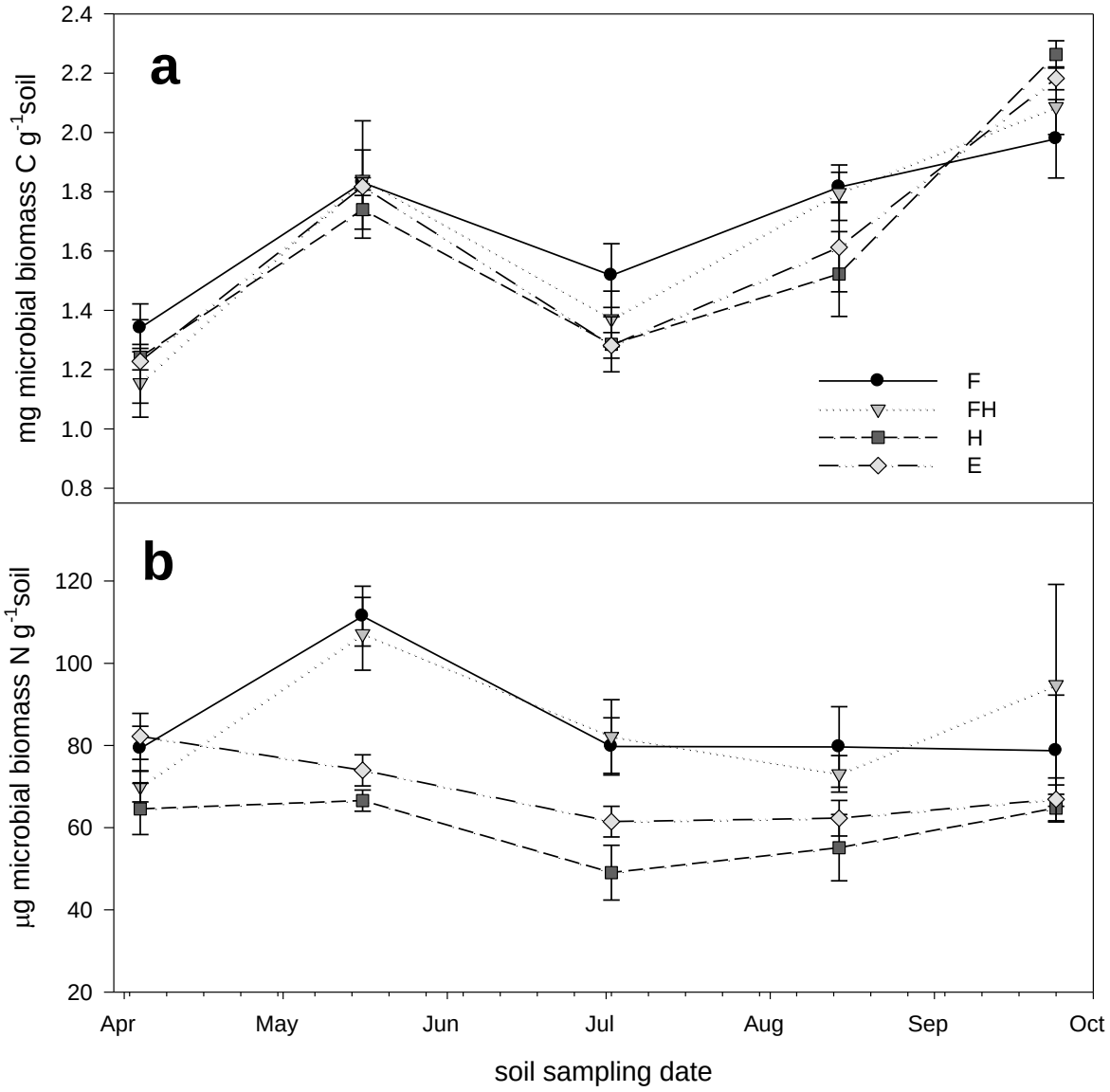


**Figure 3.** Labile C acquisition extracellular enzyme activities, BG (a), AG (b), CBH (c), and AG (d), measurements for fertilized (F), fertilized and hayed (FH), hayed (H) and early succession grassland (E) plots across the 2007 growing season. BG and CBH activity was significantly greater in F and FH plots across the growing season. AG activity was significantly greater in F and FH plots on all dates except April 4, 2007. BXYL activity was not affected by treatment. Error bars represent one standard error of the mean (n = 8).

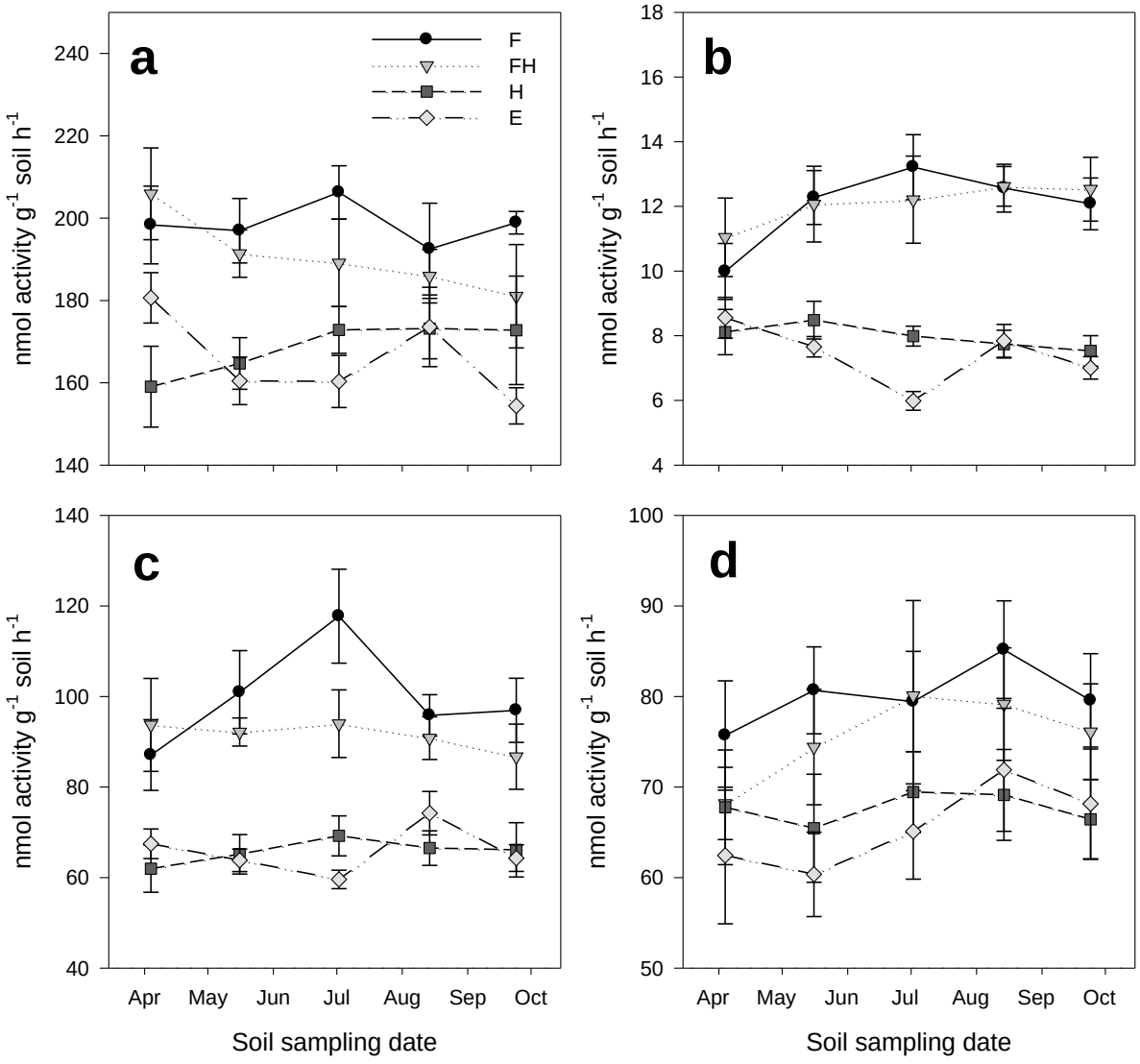


**Figure 4.** Recalcitrant C acquisition extracellular enzyme activities, phenol oxidase (a) and peroxidase (b) measurements for fertilized (F), fertilized and hayed (FH), hayed (H) and early succession grassland (E) plots across the 2007 growing season. Neither phenol oxidase or peroxidase activities were affected by treatment. Error bars represent one standard error of the mean (n = 8).

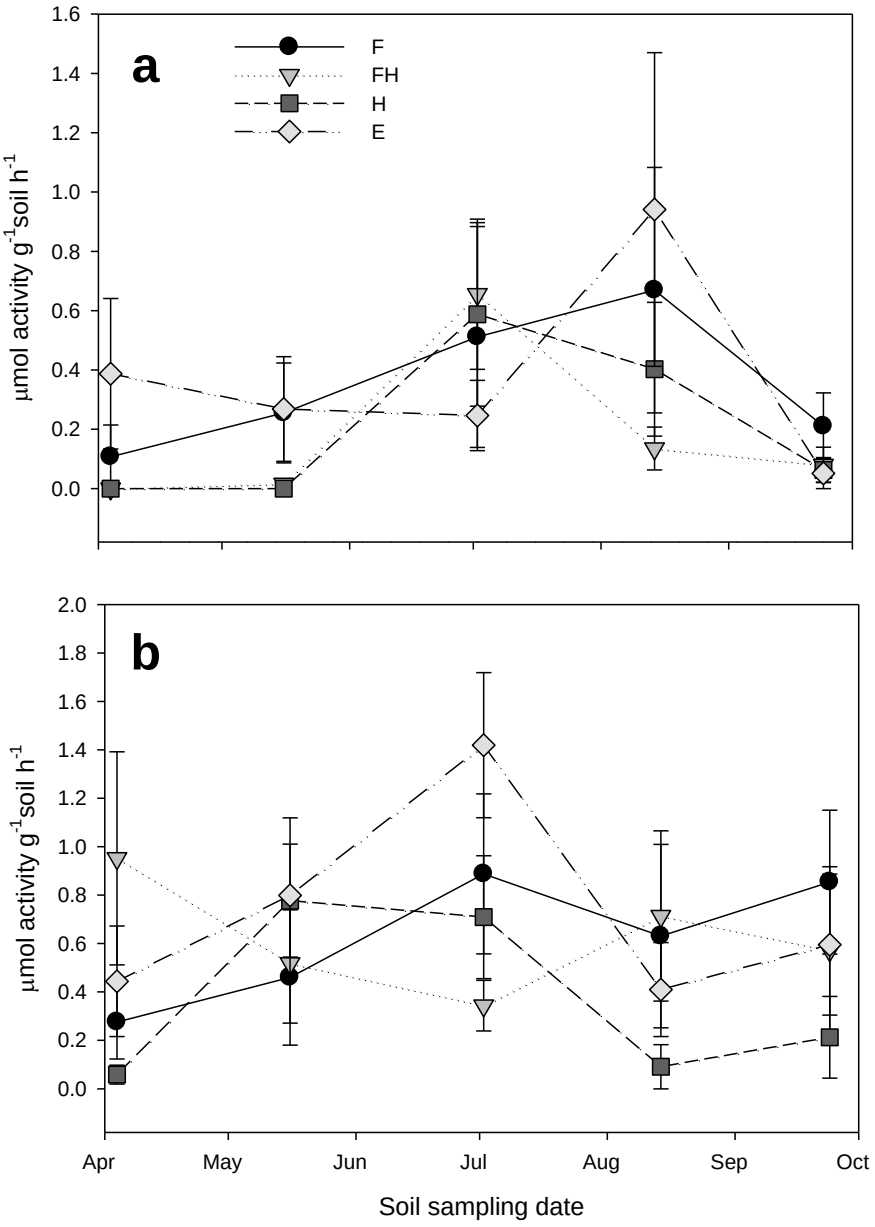


**Figure 5.** Nitrogen acquisition extracellular enzyme activities, LAP (a) and NAG (b), measurements for fertilized (F), fertilized and hayed (FH), hayed (H) and early succession grassland (E) plots across the 2007 growing season. LAP and NAG activities were not affected by treatment. Error bars represent one standard error of the mean (n = 8).

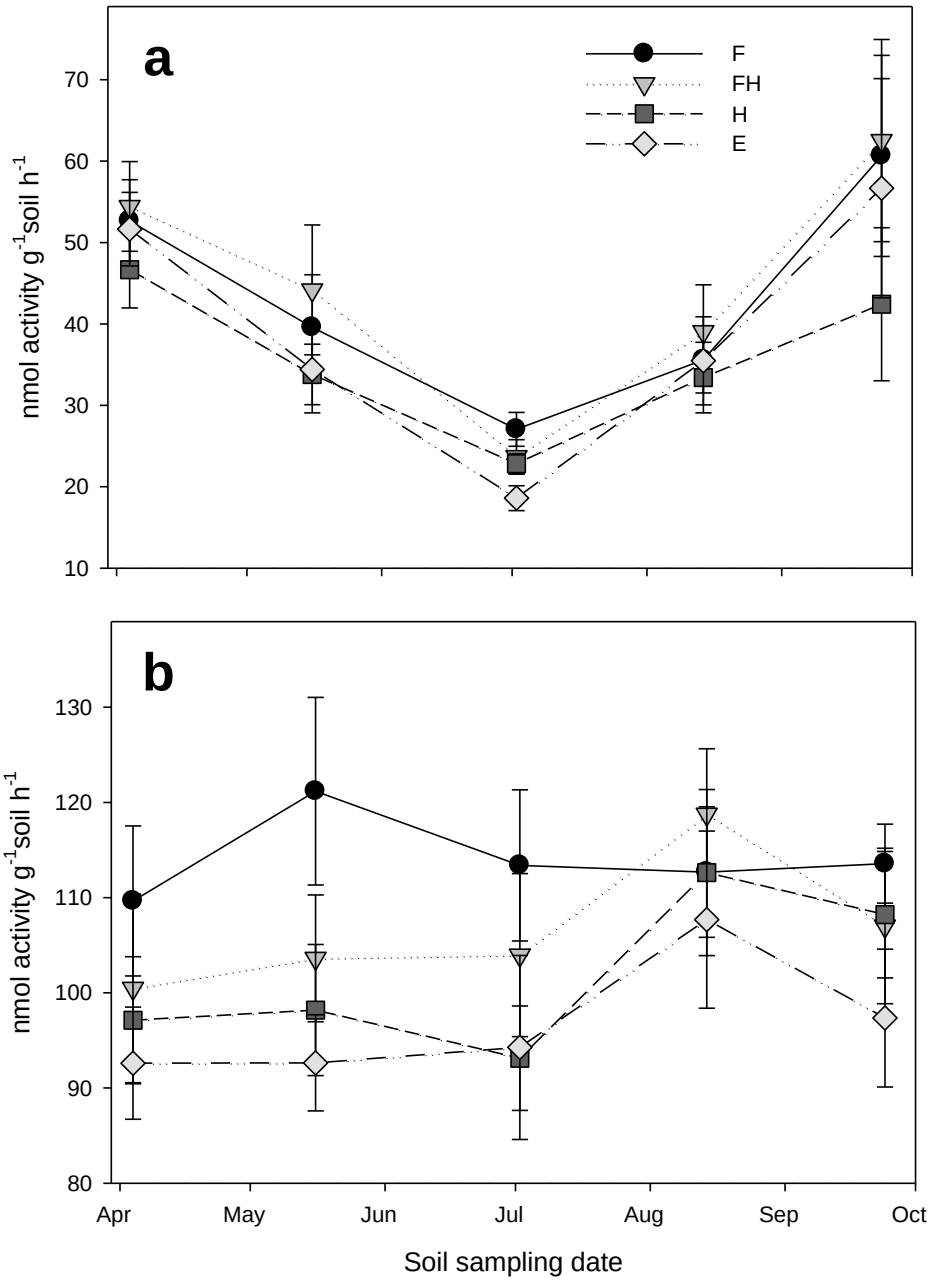
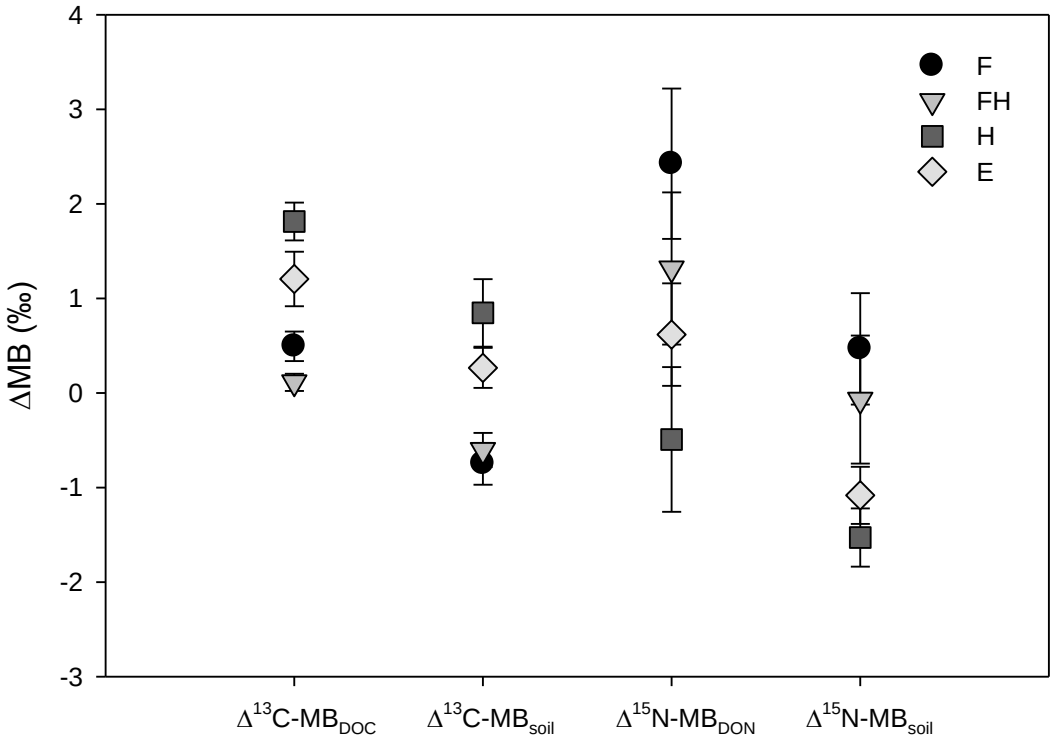


**Figure 6.** Microbial biomass $^{13}C$ and $^{15}N$ enrichments relative to the $\delta^{13}C$ and $\delta^{15}N$ of EOC, bulk soil C, EON, and bulk soil N for fertilized (F), fertilized and hayed (FH), hayed (H) and early succession grassland (E) plots at the end of the growing season. In all cases, there was a significant effect of fertilization but not haying. Error bars represent one standard error of the mean (n = 8).



## CHAPTER 2: Changes in the variability of soil moisture alters microbial community C and N resource use

*Abstract*

Grassland ecosystems contain ~12% of global soil organic carbon (C) stocks and are located in regions where global climate change will likely alter the timing and size of precipitation events, increasing soil moisture variability. In response to increased soil moisture variability and other forms of stress, microorganisms can induce ecosystem-scale alterations in C and N cycling processes through alterations in their function. We explored the influence of physiological stress on microbial communities by manipulating moisture variability in soils from four grassland sites in the Great Plains, representing a precipitation gradient of 485 to 1003 mm $y^{-1}$. Keeping water totals constant, we manipulated the frequency and size of water additions and dry down periods in these soils by applying water in two different, two-week long wetting-drying cycles in a 72 day laboratory incubation. To assess the effects of the treatments on microbial community function, we measured C mineralization, N dynamics, extracellular enzyme activities (EEA) and a proxy for substrate use efficiency. In soils from all four sites undergoing a long interval (LI) treatment for which added water was applied once at the beginning of each two-week cycle, 1.4 to 2.0 times more C was mineralized compared to soils undergoing a short interval (SI) treatment, for which four wetting events were evenly distributed over each two-week cycle. A proxy for carbon use efficiency (CUE) suggests declines in this parameter with the greater soil moisture stress imposed in LI soils from all four different native soil moisture regimes. A decline in CUE in LI soils may have been related to an increased effort by microbes to obtain N-rich organic substrates for use as protection against osmotic shock, consistent with EEA data. These results contrast with similar *in situ* studies of response to increased soil moisture variability and may indicate divergent autotrophic vs. heterotrophic

responses to increased moisture variability. Increases in microbial N demand and decreases in microbial CUE with increased moisture variability observed in this study, regardless of the soils' site of origin, imply that these systems may experience enhanced heterotrophic $CO_2$ release and declines in plant-available N with climate change. This has particularly important implications for C budgets in these grasslands when coupled with the declines in net primary productivity reported in other studies as a result of increases in precipitation variability across the region.

# INTRODUCTION

Global climate models predict altered precipitation regimes across North America in this century associated with increases in average surface temperatures, consistent with recent observations (Karl et al., 2003). Across the US Central Plains, these alterations include an increase in the number and severity of droughts and larger rainfall events between drought periods, with little to no change in annual totals (Easterling et al., 2000; Knapp et al., 2008). In this region, where annual potential evapotranspiration (PET) exceeds annual precipitation totals (Lauenroth and Burke, 1995), soil moisture is a limiting factor controlling both net primary productivity (NPP) and microbial processing of large pools of soil organic matter (SOM), and hence controlling soil carbon (C) and nitrogen (N) cycling (Harper et al., 2005; McCulley et al., 2005).

Investigators exploring how precipitation pulses drive ecosystem C and N cycling have typically focused on deserts and semi-arid grasslands, where the pulse-driven nature of the system is most evident (Austin et al., 2004). However, climate models predict that both relatively xeric and mesic grasslands of North America will experience enhanced precipitation variability in the future (Easterling et al., 2000; Knapp et al., 2008). Understanding these interactions is of particular importance in the grasslands of North America, where soil organic matter (SOM) concentrations are among the highest of all ecosystems (Scurlock and Hall, 1998; Schlesinger, 1997), and where soils have the potential to serve as either a large C source or sink with climate change (Scurlock and Hall, 1998; Knapp et al., 2008). These systems, because of their capacity to store C and their predicted future exposure to enhanced precipitation variability have received significant research attention in recent years (Knapp et al. 2002; Fay et al., 2003; Harper et al., 2005; Fay et al., 2008; Knapp et al., 2008; Heisler-White et al., 2008). However, most of these

studies focus on the influence of altered soil moisture variability on aboveground system productivity, leaving many questions about the fate of that productivity, once incorporated into soil profiles, unaddressed. For example, increased variability of rainfall events can lead to decreased total soil respiration (Harper et al., 2005), but the mechanism by which any declines in heterotrophic soil activity occurs remains unclear.

Multiple studies demonstrate the influence of soil moisture availability and its variability as a key driver of grassland soil biogeochemistry. Rates of soil respiration decrease with mean annual precipitation (MAP) in grasslands of the Great Plains (McCulley et al., 2005), and the frequency and size of precipitation events can cause ecosystem-scale alterations in C and N cycling processes (Knapp et al., 2002; Schimel et al., 2007). Precipitation events, particularly those following periods of drought, can create large flushes of nutrients and soil organic carbon (SOC) by releasing, through diffusion, drought accumulated SOM, inorganic N and microbial necromass (Austin et al., 2004; Schimel et al., 2007; Iovieno et al., 2008; Butterly et al., 2009). This flush of fresh substrate can be followed closely by large pulses of microbial respiration (Fierer et al., 2002; Iovieno et al., 2008; Butterly et al., 2009). In addition, soil moisture fluctuations can also be responsible for significant variation in N uptake and release via microbial function (Fierer and Schimel, 2002; Austin et al., 2004; Schimel et al., 2007). Soil microorganisms combat drought in several ways, all of which require an energetic investment and thus a drain on C resources (Borken and Matzner, 2009; Schimel et al., 2007). One source of drought protection is the manufacture of a layer of polysaccharide-rich mucilage that prevents desication (Borken and Matzner, 2009). Microorganisms also protect themselves against large, negative soil matric and osmotic potentials through the acquisition of protective osmolytes, generally N-rich substrates such as amino acids (Borken and Matzner, 2009; Schimel et al.,

2007). Microorganisms can release these osmolytes quickly when the soil is re-wetted to protect themselves against osmotic pressure and cell lysis. This uptake and release of N-rich resources can affect N cycling on an ecosystem scale (Schimel et al., 2007).

We explored the influence of soil moisture variability on belowground grassland C and N dynamics by conducting incubations of soils obtained from research sites across the precipitation gradient of North America's Central Plains. These soils are relatively C-rich and experience significant variability in moisture content across the growing season that is linked to variation in respiratory C losses (McCulley et al., 2005). Precipitation patterns on the eastern, mesic end of this precipitation gradient have historically exhibited relatively small but frequent rain events, while on the western end of this gradient soil moisture variability is much greater, with longer drought intervals and more extreme rainfall events (Lauenroth and Burke, 1995). As a first step towards understanding how climate change driven alterations in soil moisture variability will alter C and N dynamics in these systems, we exposed these soils to simulated moisture regimes during a laboratory incubation. Throughout the incubation, we measured $CO_2$ released. We also assessed net N mineralized, microbial biomass, and extracellular enzyme activities (EEA) associated with the degradation of C-, N-, and P-rich organic compounds, and calculated multiple proxies for microbial carbon use efficiency (CUE). We hypothesized that as variability in soil moisture increases, microbial C and N resource transformations will reflect a shift from biomass development to protection against moisture stress, and that this will be detectable via proxies for substrate use efficiency. We further hypothesized that soil microbial communities from the eastern, moister end of the precipitation gradient would experience relatively greater resource demands and lower substrate use efficiency with higher soil moisture variability than

microbial communities adapted to life on the western end of the gradient, where ambient variation in precipitation is high and soil moisture levels are typically low.

Though data obtained from *ex situ* soil must be interpreted with caution, laboratory incubations permit us to investigate heterotrophic respiratory responses without the confounding inclusion of plants, and permit environmental control difficult to attain in intact systems. We intended this study to highlight the most important potential mechanisms affecting soil C and N resource use, for further exploration *in situ*. We sought to elucidate the mechanisms by which any changes in belowground microbial transformations of SOM may occur in these systems with changes in moisture variability, and to highlight those measures of SOM transformations likely to serve as bellwethers of changes in the future.

## METHODS

### *Sites*

We chose four sites along the east-west precipitation gradient across Kansas, USA, part of the North American Great Plains (Table 1). The eastern most site, part of the Kansas University Field Station lands (KUFS, W 95°14’35” N 38°10’21”) is in an area where average annual precipitation is 1003 mm. The soils are gravelly silt loams (smectitic, thermic, Typic paleudolls) and vegetation is dominated by the tallgrass prairie species *Andropogon gerardii* and *Sorghastrum nutans*. Our second site, located at the Konza Prairie Long Term Ecological Research site (KNZ, W 96°33’18” N 39°5’2”), has an average annual precipitation of 835 mm and soils that are a mix of silty loams (smectitic mesic Typic natrusolls) and silty clay loams (fine mixed superactive mesic Pachic argiustolls). Dominant vegetation includes *A. gerardii*, *Andropogon scoparium*, *Panicum virgatum* and *S. nutans*. The third site, part of Kansas State

University's Western Kansas Agricultural Research Center, (HYS, W 99°17'46" N 38°50'13") receives an average of 578 mm precipitation yearly and the soils are silt loams (fine-silty, mixed, superactive, mesic, Cumulic haplustolls). It is a mixed grass prairie that includes *A. gerardii*, *A. scoparium*, *Bouteloua gracilis*, *Hesperostipa comata*, *B. curtipendula* and *Pascopyrum smithii*. The western most site, The Nature Conservancy's Smokey Valley Ranch, (SVR, W 100°58'55" N 38°51'50") is a short grass prairie dominated by *B. gracilis* and *Buchloë dactyloides*. It receives on average 485 mm of precipitation annually. The soils are silt loams (fine-silty, mixed, superactive, mesic, Aridic haplustolls). All sites, which are part of actively grazed rangeland, were fenced to exclude cattle and have been burned annually, with the exception of SVR, which is not burned.

*Soil collection*

Soils were collected from all four sites April 13 through April 15, 2008. We used 10 cm diameter by 10 cm beveled PVC to extract three soil cores from each site. The cores were placed in iced coolers and returned to the lab at the University of Kansas where they were stored at 4° C until the start of the incubation. Soils were well-mixed and roots > 2 mm in diameter were removed. A 15 g sub-sample from each core was placed in a 60° C oven for 48 hrs to determine gravimetric soil moisture. This dried soil was then used to determine water holding capacity (WHC) by saturating 5 g dry soil placed in Whatman #4 filter paper fitted into funnels. These were covered to prevent evaporative loss and allowed to drain overnight (Fierer and Schimel, 2002). Before the start of the incubation, soils were conditioned for one week in an incubator set at 20° C at field moisture, which was ~50% WHC for all soils.

*Soil treatments*

Soil from each of the three cores from each site was weighed into pre-weighed 5 cm diameter by 5 cm in length PVC cores fitted on the bottom with coarse filter paper (85 g dry weight). The cores were placed in 1 L Mason jars on top of a layer of glass beads to allow air circulation under the cores. The soils were separated into three treatment groups, with one sample from each core at each site per group so that there were a total of three replicates for each treatment for each site. The treatments consisted of a control, which was kept at 50% WHC throughout the incubation, and two different 2-week long wetting-drying cycles that varied in both frequency and size of the water additions and dry down periods. Long interval soils (LI) were given enough water to bring them to 75% WHC, followed by dry down periods that were two weeks in duration. Short interval soils (SI) received water equal to one quarter of the LI treatment two times per week over each two-week cycle. Thus, for every two-week cycle, both treatments received the same total amount of water, differing only in the timing and size of the individual additions.

We conducted the experiment for a total of six, two-week cycles. Water was applied using a needle and syringe for even coverage. During the dry down periods, *all* soils were gently stirred regardless of treatment, at the same time, in order to turn them over and promote homogeneity of soil moisture throughout the PVC core. While we strove to keep aggregates intact during these soil turnovers, we recognize that this may have influenced soil aggregation, C availability and the composition of the microbial communities, but we prioritized homogeneity of soil moisture within the soil cores. Because all soils were stirred regardless of water addition, we assume that any effects due to stirring were equivalent among treatments. Control jars (maintained at 50% WHC) were covered with parafilm to allow gas exchange while preventing moisture loss during the incubation; all other treatment jars were left open to permit evaporation.

All soils were weighed frequently to determine soil moisture content, and were maintained at 24° C. Sub-samples of all soils were harvested on day 58, the beginning of the last two-week cycle, for analyses described below. At the end of the final cycle (day 72), all soils received one final wetting to bring them to ~75% WHC. This moistening event included the addition of leachate derived from leaf litter, described in detail below, to provide insight into the influence of variability of soil moisture regimes on microbial CUE.

*Soil respiration*

Soil respiration was measured four times per week for all samples, on the day prior to and 2 h after any water additions, regardless of whether or not the soils received water that day. On each sampling day we sampled headspace gas twice, once right after capping the jars and a second time after a period of two to four hours. Gas samples were obtained through septa fitted into the jar lids via needle and syringe, and were analyzed for $CO_2$ concentration on a Varian gas chromatograph using a thermal conductivity detector (Varian, Walnut Creek, CA, USA). The difference between the two measures was used to calculate the rate of C respired. Total C respired over the course of the incubation was calculated by applying the average respiration rate between two sampling days to the time period between those two sampling events.

*Extracellular Enzyme Activity*

We analyzed the activity of ten extracellular enzymes in soils sampled four hours after water addition on day 58 and on day 73, 25 hours after leachate additions. We measured the activities of two cellulases and a hemi-cellulase (β-1,4-glucosidase (BG), cellobiohydrolase (CBH), β-1-4-xylosidase (BXYL)) as well as an enzyme responsible for the breakdown of starch, α-1-4-glucosidase (AG). We also measured a chitinase, β-1-4-N-acetylglucosaminidase (NAG), a peptidase, leucine amino peptidase (LAP), and the activity of phosphate-monoester

phosphohydrolase (PHOS), which releases phosphates from organic matter. The activities of these enzymes were determined using corresponding substrates fluorescently labeled with methylumbelliferone (MUB) or methyl coumarin (MC) added to soil slurries in 96-well microplates as per Saiya-Cork et al. (2002). Soils from KUFS, KNZ and HYS, which were slightly acidic, were homogenized with 50 mM sodium acetate buffer at pH of 6.5, close to the $pH_{H2O}$ of these soils. Soils from SVR, which were slightly alkaline, were homogenized in sodium bicarbonate buffer at pH 7.8. Soils were incubated at 24° C for ~18 h. Fluorescence was determined on a SpectraMax Gemini XS Fluorescence Platereader (Molecular Devices, Menlo Park, CA, USA) with 365 nm excitation and 460 nm emission filters.

We also measured the activities of peroxidase, phenol oxidase and urease using colorimetric assays set up in 96-well microplates (Saiya-Cork et al. 2002). Phenol oxidase and peroxidase activities were measured using the color change associated with the breakdown of the substrate 3,4-dihydroxy-L-phenylalanine (L-DOPA). Urease activity was assessed by measuring the ammonium accumulated through the incubation using the color change produced by addition of ammonium cyanurate and ammonium salicylate. Plates were analyzed on a SpectraMax 340PC 384 Absorbance Platereader (Molecular Devices, Menlo Park, CA, USA) at 460 nm for phenol oxidase and peroxidase and 610 nm for urease.

We group these enzymes into four categories, each representing a critical feature of SOM breakdown. Labile C acquisition enzymes – BG, CBH, BXYL and AG – are responsible for the breakdown of C rich substrates that are relatively easy to access because of their polymeric, uniform structures. Relatively recalcitrant C acquisition enzymes – phenol oxidase and peroxidase – are associated with the breakdown of substrates with polymorphic structures such

as lignin and humic acids. We group NAG, LAP, and urease as N acquisition enzymes, and consider PHOS, a phosphorus acquisition enzyme, separately.

*Soil microbial biomass and extractable soil N*

We estimated microbial biomass C and N (MBC and MBN) using the fumigation-extraction method (Brookes et al., 1985; Doyle et al., 2004). In addition to analyses conducted on pre-incubation soils from each site, soil sub-samples from each treatment and each site were removed 4-6 hours after wetting on day 58 and 25 h after leachate addition at the end of the incubation. We immediately extracted 2.5 g of this soil with 12.5 ml of 0.5 M $K_2SO_4$ while another 2.5 g was directly exposed to chloroform for 24 h. After venting, these soils were also extracted with 12.5 ml of 0.5 M $K_2SO_4$. Fumigated and un-fumigated extracts were subjected to persulfate digestion (Doyle et al., 2004). We used a NaOH concentration of 0.50 M to increase the final digest pH, ensuring the retention of dissolved inorganic carbon (DIC) in solution until analysis. We quantified the concentration of DIC in the digested extracts using a diffusion block (Doyle et al., 2004) on a Lachat auto-analyzer. Nitrate and nitrite concentrations in the extracts were determined via cadmium reduction. Microbial biomass C and N were calculated as fumigated extractable organic C (EOC) or total dissolved N (TDN) minus un-fumigated EOC and TDN, divided by an efficiency factor of 0.45 (Jenkinson et al., 2004). Glycine and nicotinamide standards were included in each analysis to test for digestion efficiency. Extractable organic C concentrations were corrected when necessary using the above standards. We determined soil inorganic nitrogen by quantifying nitrate and nitrite concentrations in un-fumigated soil extracts on a Lachat auto-analyzer using cadmium reduction, and ammonium concentrations on a diffusion block (Willason et al., 1986; Doyle et al., 2004). Extractable organic N (EON) was calculated by subtracting total inorganic N from TDN.

*Carbon use efficiency*

At the end of the incubation, we wanted to determine how exposure to soil moisture variability treatments influenced the efficiency with which microbial communities used an added C substrate. To this end, we added water amended with C substrates from leaf litter leachate (LLC), bringing all soils to 75% WHC regardless of their previous moisture regime (LI or SI). The LLC was obtained by soaking deciduous tree litter, predominantly sugar maple and sweet gum trees, in de-ionized water for 14 days then filtering the leachate through a Whatman #4 filter. The resulting LLC contained 3164 mg $l^{-1}$ dissolved organic C and < 0.1 mg $l^{-1}$ dissolved organic N. We diluted this LLC solution so that final C additions totaled 0.72 mg LLC $g^{-1}$soil with a negligible amount of N (< 0.04 μg $g^{-1}$soil). This amount of added C was enough to replace approximately one-half the total C respired during the previous 72 days with relatively labile, C-rich compounds and enhanced our ability to track changes with previous LI vs. SI treatment in respiration rates and MBC. We consider these LLC additions to more accurately represent features of the complex substrate landscape experienced by soil microorganisms than addition of individual C compounds such as glucose, as is often used in incubation experiments (Rousk and Baath, 2007; Ziegler and Billings, *in press*). After LLC addition at the end of the incubation, soil respiration rates were measured at intervals of 0.5, 1.5, 2.5, 4.5, 6.5, 8.5, 12.5, and 24.5 hours while the jars remained capped.

We used three different methods to estimate microbial CUE after LLC additions. The first method is based on the amount of LLC utilized by microorganisms with the assumption that all leachate-derived substrate used, less what is respired, is incorporated into biomass (Frey et al., 2001; Thiet et al., 2006). This LLC based CUE is calculated as

$$CUE_{LLC} = \frac{(\Delta LLC - \sum CO_2 - C)}{\Delta LLC},$$

where ΔLLC is the change in the concentration of extractable organic C and $\Sigma CO_2$-C is the cumulative amount of C lost through respiration. Here, we assume that extractable organic C in these soils is dominated by the added LLC and that microbial activity after LLC addition is fueled primarily by the C contained in the leachate solution. In another approach, it is assumed that the sum of the MBC measured and the cumulative C lost via respiration is equal to the amount of substrate utilized (Frey et al., 2001; Thiet et al., 2006). This CUE measure is based on the change in MBC and is calculated as

$$CUE_{MBC} = \frac{\Delta MBC}{(\Delta MBC + \sum CO_2 - C)},$$

where ΔMBC is the amount of microbial biomass produced. Finally, we calculated a ratio of the amount of MBC produced per amount LLC consumed,

$$CUE_{ratio} = \frac{\Delta MBC}{\Delta LLC}.$$

Because we cannot estimate gross rates of microbial production without concerns about microbial recycling, a direct measure of microbial efficiency is not feasible. Though the absolute values of these proxies may be biased depending on the method of calculation (Thiet et al., 2006), the relative differences between treatments permit comparison between soils exposed to our experimental treatments, and provide informative measures of C use by these soils' microbial communities.

*Statistical Analyses*

The analysis of soil water content (SWC) data measured multiple times over the course of the incubation required the use of 2-way repeated measures ANOVA in SAS PROC MIXED (SAS Institute, Cary, SC., USA) to determine day, treatment and day*treatment effects. Because the sampling dates were un-evenly spaced, we used the spatial power law to model the

covariance structure. We used Differences of Least Squares Means (LSM) for pairwise comparisons with a Tukey-Kramer *P*-value adjustment (Tukey, 1953; Kramer, 1956) to control the maximum, experimentwise error rate (Hayter, 1989). In order to compare data between soils from different sites along the precipitation gradient where texture, pH and [SOM] differ, we normalized all LI and SI treatment data by dividing by the control treatment values. We present all data, except SWC, from LI and SI treatments as percentages of the control treatment. We used a 2-way ANOVA in SAS PROC GLM to determine effects of treatment, soil origin (site) and treatment*site on total C respired, respiration rates, soil moisture variability (coefficient of variation, CV), EEA, inorganic N, MBC, MBN and measures of CUE. When data were non-normally distributed, we rank ordered the data and performed the same 2-way ANOVA as above on the ranks. This was required for day 58 measures of PHOS, phenol oxidase, peroxidase, MBC, MBN and inorganic N, and day 73 measures of LAP, urease, PHOS, phenol oxidase, peroxidase and $CUE_{MBC}$. All effects are considered significant at $P < 0.05$ unless noted.

When we harvested sub-samples on day 58, the beginning of the last wetting and drying cycle, SWC was greater in LI soils compared to SI. Though this permitted us to assess differences in microbial function with varying SWC, we were primarily interested in the influence of legacy effects of soil moisture *variability* on microbial functioning, not SWC *per se*. We address this issue by analyzing for and focusing much of our discussion on treatment effects on day 73, when SWC was equivalent among treatments, and thus any differences in measures of microbial community function on this sampling date reflect legacy effects of months of imposed differences in soil moisture variability.

## RESULTS

### *Soil moisture content and variability*

Long interval and SI soils received the same amount of water in total over the course of the incubation, but the variation in timing and magnitude of these water additions resulted in significant differences in the coefficient of variation (CV) in soil moisture between treatments, and in soil water content (SWC, Fig. 1). The CV in soil moisture across the incubation was approximately twice as great in LI compared to SI soils for all sites (KUFS, 76.0 ± 3.5 vs. 38.2 ±0.7, $P = 0.0005$; KNZ, 63.2 ± 3.4 vs. 32.9 ± 2.5, $P = 0.002$; HYS, 71.0 ± 2.1 vs. 36.1 ± 2.0, $P = 0.0003$; SVR, 76.9 ± 3.4 vs. 39.5 ± 0.7, $P = 0.0004$). Treatment, day and the interaction between treatment and day had significant effects on SWC. Within the interaction, SWC was not significantly different between LI and SI treatments on a majority of the dates measured, with the exception of the HYS soils. The number of days in which SWC in LI was significantly greater than in SI treatments was similar to the number of days in which SWC was significantly lower in LI compared to SI treatments.

*Respiration and microbial C use efficiency*

Control soils, kept at a constant soil moisture level (50% WHC), released approximately three to four times more $CO_2$-C than those undergoing wetting-drying treatments (Fig. 2). Total C mineralized in the LI soils was 1.4 to 2.0 times higher than soils undergoing the SI treatment. Normalizing respiration data by the control soils to permit comparison across sites, we observed that LI soils averaged across sites mineralized significantly more C (39.0 ± 2.7% vs. 27.1 ± 2.1% of control soils; $P = 0.001$) and had higher respiration rates (48.9 ± 3.3% vs. 29.9 ± 1.8% of control soils; $P < 0.0001$) than microorganisms in SI soils through day 58. Soils from KUFS and KNZ mineralized more C and had higher respiration rates than soils from SVR ($P = 0.04$) through day 58.

Respiration rates in the LI and SI soils on day 72 were 1.2 to 4.6 times higher following the addition of LLC compared to the addition of water alone (Fig. 2). Total C mineralized between day 72 and 73 was equal to one-third to one-half of the total C mineralized in the previous 72 days, or 65 – 114% of the LLC added. LI soils had greater and SI lower respiration rates and total C mineralized in the 24 h after LLC addition than control soils. Microorganisms in LI soils across sites mineralized significantly more C over the 24.5 h following LLC addition than in SI soils (145.1 ± 14.4% vs. 96.3 ± 9.4%), and LI soils had higher respiration rates than SI soils after LLC addition (144.5 ± 16.0% vs. 94.8 ± 9.9%), consistent with greater respiratory losses from LI soils throughout the incubation.

*Extractable N and microbial biomass*

By day 58, soils undergoing wetting-drying treatments had higher inorganic N availability relative to controls, with the exception of SVR LI soils (i.e. > 100%; Fig. 3a). In addition, by day 58 SI soils appeared to have accrued more inorganic N ($NO_3^-$ plus $NH_4^+$) than LI soils ($P = 0.05$), and net N mineralization in SVR soils across treatments was lower than in soils from all other sites ($P = 0.05$; Fig. 3a) . Similarly, on day 73, inorganic N availability was greater in SI than LI soils ($P = 0.0002$) and SVR soils had significantly less N relative to their controls when compared to HYS, KNZ and KUFS soils ($P = 0.002$; Fig. 3b). We were unable to detect EON in any of the soils sampled on either day 58 or day 73.

In all soils and treatments, absolute values of MBC ranged from 0.28 – 2.20 mg C $g^{-1}$ soil while MBN ranged from 22.99 to 227.79 μg N $g^{-1}$ soil. Microbial biomass C on day 58 was not significantly different between treatments or sites (Fig. 3c). On day 73, after LLC additions, there were significant effects of treatment and site on MBC relative to control soils (Fig. 3d). SI soils had greater MBC than LI soils ($P = 0.05$), and MBC relative to control soils was higher in

soils from SVR than in soils from HYS, which in turn was greater than MBC in soils from KNZ and KUFS ($P < 0.0001$; Fig. 3d).

On day 58, we found a significant effect of treatment on MBN with greater MBN in SI compared to LI soils, but there was also a significant treatment*site interaction such that in HYS soils we saw the opposite effect, with greater MBN in LI than SI soils (Fig. 3e, $P = 0.03$). There were no differences in MBN between treatments or sites on day 73 (Fig. 3f).

*Extracellular enzyme activities*

Extracellular enzyme activities exhibited great variation between sites and with moisture treatment (Table 2). On day 58, EEA was generally greater in LI soils than in SI soils, as percent of controls (top panels, Figs. 4 & 5). As a percent of the controls, LI soils exhibited greater labile C acquisition EEA (BG, $P < 0.0001$; AG, $P = 0.0003$; CBH, $P < 0.0001$; BXYL, $P < 0.001$) relative to SI soils. In addition, LI soils also exhibited higher N acquisition (NAG, $P = 0.008$; LAP, $P < 0.0001$) and P acquisition (PHOS, $P < 0.0001$) enzyme activity compared to SI soils. Site of origin also governed EEA response to moisture treatment; activities of BG ($P < 0.0001$), CBH ($P < 0.0001$), BXYL ($P = 0.0001$), and LAP ($P = 0.02$) were higher in SVR LI and SI soils, relative to their controls, than in KUFS and KNZ soils relative to their control soils (top panels, Figs. 4 & 5). Recalcitrant C acquisition EEA (phenol oxidase and peroxidase) was not affected by site and the only detectable treatment effect was found in the HYS soils, in which peroxidase activity was higher in LI than SI soils ($P = 0.03$).

After LLC additions, when SWC was equivalent across treatments, labile C acquisition, NAG and PHOS activities in LI and SI soils were statistically equivalent (bottom panels in Fig. 4 & 5). LAP activity was greater in LI compared to SI soils ($P = 0.002$) and was affected by site such that activity in SVR soils was greater than that in KUFS soils, which was greater than

activity in HYS and KNZ soils ($P < 0.0001$; Fig. 5f). PHOS, also affected by site, was greater in KUFS, KNZ and HYS soils than in SVR soils ($P = 0.0002$; Fig. 5h). Phenol oxidase activity was higher in the LI than the SI treatment for KUFS and KNZ soils ($P = 0.05$) and was undetectable in HYS and SVR soils for both treatments. Peroxidase activity did not vary between LI and SI treatments but was greater in SVR soils than in KUFS, KNZ and HYS soils (data not shown; 389.6 ± 52.1% vs. 0.28 ± 0.07%; $P = 0.0003$).

*Carbon use efficiency proxies*

All three estimates of microbial CUE indicate that relative to control soils, SI soil microbial communities were generally more efficient at retaining C as biomass than those in LI soils, relative to C lost as respiration ($CUE_{LLC}$, $P$ =0.0004; $CUE_{MBC}$, $P < 0.0001$; $CUE_{ratio}$, $P$ =0.06; Fig. 6). Differences by site depended on the method used to calculate CUE, but all proxies suggest that microbial communities in SVR soils had relatively high CUE. Calculation of CUE based on LLC accrual ($CUE_{LLC}$) indicate significant ($P = 0.01$) effects of site such that HYS soil communities show lower efficiency than SVR, KNZ and KUFS soil communities. There was a significant treatment*site interaction for measures of $CUE_{MBC}$ and within this interaction, SI soils had greater CUE than LI soils from all sites (SVR, $P = 0.09$; HYS, $P = 0.03$; KNZ, $P < 0.0001$; KUFS, $P = 0.08$; Fig. 6), while site differences were only evident in the LI soils. In the LI soils, $CUE_{MBC}$ was greater in SVR soils compared to HYS and KNZ soils ($P = 0.05$; Fig. 6). $CUE_{ratio}$ was marginally greater in SI than LI soils ($P = 0.06$) and was higher in SVR soils compared to KUFS soils ($P = 0.05$; Fig. 6).

## DISCUSSION

We sought to determine how the stress induced by soil moisture variability affects microbial C and N resource transformations. We hypothesized that increases in soil moisture variability would decrease substrate use efficiency. We found that regardless of the native soil moisture regime, the activities of soil microbial communities were similarly influenced by increased soil moisture variability, with apparent decreases in CUE. In addition, we wanted to investigate how microbial communities, adapted to different *in situ* soil moisture regimes, would respond to similar patterns of soil moisture variability. We hypothesized that in order to cope with high soil moisture variability stress, we would see microbes shift their patterns of resource allocation, and that these shifts would be of higher magnitude in soils from the mesic end of the gradient than in soils from the western end of the precipitation gradient, where microorganisms are adapted to higher soil moisture variability stress. We found patterns of C use in response to treatment that were similar across sites, but differences in N acquisition and use between sites.

*Effects of soil moisture variability on microbial function*

Respiration rates and the total amount of C mineralized after LLC additions varied with treatment, suggestive of changes in microbial community functioning induced by altered SWC variability. Although we have no direct measure of the amount of LLC incorporated in biomass or mineralized, the relatively large differences in MBC accrual and respiration rates after LLC addition compared to the addition of water alone supports our assumption that the EOC in these soils was dominated by the added LLC and that microbial activity after LLC addition was fueled by that sudden availability of EOC, though some priming of indigenous SOC may have also occurred (Kuzyakov, 2010). The greater release of $CO_2$ from LI soils, in concert with equivalent labile C acquisition enzyme activities in LI and SI soils at the end of the incubation, imply that increasing SWC variability can induce declines in microbial CUE. This is consistent with our

calculated estimates of CUE. Lower CUE in LI soils may result from higher levels of physiological stress experienced by all LI microorganisms, regardless of their native precipitation regime. Both longer drought intervals and larger pulses of water such as those imposed by the LI treatment are associated with a higher potential for cell lysis due to osmotic pressure (Iovieno et al., 2008; Schimel et al., 2007; Fierer et al., 2002). Microorganisms undergoing the LI treatment thus may have devoted more of their C resources to survival mechanisms such as mucilage production, membrane transport proteins and protective osmolyte production and the respiratory costs associated with these functions rather than to biomass accrual (Borken and Matzner, 2009; Schimel et al., 2007; Fierer and Schimel, 2002).

Several observations of N cycling in these soils are consistent with microbial communities in LI soils, regardless of native precipitation regime, experiencing greater physiological stress. Soil N dynamics can be an important indicator of the physiological status of a microbial community because the protective osmolytes used by microorganisms to combat physiological stress tend to be N-rich organic compounds such as amino acids (Schimel et al., 2007). Both SI and LI soils from HYS, KNZ and KUFS accrued inorganic N relative to control soils (385 – 5500%, Fig. 3a,b), which were kept at a constant soil moisture level over the course of the incubation. This suggests that N demand was greater with wetting and drying regardless of treatment. There were also differences in N dynamics between the treatments; microbes in SI soils appeared to have greater net N mineralization than microbes in LI soils throughout the incubation as inorganic N levels were higher in SI soils than in LI soils on both day 58 and 78 (Fig. 3a,b). Further, when SWC was similar N acquisition enzyme activity (LAP) was greater in LI soils relative to SI soils (Fig. 5f), while PHOS activity was not different suggesting that LI microorganisms invested more resources in obtaining organic N in excess of basic nutrient

requirements. This in turn is consistent with studies suggesting that N-rich osmolytes are an important means of protection from moisture stress for microbes (Borken and Matzner, 2009; Schimel et al., 2007). When soils were sub-sampled after water addition on day 58, we found no differences in MBC but greater MBN in SI than in LI soil microorganisms (with the exception of HYS soils). This is also consistent with the idea that with a larger pulse of water and thus higher osmotic potential stress, LI microorganisms released more organic N-rich osmolytes in order to prevent cell lysis. We were unable to detect a concurrent increase in EON that would support this, but it is unclear whether or not microorganisms release protective osmolytes to the extracellular environment or mineralize them (Williams and Xia, 2009; Fierer and Schimel, 2003; Halverson et al., 2000; Kempf and Bremer, 1998). The lower level of MBN right after wetting coupled with the higher N acquisition EEA in LI soils suggest that LI soil microbial communities may be less efficient in their use of N because of their use of N-rich osmolytes, while their SI counterparts appear to have more readily been able to perform net N mineralization as well as N immobilization.

Obtaining and then employing N-rich organic compounds to protect against the stresses imposed by high moisture variability also incurs a C cost. Enhanced LAP activity in LI soils, for example, can only occur with a greater C investment in LAP production. In addition, transporting soluble N osmolytes such as amino acids to and from the cytoplasm incurs a respiratory cost of C that otherwise could be used for growth (Schimel et al., 2007; Iovieno et al., 2008). All else equal, the enhanced C costs and higher respiratory C losses that must accompany greater N acquisition efforts by a microbial community would impose a lower CUE on that community. These concepts are consistent with LI soils in the current experiment experiencing

greater respiratory losses and exhibiting associated lower CUE as estimated by three different proxies.

*Effects of soil origin on response to soil moisture variability*

Soil microbial community structure varies with precipitation across the Great Plains of North America (McCulley et al., 2004). In spite of these apparent differences in community composition, we observed a convergence in microbial community response to water treatment, regardless of soil origin. We observed very few differences between sites in the direction of microbial response of respiration and enzymatic activity to LI and SI treatments. The only significant interactions between treatment and site were in MBN on day 58 and LAP activity after LLC additions. In all other analyses, treatment effects shared the same directionality, if not the same magnitude, between sites. In other stressful situations, such as heat shock, starvation or N limitation, bacteria appear to respond universally through global regulation of multiple operons and regulons (Kim and Gadd, 2008). Such a universal microbial response to shock or stress may have masked any inherent differences in community composition existing in soils in the current study.

Even though responses of microorganisms to varying levels of soils moisture stress seem to follow similar patterns between treatments, the magnitude of these responses varies, though not in the manner we hypothesized. Instead of finding a greater magnitude of response in soils from the mesic end of the gradient, differences were found between the westernmost site, SVR, and all other sites. For example, we found that on day 58 inorganic N in SVR soils was more similar to controls than inorganic N in soils from other sites. In addition, we saw higher EEA (BG, CBH, BXYL, LAP) in SVR soils compared to all other sites, as well as relative to control soils averaged across treatments. This higher activity, relative to control soils in particular,

suggests that SVR soil communities are extremely well adapted to highly variable and low levels of soil moisture. This level of adaptation allows them to carry out higher levels of these activities under more variable and limiting conditions than when presented with constant, non-limiting soil moisture conditions. If SVR soils are representative of other semi-arid grassland soils, it suggests that these soils may be better able to maintain function under higher levels of precipitation variability than soils native to more mesic areas.

Although our laboratory manipulations are difficult to compare to *in situ* studies, it is interesting to note that the convergence of microbial community responses to imposed changes in moisture variability observed in this study, regardless of native precipitation regimes, is not consistent with field studies (Knapp et al., 2002; Harper et al., 2005; Knapp et al., 2008; Heisler-White et al., 2008). Rainfall manipulations in relatively xeric grasslands that simulate larger precipitation events with lengthened intervals between them report generally increasing aboveground NPP (Heisler-White et al., 2008), while at more mesic sites similar treatments can result in decreased aboveground NPP (Knapp et al., 2002; Harper et al., 2005; Knapp et al., 2008; Heisler-White et al., 2008). Based on these studies, we anticipated that the precipitation regime at a soil's site of origin – xeric or mesic – would be a key determinant of microbial community response to changes in moisture variability. Instead, we found that soils from all sites mineralized significantly more C with LI treatment. These studies suggest the importance of native precipitation regime as a driver of ecosystem C flux responses to altered rainfall timing in North American grasslands, and highlight the potential for divergent autotrophic vs. heterotrophic responses to increased moisture variability.

*Conclusions*

Increases in soil moisture variability impose increased physiological stress on soil microorganisms that can create an increase in N demand, concurrently lowering CUE and increasing C losses through respiration. This decrease in CUE regardless of the soils' native precipitation regime, particularly when coupled with decreases in NPP as variability in precipitation patterns increase across the region (Knapp et al., 2002; Knapp et al., 2008; Heisler-White et al., 2008), may lead to increased C losses from these grassland systems. In this study, we found that an increase in soil moisture variability could increase heterotrophic C losses by up to 200% while also decreasing net N mineralization by up to 49%, a feature that ultimately would limit the N available for NPP if realized *in situ*.

Though several studies are consistent in their conclusions about productivity responses to altered rainfall timing, these studies do not explore heterotrophic responses in isolation (Knapp et al., 2002; Knapp et al., 2008; Heisler-White et al., 2008). Plant communities tend not to respond as quickly to resource pulses as microbial communities (Austin et al., 2004) and this phenomenon can be especially prevalent after dry periods (Harper et al., 2008). The findings in the current study indicate that increased moisture variability enhances heterotrophic soil respiratory losses regardless of those soils' native precipitation regime. As such, our results point to divergent patterns in autotrophic versus heterotrophic responses to increased variability in soil moisture regimes, and highlight the need for more belowground studies that explore the mechanisms driving these differences.

**Table 1.** Characteristics of the four grassland study sites located across an east-west precipitation gradient in Kansas, USA. Clay, silt, SOM, pH and bulk density are means ± standard error (n = 3).

| Site | Kansas Field Station and Ecological Reserves | Konza Prairie LTER | K-State Western Kansas Agricultural Research Center | Nature Conservancy Smokey Valley Ranch |
|---|---|---|---|---|
| Abbreviation | KUFS | KNZ | HYS | SVR |
| Average annual precipitation | 1003 mm | 850 mm | 579 mm | 498 mm |
| soil type | Typic paleudolls | Typic natrusolls | Cumulic haplustolls | Aridic haplustolls |
| % clay | 26.33 ± 0.66 | 27.63 ± 0.69 | 31.33 ± 0.84 | 24.98 ± 1.05 |
| % silt | 61.93 ± 0.27 | 65.17 ± 1.18 | 58.38 ± 0.29 | 57.60 ± 0.87 |
| % SOM | 4.69 ± 0.22 | 5.86 ± 0.17 | 7.92 ± 0.55 | 3.13 ± 0.22 |
| pH | 5.99 ± 0.06 | 6.11 ± 0.11 | 6.59 ± 0.11 | 7.83 ± 0.06 |
| Bulk Density | 1.30 ± 0.01 | 1.08 ± 0.06 | 1.07 ± 0.05 | 1.32 ± 0.03 |

**Table 2.** Minimum and maximum values of extracellular enzyme activities across all sites (SVR, HYS, KNZ, and KUFS) in control, long interval (LI), and short interval (SI) treatments (ng activity $h^{-1}$ $g^{-1}$ soil). See text for site and enzyme abbreviations.

| Enzyme | sample day | control soils (ng activity $g^{-1}$soil $h^{-1}$) | | LI soils (ng activity $g^{-1}$soil $h^{-1}$) | | SI soils (ng activity $g^{-1}$soil $h^{-1}$) | |
|---|---|---|---|---|---|---|---|
| | | *min* | *max* | *min* | *max* | *min* | *max* |
| BG | 58 | 88.05 | 300.08 | 126.53 | 333.53 | 88.75 | 274.56 |
| | 73 | 65.52 | 264.51 | 80.71 | 282.37 | 80.06 | 276.27 |
| CBH | 58 | 22.72 | 187.40 | 37.21 | 182.18 | 26.88 | 161.16 |
| | 73 | 14.06 | 157.12 | 20.31 | 177.41 | 18.19 | 141.13 |
| BXYL | 58 | 39.97 | 124.12 | 57.41 | 123.77 | 40.71 | 113.84 |
| | 73 | 22.33 | 88.93 | 24.46 | 100.62 | 29.61 | 89.54 |
| AG | 58 | 17.30 | 64.53 | 30.90 | 89.69 | 26.53 | 84.94 |
| | 73 | 11.86 | 54.12 | 22.47 | 74.09 | 19.20 | 57.05 |
| NAG | 58 | 22.07 | 226.14 | 23.38 | 187.40 | 16.10 | 140.24 |
| | 73 | 20.66 | 221.35 | 12.07 | 200.31 | 12.23 | 137.82 |
| LAP | 58 | 88.22 | 235.06 | 123.35 | 349.38 | 47.57 | 215.60 |
| | 73 | 78.13 | 180.05 | 56.11 | 214.50 | 42.44 | 255.68 |
| Urease | 58 | 353.91 | 1233.05 | 0 | 1802.52 | 0 | 1120.04 |
| | 73 | 124.33 | 2128.18 | 0 | 775.0 | 0 | 821.94 |
| PHOS | 58 | 189.76 | 355.69 | 219.36 | 440.24 | 151.73 | 309.12 |
| | 73 | 161.98 | 296.83 | 142.30 | 321.84 | 104.17 | 317.53 |
| Phenol oxidase | 58 | 0 | 25299.49 | 0 | 30608.80 | 0 | 49788.48 |
| | 73 | 0 | 67735.33 | 0 | 3497.40 | 0 | 1490.01 |
| Peroxidase | 58 | 171.61 | 84276.33 | 0 | 70477.52 | 0 | 81061.84 |
| | 73 | 0 | 42692.05 | 701.10 | 177870.67 | 1144.22 | 176348.20 |

**Figure 1**. Gravimetric soil water content throughout the incubation for LI (●) and SI (▽) treatments in soils from a) SVR, b) HYS, c) KNZ and d) KUFS. Horizontal, dashed line represents soil moisture level of control soils. Asterisks indicate significant treatment effects and error bars represent ± SE of the mean.

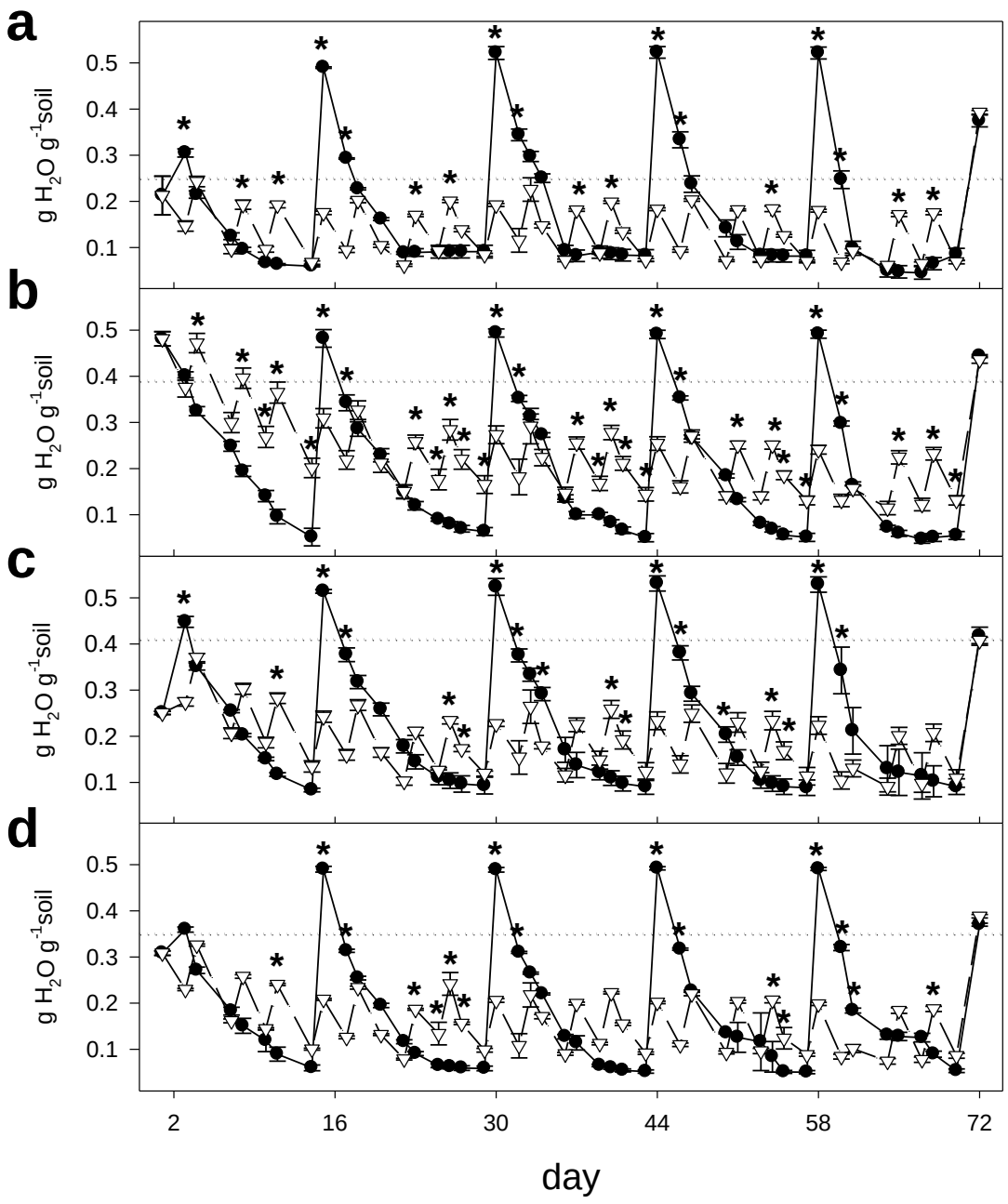


**Figure 2**. Cumulative C respired over the entire 73 day incubation from control (■), LI (●) and SI (▽) soils from a) SVR, b) HYS, c) KNZ and d) KUFS. Arrows indicate soil analyses on day 58 and day 73 of the incubation. Error bars represent ± SE of the mean.

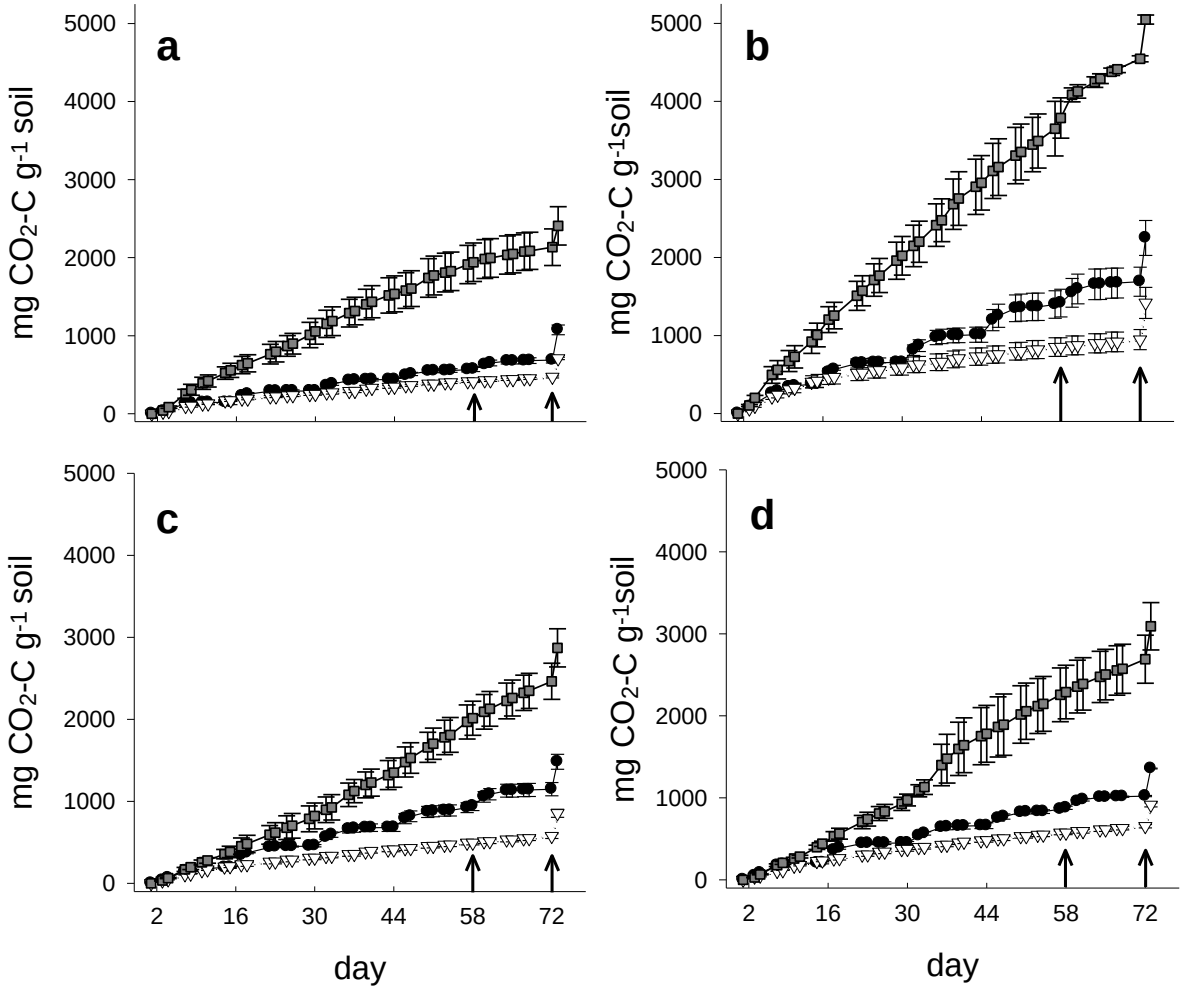


**Figure 3**. Inorganic N availability in LI (●) and SI (▽) soils as a percentage of the control soils for day 58 (a) and day 73 (b), MBC in LI and SI soils as a percentage of the control soils for day 58 (c) and day 73 (d), and MBN in LI and SI soils as a percentage of the control soils for day 58 (e) and day 73 (f). Lower case letters indicate significant differences between sites. Dashed line is a reference for 0% difference between control and treatment soils and error bars represent ± SE of the mean.

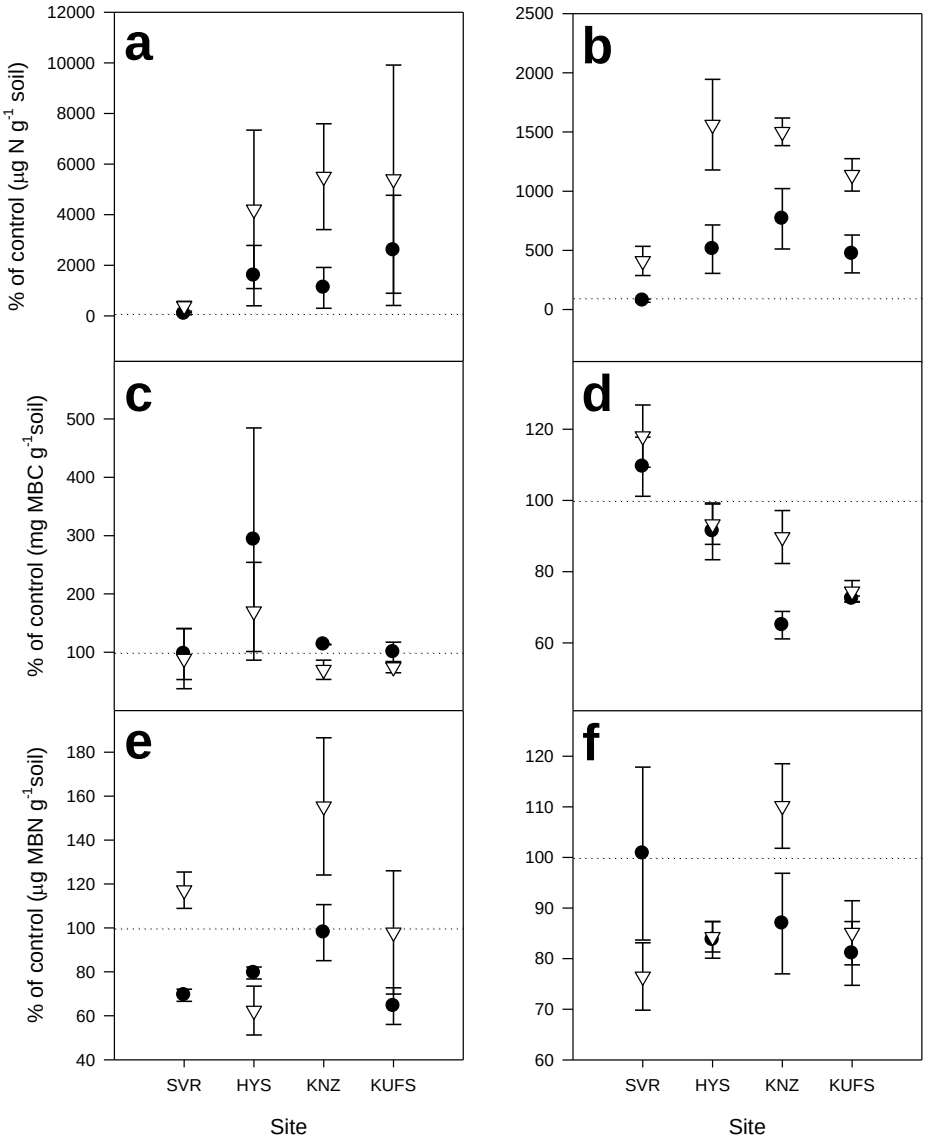


**Figure 4**. Extracellular enzyme activities targeting labile C substrates for LI (●) and SI (▽) treatments as a percentage of control soils from assays on day 58 a) BG , b) CBH, c) BXYL, and d) AG and on day 73 e) BG, f) CBH, g) BXYL and h) AG. Lower case letters indicate significant differences between sites. Dashed line is a reference for 0% difference between control and treatment soils error bars represent ± SE of the mean.

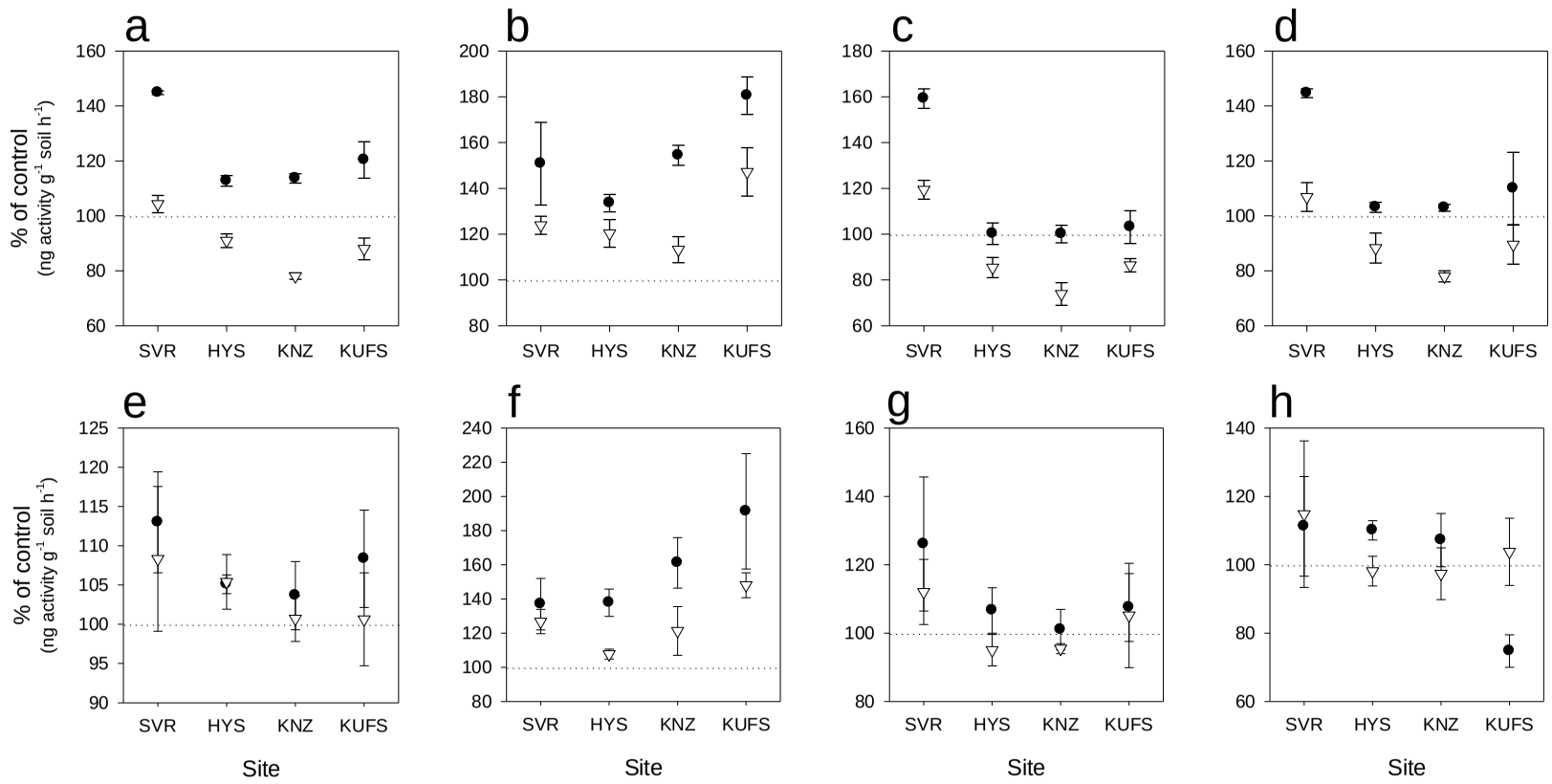

a
b
c
d
e
f
g
h
% of control
(ng activity g-1 soil h-1)
SVR
HYS
KNZ
KUFS
Site

**Figure 5**. Extracellular enzyme activities targeting N and P rich substrates, for LI (●) and SI (▽) treatments as a percentage of control soils from assays on day 58 a) NAG , b) LAP, c) urease, and d) PHOS and on day 73 e) NAG, f) LAP, g) urease and h) PHOS. Lower case letters indicate significant differences between sites. Dashed line is a reference for 0% difference between control and treatment soils and error bars represent ± SE of the mean.

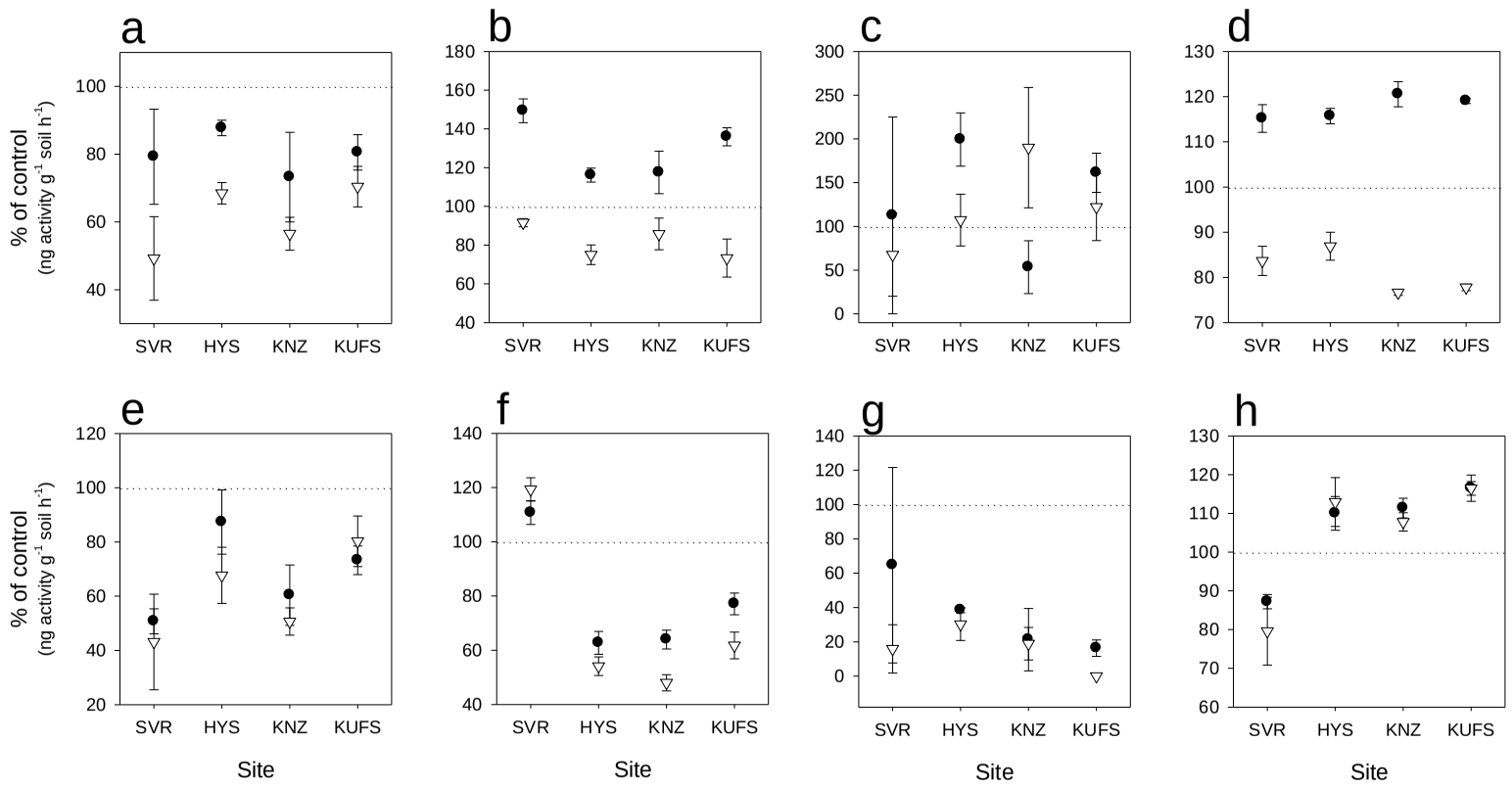
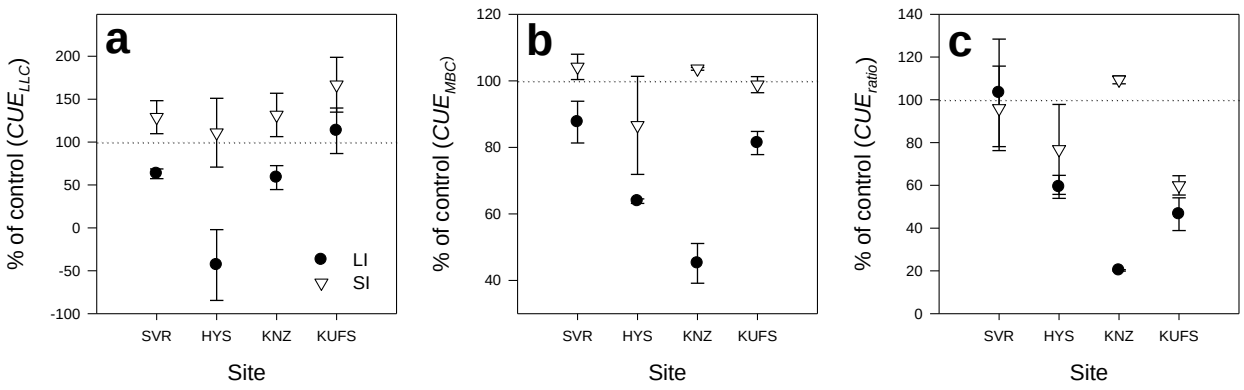

a
b
c
d
e
f
g
h
% of control
(ng activity g-1 soil h-1)
SVR
HYS
KNZ
KUFS
Site

**Figure 6**. Estimates of CUE based on a) LLC utilization, $CUE_{LLC}$ b) MBC accrual, $CUE_{MBC}$ and c) a ratio between the two, $CUE_{ratio}$ in LI (●) and SI (▽) soils from all four study sites presented as a percentage of the efficiency in control soils. Dashed line is a reference for no difference between control and treatment soils and error bars represent ± SE of the mean.



## CHAPTER 3: Tracking C and N flows through microbial biomass with increased soil moisture variability

*Abstract*

Changes in soil moisture with cycles of soil wetting and drying are associated with shifts in osmotic potentials that induce physiological stress for microbial communities. These instances of soil moisture stress can alter flows of C and N at an ecosystem scale. In this study we manipulated the length and severity of soil moisture stress and disturbance in grassland soils from four sites along a precipitation gradient. After subjecting soils to a two-month long incubation under two different wetting-drying regimes, one of high and one of low stress and disturbance, we moistened soils with $^{13}C$ and $^{15}N$ labeled glycine solution to trace C and N though the soils as they dried. We predicted that microorganisms in these soils would preferentially use N-rich protective solutes to guard against osmotic upshock, and hypothesized that we would observed higher specific respiration rates in soils experiencing relatively greater moisture variability associated with the energetic costs of obtaining these N-rich osmolytes. We further hypothesized that the $^{15}N$ tracer would reveal less influence of moisture stress on nitrification than on processes associated with denitrification. Contrary to our predictions, we found evidence for preferential use of N-free osmolytes with increased soil moisture stress in soils from the mesic end of the precipitation gradient. Soils from the western, semi-arid end of the gradient were less sensitive to soil moisture stress and did not differ in N demand under high and low stress. Specific respiration rates were higher in all soils under greater soil moisture stress immediately after re-wetting, then returned to levels equal to or below rates in soils under low soil moisture stress regimes. Nitrification was more dominant in soils under the highest levels of

stress. If climate change does increase the magnitude of soil moisture stress in this region, then these results suggest increases in both soil C release and N losses on the mesic end of this precipitation gradient.

## INTRODUCTION

Microbial activity in grassland soil systems is primarily limited by the transitory nature of the resource pulses that follow precipitation events and the physiological stress of the resulting fluctuations in osmotic potentials and drought periods between precipitation events (Austin et al., 2004). Variability in soil moisture conditions can be particularly stressful for microorganisms in grasslands given that these systems typically occur where potential evapotranspiration (PET) is equal to or greater than precipitation totals, limiting water availability (Lauenroth et al., 1999). Across a precipitation gradient in the Great Plains of North America, deficits between annual PET and mean annual precipitation (MAP) increase in concert with variability in the magnitude and timing of precipitation, thus increasing the level of physiological stress imposed upon soil microorganisms (Williams and Rice, 2007; Kempf and Bremer, 1998). Similar to plant communities, microbial communities native to the most stressful regions within this gradient are likely dominated by organisms whose life strategies are inherently resistant to these stresses. For example, Gram positive bacteria and fungi possess physiological adaptations such as thick cell walls or slow growth rates that allow them to survive high levels of soil moisture stress (Schimel et al., 2007; Voroney, 2007; Grime, 1977), and have been found in relatively higher abundance in semi-arid versus more mesic regions of the Great Plains (McCulley et al., 2004). These physiological adaptations may have tradeoffs however, requiring an extra investment of nutrients and energy for cell wall construction or loss of the ability to fully capitalize on transient resource

pulses Schimel et al., 2007; Voroney, 2007; Kempf and Bremmer, 1998). In contrast, microbes that dominate areas where PET is equal to MAP and soil moisture variability is relatively low are likely fast growing and less efficient organisms (Fierer et al. 2007; Grime 1977) that, when faced with increased levels of soil moisture stress, must acclimate by inducing a physiological response, requiring an investment of resources (Schimel et al., 2007; Kim and Gadd, 2008).

Because climate change in the Great Plains region is expected to promote increases in rainfall variability that will lead to longer, more severe droughts (Easterling et al., 2000), predictions of future functioning of soil microorganisms responsible for biogeochemical processes rely on our understanding of how soil moisture stress controls those microbes' resource demands. One such resource, soil N, serves multiple roles for soil microorganisms. For example, in addition to its importance as a growth limiting nutrient, reduced forms of N can be used as electron donors by nitrifiers and oxidized forms as electron acceptors by denitrifiers. Autotrophic nitrifying bacteria tend to be slow growing and able to survive periods of extremely low soil moisture and to even thrive under soil wetting-drying stress (Fierer and Schimel, 2002; Stark and Firestone, 1995; Allison and Prosser, 1991). This makes it seem likely that nitrifiers would be well suited to cope with soil moisture stress. In contrast, denitrification, an anaerobic process, requires relatively water saturated, and thus $O_2$ limited, conditions. Additionally, heterotrophic denitrifiers must rely upon water to deliver both C and N substrates and they are primarily Gram negative bacteria, thus lacking a thick protective cell wall (Shapleigh 2006). These limitations imply that denitrifiers would be poorly suited to cope with high soil moisture stress. In fact, soil moisture stress has been linked to decreases in $NH_4^+$ availability and increases in $NO_3^-$ accumulation that may be due to shifts in nitrification versus denitrification potential (Fierer and Schimel, 2002; Tiemann and Billings, *in press* and unpublished data). Although

nitrification and denitrification rates have been linked to soil water potentials, in general little is known about the effects of changes in soil moisture stress, particularly increases in soil moisture variability on the coupling of N cycling processes such as nitrification and denitrification (Borken and Matzner, 2009).

In addition to its roles as nutrient and metabolite, N is also an important component of many of the solutes that serve as protective osmolytes during periods of drought. In systems with fluctuating osmotic potentials, microorganisms must guard against cellular water loss. While Gram positive bacteria have a thick outer cell wall that allows them to withstand turgor pressures up to $2 \times 10^6$ Pa (Kempf and Bremer, 1998), they and non-Gram positive organisms must also regulate osmotic pressures with protective solutes. Microorganisms, particularly bacteria, utilize a narrow range of N-rich organic protective solutes, such as amino acids and glycine betaine, that are compatible with cell function (Yancey et al., 1982; Kempf and Bremer, 1998; Schimel et al., 2007). As a consequence, it has been estimated that the acquisition of organic solutes for use as protective osmolytes during just one drought period is equivalent to 3-6% of annual net primary productivity (ANPP) and 10-40% of annual net N mineralization in grassland ecosystems (Schimel et al., 2007). When microorganisms are relieved of osmotic stress, the fate of the protective osmolytes is unclear. Some researchers suggest that these protective solutes are rapidly and selectively released to the extracellular environment (Halverson et al., 2000; Kempf and Bremer, 1998), while others suggest that rather than losing this pool of C and N resources to their surroundings, microorganisms mineralize the solutes (Fierer and Schimel, 2003; Williams and Xia, 2009). In either scenario, as soils dry again and osmotic stress returns, a considerable investment must be made by microbes to regain these N-rich protective solutes. In a previous study investigating the effects of soil moisture variability on microbial resource use, we found

360 – 4800% more N mineralized and evidence for a decrease in C use efficiency in grassland soils undergoing wetting-drying cycles compared to soils kept at a constant soil moisture level (Tiemann and Billings, *in press*). This study prompted us to ask if these relatively high rates of N mineralization coupled with low C use efficiency were the result of increases in the acquisition and release of N-rich osmolytes. Because the acquisition and release of protective solutes are apparently of sufficient magnitude to influence ecosystem level N and C fluxes (Schimel et al., 2007; Tiemann and Billings, *in press*), understanding the mechanism behind these fluxes is critical for predicting not only microbial community structure, but also ecosystem responses to global climate change.

In the current study, we manipulated the severity of soil moisture stress that microorganisms must overcome by altering the magnitude and frequency of soil wetting events to investigate how microbial communities utilize N resources under varying levels of soil moisture stress. As in Tiemann and Billings (*in press*), we conducted these manipulations using grassland soils collected from four different native precipitation regimes, along part of the Great Plains precipitation gradient in Kansas, USA. For two months we treated these soils with either a high frequency, low water addition treatment that induced relatively low levels of osmotic and water availability stress, or a low frequency, high magnitude water addition treatment that induced relatively high osmotic and water availability stresses. While the design is similar to the previous study (Tiemann and Billings, *in press*), here we have accentuated the difference between the high and low stress treatments. Additionally, at the end of two months, after soil microorganisms had presumably acclimated to the different wetting-drying regimes, we applied a final water amendment to the soils that included $^{13}C$ and $^{15}N$ labeled glycine. To our knowledge this is the first use of a dual labeling approach to track microbial resource use during soil

wetting-drying cycles. We selected glycine as a tracer compound because it represents a simple organic C and N source that can be directly incorporated into proteins, easily deaminated for use as an energy source or for biosynthesis, and it can be methylated to form one of the most preferred bacterial osmolytes, glycine betaine (Kimura et al., 2010; Kim and Gadd, 2008; RoeBler and Muller, 2001; Kempf and Bremer, 1998). We measured the size of N pools considered easily accessible to microorganisms, the total carbon (C) respired, and N lost as $N_2O$, and traced the isotopic labels through these pools. Although we cannot know the intended use of resources taken into microbial cells, this approach allowed us to track the fate of added C and N as *potential* metabolites or protective solutes as the soils dried and osmotic stress increased. We used these data to answer the following questions: 1) Does microbial N demand reflect the severity of soil moisture stress and is this relationship variable in soil collected from locations across a precipitation gradient? 2) Is an increase in N demand associated with an increase in soil C losses through higher microbial respiration rates? 3) Are protective solutes returned to the extracellular environment during osmotic downshock, or are these substrates rapidly mineralized to support cell maintenance and activity? 4) Do N cycling processes such as nitrification and denitrification differ in their responses and thus become uncoupled as soil moisture stress increases?

Microorganisms in soils from the eastern, mesic end of the precipitation gradient in Kansas are adapted to a native precipitation regime of relatively equally sized, more evenly distributed rainfall events such that overall soil moisture deficits are lower (Lauenroth and Burke, 1995). On the western, semi-arid end of the precipitation gradient, microorganisms contend with lower precipitation totals associated with greater ambient variation in both the size and frequency of precipitation events (Lauenroth and Burke, 1995). We hypothesized that as we

increased soil moisture stress – both in terms of moisture content and variability – through our laboratory manipulations:

- soil microorganisms from more eastern sites would exhibit relatively low N demands when exposed to low moisture stress and high N demands when subjected to high levels of soil moisture stress due to increased use of N-rich osmolytes
- in western soils, we would find relatively lower N demands overall because of physical adaptations, although we still expected to see some increase in N demand with increasing soil moisture stress as these organisms increasingly relied on additional protection though osmolyte accumulation
- microbes from the western end of our gradient, likely better adapted to high levels of soil moisture stress, would exhibit smaller differences between soil moisture stress treatments compared to treatment differences in soils from the mesic end of the gradient
- any increase in N demand associated with an increase in the level of moisture stress would coincide with decreases in microbial substrate use efficiency, exhibited via increases in specific respiration rates, regardless of the soil's native precipitation regime
- microbial nitrification would outpace and be decoupled from denitrification
- we would observe tracer flows consistent with protective osmolytes being recycled, while under lower stress conditions, evidence would suggest osmolyte release into the extracellular environment.

## METHODS

### *Study Sites*

Soils used in this study were collected from four sites across a precipitation gradient in Kansas, USA, which contains mesic tallgrass prairie in the east and semi-arid mixed grass and shortgrass steppe in the west (Table 1). The eastern most site, part of the Kansas University Field Station lands (KUFS), (W 95°14'35" N 38°10'21") receives an average of 1003 mm of precipitation annually. The soils at this location are gravelly silt loams (smectitic, thermic, Typic paleudolls). Moving west, the second site, located at the Konza Prairie LTER (KNZ, W 96°33'18" N 39°5'2"), averages 835 mm precipitation annually and soils are a mix of silty loams (smectitic mesic Typic natrusolls) and silty clay loams (fine mixed superactive mesic Pachic argiustolls). Our third site, part of Kansas State University's Western Kansas Agricultural Research Center, (HYS, W 99°17'46" N 38°50'13") receives an average of 578 mm precipitation annually and the soils are silt loams (fine-silty, mixed, superactive, mesic, Cumulic haplustolls). The final and western most site, The Nature Conservancy's Smokey Valley Ranch, (SVR, W 100°58'55" N 38°51'50") receives on average 485 mm of precipitation annually. The soils are silt loams (fine-silty, mixed, superactive, mesic, Aridic haplustolls). All sites are part of actively grazed rangeland and are burned annually, with the exception of SVR, which is not burned.

*Soil Collection*

We collected soils from each of the four study sites on May 28 and May 29, 2009. We used PVC cores (10 cm diameter, 10 cm long) to collect three soil cores, approximately 10 m apart, from established plots at each location. On the day of collection, soils were returned to the lab at the University of Kansas where roots greater than 2 mm in diameter were removed and soil

from each core was homogenized. Immediately after this processing, we weighed sub-samples of each soil and dried them at 60° C for > 48 h to determine gravimetric soil water content (SWC).

*Soil wetting and drying incubation*

We weighed the equivalent of 100 g dry soil from each soil sample into two separate, pre-weighed, 5 cm diameter by 5 cm long PVC collars fitted with two sheets of coarse filter paper on the bottom. The soils inside the PVC collars were then placed into 1 L jars on top of a wire mesh support that allowed for air and water flow through the filter paper bottoms. Each soil core collected had two replicate jars, one for each of our two different soil wetting-drying regimes. The first treatment, a large water pulse followed by a long drying interval (LI), was achieved by applying enough water to bring the soils to 100% water holding capacity (WHC) and allowing the soils to dry for two weeks. For the second treatment, we applied a water pulse $^1/_4$ the size of the LI treatment at a short time interval (SI), every 3-4 days, so that these soils, over a two week period, received the same total amount of water as the soils undergoing the LI treatment. Because of the longer and more severe drought interval, followed by a more extreme wetting event, the LI treatment created an environment of generally higher osmotic stress compared to SI soils. We imposed more severe differences in moisture stress regimes than those applied in our earlier study by increasing the amount of water each soil received during each two-week cycle. This decreased variability in SI treatment relative to that imposed in the earlier study, and was intended to increase osmotic downshock in our LI treatment while still resulting in extremely low water potentials, and thus high levels of osmotic upshock, at the end of each drying cycle. The incubation was conducted for a total of five, two-week long wetting-drying cycles, or 72 days, which based on results from our previous study, was enough time for microbial communities to adapt to the different soil moisture regimes (Tiemann and Billings, *in*

*press*). We applied the water using a needle and syringe to assure even coverage. All soils, regardless of treatment, were gently mixed twice a week during the dry down periods to achieve homogeneous soil moisture.

During the incubation, soil respiration and $N_2O$ production were measured by capping the jars with lids fitted with septa and removing a 14 ml gas sample from the jar headspace after 2-4 h. The gas samples were placed in pre-evacuated 12 ml glass vials capped with butyl rubber septa. Gas samples were analyzed for $CO_2$ and $N_2O$ on a gas chromatograph equipped with a thermal conductivity detector and an electron capture detector (Varian, Inc., Walnut Creek, CA). Samples of lab air taken prior to capping the jars were used to determine $CO_2$ or $N_2O$ accrual while the jars were capped. Soils within the PVC collars were weighed frequently to determine soil moisture content, and were kept at 24° C throughout the incubation.

On day 72 of the incubation, the end of the final wetting-drying cycle, we sub-sampled the soils to measure microbial biomass C and N (MBC and MBN), extractable organic C and N (EOC and EON) and inorganic N. We used a sequential extraction procedure following Finzi et al. (2005). In the first step of this procedure, 8 g soil was extracted with 40 ml of 0.5 M $K_2SO_4$ on an orbital shaker for 1 h. The resulting soil slurry was centrifuged and the supernatant passed through a 0.45 μm filter attached to a syringe to separate inorganic N and EOC and EON from MBC and MBN. Next, we cut the syringe top filters in half and returned them to the centrifuge tubes with the previously extracted soils, added 2 ml chloroform, and capped the tubes tightly for 24 h. After the chloroform was vented, the remaining soil and cut filters were again extracted with 40 ml of 0.5 M $K_2SO_4$ for 1 h, centrifuged and the supernatant filtered through Whatman #4 filter paper. We assumed that all EOC and EON in these final extracts originated from MBC and MBN only. We determined inorganic N ($NH_4^+$ and $NO_3^-$) colorimetrically on a Lachat

QwikChem autoanalyzer (Hach Co., Loveland, CO, USA). Extracts containing inorganic N and EOC and EON, and extracts containing MBC and MBN, were digested with persulfate and the resulting concentrations of inorganic C and N were again determined colorimetrically on the Lachat (Doyle et al., 2004). Microbial biomass estimates were corrected using a chloroform efficiency factor of 0.45 (Jenkinson et al., 2004).

*Isotopically labeled glycine addition*

On day 73 of the incubation, we added enough water to soils to bring them all to 75% WHC. In one of the two duplicates for each soil sample, we amended the water with glycine enriched with 98-atom% $^{15}N$ and 99-atom% $^{13}C$. Glycine additions were based on measures of the inorganic N pool size obtained on day 72 as described above. We added enough glycine so that if it was all transformed into inorganic N, that pool would have a $\delta^{15}N$ of ~1500‰. Because the inorganic N pool size varied between treatments and soil origin, we added glycine at a rate of between 0.21 and 1.01 ug glycine $g^{-1}$ soil. When all water and water plus glycine additions had been completed, after ~1 h, we began sub-sampling the soils again for sequential extraction as described above. The first set of extracts was thus filtered ~4 h after the water plus glycine addition. When this procedure was completed, jars were capped for 4 h and gas samples obtained as described above with the addition of two extra samples, injected into 12 ml Exetainers (Labco Ltd., High Wycombe, UK) for $\delta^{15}N_2O$ and $\delta^{13}CO_2$ analyses. This procedure was repeated twice more, 24 h after the glycine addition and 120 h (or 5 d) after glycine addition. During this 5 d period, soils were weighed daily to determine SWC.

*Determining $\delta^{15}N$ and $\delta^{13}C$*

We used an acid diffusion technique to determine $\delta^{15}N$ values of the inorganic N ($NH_4^+$ and $NO_3^+$), EON and MBN pools (Stark and Hart 1996). Briefly, for $^{15}NH_4^+$ analysis, 10 ml of soil extract from the first step in the sequential soil extraction was added to a specimen cup containing 30 ml of 1.0 M KCl and enough $NH_4Cl$ to bring the total N to 60 μg in each cup. Next, we added ~0.2 g MgO to volatilize $NH_3$, and an acid trap before immediately sealing the cups. The acid traps consisted of two pre-leached glass fiber filter discs (7 mm diameter), each acidified with 10 μl of 2.5 M $KHSO_4$ and sealed between two pieces of Teflon (PTFE) tape. For $^{15}NO_3^-$ analysis, cups were setup as above, but when the MgO was added the cups were not capped and instead left open for six days to allow the $NH_4^+$-derived $NH_3$ to escape. Then we added the acid trap, along with Devarda's alloy, and the cups were sealed. For EON and MBN, extracts from the first (EON) and second (MBN) sequential extractions were digested with persulfate, added to cups as above and sealed with acid traps and both MgO and Devarda's alloy. All samples were diffused for 6 days before harvesting the acid traps. We removed the discs from the Teflon tape and placed them into tin capsules in a desiccator for drying. The samples were then analyzed using continuous-flow direct combustion and mass spectrometry on a Europa 20-20 Mass Spectrometer (Europa Scientific, Crewe, UK) at the Utah State University Stable Isotope Laboratory. Gas samples were analyzed for $\delta^{13}CO_2$ at Kansas State University's Stable Isotope Mass Spectrometry Laboratory on a Thermo Finnigan GasBench II with CombiPAL autosampler (Thermo Finnigan, Bremen, Germany), and for $\delta^{15}N_2O$ at the University of California, Davis, Stable Isotope Facility on a SerCon Cryoprep trace gas concentration system interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK).

*Statistical Analyses*

For soils from each site, we compared SWC, $NH_4^+$, $NO_3^-$, EON, MBN, net $N_2O$ efflux and total N pool sizes as well as the proportion of $^{15}N$ recovered in each N pool ($NH_4^+$, $NO_3^-$, EON, MBN) in LI versus SI treatments. We compared these parameters on day 72, 24 hours before water plus glycine addition, day 73, 4 h after water plus glycine addition, day 74, 24 hours after water plus glycine addition and day 78 using repeated measures ANOVA in SAS PROC MIXED (SAS Institute, Cary, SC., USA). Post hoc, pairwise comparisons were made using differences of least squared means. When the treatment*time interaction was significant we compared differences of LS means with Tukey-Kramer adjusted p-values to control the maximum experimentwise error rate (Hayter, 1989). To compare N demand between treatment and sites, we created an index of the proportion of total N ($NH_4^+$ + $NO_3^-$ + EON + MBN) found at each sampling time point relative to that found only in the microbial biomass. We considered the $NH_4^+$, $NO_3^-$ and EON captured in our extracts as relatively accessible N, and assumed that our measured MBN originally came from some combination of these pools. Thus, this index represents the amount of N incorporated into biomass (N demand) relative to the total amount of relatively accessible N that was available when the MBN pool was formed. To address questions about substrate use efficiency we calculated specific activity, dividing respiration rates by both MBC and MBN. These specific activities can be used as proxies for substrate use efficiency (Anderson and Domsch, 1993; Wang et al., 2003). To determine treatment effects on total C respired and SWC's coefficient of variation (CV) we employed ANOVA, using SAS PROC GLM. Nitrogen demand and proxies for substrate use efficiency were analyzed using repeated measures ANOVA as described above. To further assess how the relationship between native soil moisture regimes and our imposed soil moisture regimes we created an index that represents a gradient of soil moisture stress. This index was calculated by multiplying the MAP at each

study site by the imposed soil moistures regime's coefficient of variation (CV). As the index increases, the level of soil moisture stress increases. We analyzed the relationship between this index of soil moisture stress and N demand, or changes in $^{15}N\text{-}NH_4^+$ and $^{15}N\text{-}NO_3^-$ by correlation using SAS PROC CORR.

## RESULTS

We observed no overall treatment effect on average SWC across the incubation in any soil, but there was a significant interaction between treatment and day at each site ($P < 0.0001$ for all sites; Fig. 1). At the beginning of each two-week cycle, just after water addition, SWC was greater in LI compared to SI soils. SWC was equivalent in LI and SI soils on one day per two-week cycle – the first measurement after water addition – across all sites. On all other days, SI SWC was consistently greater than LI SWC. On day 72, the first day of soil N pool measurements, SI treatment SWC was between 8 and 13% greater than LI SWC across all sites. To assess variation in SWC imposed by our moisture treatments, we calculated the CV for SWC over the course of the initial 72 day incubation. At all sites, our soil moisture manipulations created a CV for SWC in LI soils slightly more than double the CV in SI soils at all sites ($P < 0.0001$; SVR 90.6 ± 4.2 vs. 34.2 ± 1.3; HYS, 57.7 ± 3.1 vs. 25.8 ± 0.7; KNZ, 73.7 ± 6.0 vs. 28.0 ± 1.5; KUFS, 77.4 ± 4.7 vs. 26.8 ± 1.4). Over the course of the initial 72 day incubation, SI soil microbes respired less C than those in LI treatments in SVR (670.4 ± 143.1 vs. 772.6 ± 66.3 µg $CO_2$-C $g^{-1}$ soil), KNZ (1432.8 ± 73.5 vs. 1627.1 ± 65.4 µg $CO_2$-C $g^{-1}$ soil) and KUFS (1152.4 ± 228.5 vs. 1455.5 ± 253.1 µg $CO_2$-C $g^{-1}$ soil) soils, but these differences were not significant, and soils from HYS exhibited the opposite trend (SI, 1715.92 ± 179.2 vs. LI, 1494.28 ± 131.2 µg $CO_2$-C $g^{-1}$ soil).

*Nitrogen pools through time*

Day 72 was the final day of the last dry down period for all soils before the water plus glycine addition. On day 72, SVR LI soils exhibited higher $NH_4^+$ availability ($P = 0.0003$) but lower $NO_3$ ($P = 0.0002$) and EON (P = 0.0005) concentrations than SVR SI soils (Fig. 2a,b). We found no significant differences in MBN between treatments on day 72, but recovered more N across all pools measured from SVR SI than from SVR LI soils ($P = 0.001$; Fig. 2a,b). On days 73 (4 h after glycine addition), 74 and 78, there were no treatment effects on soil $NO_3^-$, EON or MBN and there was very little to no detectable $NH_4^+$ availability in either treatment. In both SI and LI soils we found higher concentrations of $NH_4^+$ ($P < 0.0008$), $NO_3^-$ ($P < 0.0004$), EON ($P < 0.0001$) and MBN ($P < 0.0001$) on day 72 compared to days 73, 74 and 78 (Fig. 2a,b). Rates of $N_2O$ efflux did not differ between treatments, but were greater on day 74 compared to day 72 ($P = 0.05$), and day 78 ($P = 0.007$). Four hours after glycine addition, we recovered more $^{15}NO_3^-$ from LI sols than SI soils ($P = 0.03$) and found more $^{15}N$ incorporated into SI than the LI microbial biomass N ($P = 0.01$), with proportions of $^{15}N$ recovered in $NO_3^-$ and MBN pools on days 74 and 78 equal between treatments (Fig. 2c,d). The $^{15}N$ recovered from $NH_4^+$ and EON pools was equal between treatments on all sampling days (Fig. 2c,d).

There were no treatment differences in total N extracted from HYS soils on day 72 (Fig 3a,b). We did, however, find marginally greater MBN in HYS SI compared to HYS LI soils on day 72 ($P = 0.06$). We found little to no $NH_4^+$ and no other treatment effects on any other N pool on day 72 or any of the subsequent sampling days. We found differences in N pool size by sampling time, with greater $NH_4^+$ ($P < 0.0001$), EON ($P = 0.02$) and MBN ($P = 0.0002$) on day 72 compared to days 73, 74 and 78 (Fig. 3a,b). We also saw higher net $N_2O$ production on day

74 compared to day 72 (P = 0.008), day 73 (P = 0.006) and day 78 (P = 0.0003). LI soils $^{15}N$ recovery was lower in the MBN (*P* = 0.009) and greater in the EON (*P* = 0.04) pools compared to SI soils (Fig. 3c,d). We also found more $^{15}N$ in the LI compared to SI $NO_3^-$ pool (*P* = 0.006; Fig. 3c,d).

We recovered an equal amount of total N from KNZ LI and SI soils on day 72, but LI soils had more $NH_4^+$ (*P* = 0.002), $NO_3$ (*P* = 0.004) and EON (*P* = 0.008) with marginally lower MBN (*P* = 0.06) compared to SI soils (Fig. 4a,b). Both LI and SI soils had higher total N prior to water addition than on day 73 (*P* = 0.03) or day 74 (*P* = 0.004) with only SI soils exhibiting significant losses of MBN between day 72 and 74 (*P* = 0.003) or 78 (*P* = 0.05; Fig. 4a,b). LI soil $NH_4^+$ concentrations increased (*P* = 0.003) while $NO_3^-$ concentrations decreased (*P* = 0.0005) immediately after water addition, followed by decreases in $NH_4^+$ (*P* = 0.0002) and increases in $NO_3^-$ (*P* = 0.007) as the soils dried over 5 days (Fig. 4a,b). There were also differences in net $N_2O$ production with higher rates of efflux on day 74 compared to day 72 (*P* = 0.0007), day 73 (*P* = 0.001) and d78 (*P* = 0.005). More $^{15}N$ was recovered in LI than SI $NH_4^+$ pools on day 73 (*P* = 0.0001), and then recovery of $^{15}N$-$NH_4^+$ from LI soils decreased between day 73 or 74 (*P* = 0.0002) and day 78 (*P* < 0.0001; Fig. 4c,d). As $^{15}N$-$NH_4^+$ levels decreased, we concurrently observed increases in $^{15}N$-$NO_3^-$ in the LI soils between day 73 and days 74 (*P* = 0.005) or 78 (*P* = 0.0002; Fig. 4c,d). These declines through time in $^{15}N$-$NH_4^+$ with increasing $^{15}N$-$NO_3^-$ in LI soils were significantly correlated (r = 0.95, *P* < 0.0001). By treatment, we recovered more $^{15}N$ in the LI $NO_3^-$ pool on days 74 (*P* = 0.009) and 78 (*P* = 0.03) and across all sampling times more EO$^{15}N$ (*P* = 0.03) and less MB$^{15}N$ (*P* = 0.006) compared to SI soils. There were declines in $^{15}N$ recovered within MBN in both treatments as the soils dried (day 73 vs. 74, *P* = 0.0004; day 73 vs. 78, *P* = 0.0003).

We found greater total N in KUFS LI compared to SI soils on days 72 ($P$ = 0.002) and 74 ($P$ = 0.01; Fig. 5a,b). This difference was due to higher $NH_4^+$ ($P$ = 0.03) and EON ($P$ = 0.0006) in LI versus SI soils but equivalent $NH_4^+$ and MBN pools between treatments (Fig. 5a,b). We found no differences in net $N_2O$ efflux by day or treatment. A greater proportion of $^{15}N$ was recovered in the $NH_4^+$ ($P$ = 0.02) and EON ($P$ = 0.0006) pools of LI compared to SI soils, in concert with a greater proportion of $^{15}N$ recovered in the MBN of SI compared to LI soils ($P$ = 0.0006; Fig. 5c,d). Regardless of treatment, the proportion of $^{15}N$ recovered as $NH_4^+$ ($P$ = 0.0005) or EON ($P$ = 0.02) decreased as soil dried, while recovery increased in the $NO_3^-$ ($P$ = 0.008) and MBN ($P$ = 0.01) pools (Fig. 5c,d). As with KNZ soils, declines through time in $^{15}N$-$NH_4^+$ were significantly correlated with increases in $^{15}N$-$NO_3$ in the LI soils (r = 0.99, $P$ < 0.0001).

*Nitrogen demand and specific respiration rates*

Nitrogen demand was calculated as the proportion of all readily available N ($NH_4^+$ + $NO_3^-$ + EON + MBN) found in the microbial biomass. In SVR soils, N demand was higher in LI compared to SI soils on day 72 and equivalent on all days after glycine and water addition (Fig. 6a), and in HYS soils we found no significant differences in N demand between treatments at any sampling time (Fig. 6b). In contrast with soils from drier sites, we found greater and more consistent treatment differences in N demand in KNZ and KUFS soils. On days 72, 73 and 74 SI soils from KNZ had greater N demand than LI soils ($P$ = 0.0002; $P$ = 0.07; $P$ = 0.01; Fig. 6c). The largest treatment effects on N demand were found in KUFS soils, with LI demand greater than SI demand at all sampling times (days 72, $P$ = 0.0002; 73, $P$ = 0.0008; 74, $P$ = 0.0008; and 78, $P$ = 0.0002; Fig. 6d).

Measures of specific activity can serve as proxies for microbial substrate use efficiency (Anderson and Domsch, 1993; Wang et al., 2001). In SVR soils, we found a significant interaction between treatment and time for respiration rates per unit MBC and per unit MBN. On day 72, prior to glycine and water addition, SI microorganisms were less efficient per unit of MBC than LI microorganisms ($P = 0.01$), while on day 73, efficiency per unit MBN ($P = 0.01$) and per unit MBC ($P = 0.10$) was higher in SI compared to LI soils. In HYS soils on day 72, SI microbes appeared to be less efficient than LI microbes on a per unit MBC ($P = 0.02$) and MBN ($P = 0.01$) basis. As with SVR soils, after water addition this treatment effect was reversed and efficiencies per unit MBC on day 73 ($P = 0.08$) and per unit MBN on days 73 ($P < 0.0001$) and 74 ($P = 0.01$) were lower in LI than in SI soils. The soils from KNZ and KUFS exhibited similar trends in specific respiration rates as HYS and SVR soils. KNZ soils had higher respiration rates per unit MBC ($P = 0.03$) or MBN ($P = 0.07$) in SI compared to LI soils on day 72, and the opposite relationship on day 73 (per unit MBC, $P = 0.005$; per unit MBN, $P < 0.0001$). In KUFS soils proxies for substrate use efficiency were also lower in SI compared to LI soils on day 72 (per unit MBC, $P = 0.0002$; per unit MBN, $P = 0.01$), then higher in SI compared to LI soils on day 73 (per unit MBC, $P = 0.07$; per unit MBN, $P < 0.0001$).

## DISCUSSION

In addition to fundamental N requirements, including the N demands associated with nitrification and denitrification, we assumed microorganisms in these soils also would use N-rich compounds as compatible solutes to combat osmotic stress during drought and osmotic downshock following wetting of a dry soil. We hypothesized that the magnitude of the osmotic stress would increase or decrease N demands and $CO_2$-C losses accordingly, above the

background of fundamental N requirements. Specifically, as the soils dried subsequent to the final water and glycine addition, we expected to find higher levels of MBN and greater relative incorporation of the $^{15}N$ label into MBN in the LI compared to the SI soils, with increases in C mineralization rates in LI compared to SI soils. Further, we hypothesized that soils native to environments typically imposing long periods of high moisture stress on soil microorganisms (SVR and HYS) would have relatively smaller differences in N demand between treatments when compared to soils native to environments with generally lower soil moisture stress (KUFS, KNZ). Additionally, we hypothesized that treatment differences in eastern soils would be more pronounced so that the changes with the LI treatment in MBN, C mineralization and N cycling, would be greater in KUFS and KNZ soils relative to HYS and SVR soils. With increased soil moisture stress, we expected to see an increase in net nitrification with concurrent decreases in denitrification as evidenced by a greater proportion of both total extractable N and $^{15}N$ found in the $NO_3^-$ pools of LI compared to SI soils across all sites. Finally, we hypothesized that under higher levels of stress, there would be evidence of osmolyte recycling in LI soils and osmolyte release in SI soils. We predicted that LI soils would exhibit higher respiration rates during osmotic downshock and smaller decreases in biomass C and N that reflected osmolyte recycling or mineralization when compared to SI soils.

*Osmolytes and N demand*

Microorganisms typically employ a variable suite of osmolytes based upon what they can obtain from their extracellular environment or synthesize, and the composition of this suite of osmolytes is dependent on growth conditions as well as microbial community composition (Kempf and Bremer, 1998; RoeBler and Muller, 2001). For example, bacteria and archaea seem

to prefer N-rich osmolytes, such as amino acids and glycine betaine, which they obtain from the extracellular environment through specialized transport systems when possible, or through synthesis (Yancey et al., 1982; Kempf and Bremer, 1998; RoeBler and Muller, 2001). As a secondary option, they synthesize N-free osmolytes such as the disaccharide trehalose (Yancey et al., 1982; Kempf and Bremer, 1998; Roebler and Muller, 2001). In contrast, fungi prefer N-free osmolytes such as alcohols and polyols (Yancey et al., 1982). While it is recognized that microorganisms concurrently use a variety of osmolytes, particularly at the community scale, patterns in microbial community selection of resources for osmolyte use or generation are unclear (Williams and Xia, 2009; Bapiri et al., 2010). Our data help clarify how some soil microbial communities may preferentially select between N-rich vs. N-free resources for osmolyte generation.

In the current study, contrary to our hypothesis, microorganisms undergoing the LI treatment in soils from KUFS and KNZ exhibited *lower* N demands than their SI counterparts in both wet and dry conditions. Upon re-wetting of these soils on the final day of the incubation, SI microorganisms lost considerably more MBN than LI microorganisms, and as the soils began to dry again, significantly more added $^{15}$N-glycine resided in MBN of SI soils compared to LI soils. Further, respiration rates and quantity of $^{13}$C-labeled glycine respired were higher in LI soils just after glycine additions. Combined, these data are consistent with microbial communities in KUFS and KNZ soils, when subjected to greater soil moisture stress as in the LI treatment, relying on predominantly N-free osmolytes, and mineralizing those osmolytes when experiencing osmotic downshock rather than releasing them to the extracellular environment. We find support for this concept from a study using soils from another site at Konza Prairie. Williams and Xia (2009) characterized the composition of the soluble organic pool following the

rewetting of dry Konza soils and found no evidence for the release of N-rich osmolytes such as glycine betaine. Instead, oligosaccharides constituted the majority of the soluble C released upon wetting of a dry soil (Williams and Xia, 2009). Further support comes from Fierer and Schimel (2003), who found evidence for rapid mineralization of cytoplasmic solutes upon the wetting of a dry soil from a semi-arid region. Similar to these findings, in the LI soils native to areas with low soil moisture stress, we saw evidence for increased mineralization but no evidence for the release of large stores of N-rich osmolytes upon osmotic downshock, or preferential acquisition of N-rich osmolytes as soils dried and osmotic upshock increased.

One mechanism that could account for differences in N-rich versus N-free osmolyte preference is shifting microbial community composition with moisture regime. An increase in the relative abundance of fungi with LI treatment compared to SI might explain the apparent increase in N-free osmolyte use in LI KUFS and KNZ soils. In a grassland study of bacterial and fungal growth rates during drying-wetting cycles, comparable to the LI treatment in the current study, Bapiri et al. (2010) found an increase in fungal growth relative to bacteria that became more pronounced with more drying-rewetting cycles. Additional evidence comes from a study conducted in the same grassland areas as the current study, where higher fungal abundance was observed in areas of high native soil moisture variability (HYS and SVR) compared to areas with historically lower soil moisture variability (KNZ; McCulley et al., 2004). Bapiri et al. (2010) also suggest that interactions between the original microbial community composition, soil properties and soil moisture legacy prior to drying-rewetting may control the recovery rates of different organisms (i.e. fungi or bacteria) such that responses across different soils and studies will vary. This is supported by findings in the current study in which only LI soils from KUFS and KNZ

experienced variation in N demand consistent with changes in osmolyte usage that may be linked to changes in fungal activity or abundance.

Shifts in osmolyte preferences can also be explained on the basis of microbial energetics. Resource transformations in soils can be viewed very simply as the result of microorganisms' search for energy (McGill, 2007). In this context, there are two reasons why microorganisms may prefer to utilize N-free osmolytes. First, the uptake, processing and storage of simple sugars is ever-present, suggesting that accumulation of simple sugar osmolytes such as trehalose or other oligosaccharides requires a minimal investment of additional energy (Kim and Gadd, 2008). In contrast, the uptake or synthesis of something like glycine betaine would require a much larger energy investment because of the costs associated with the additional transcription and translation of genes responsible for specialized transporter systems or synthesis (ReoBler and Muller, 2001; Kempf and Bremer, 1998). Second, it would seem to be more energetically favorable for microorganisms to mineralize osmolytes rather than release and lose them to the extracellular environment, particularly when combating high levels of stress (Fierer and Schimel, 2003; Williams and Xia, 2009). Assuming this is the case and microorganisms preferentially mineralize osmolytes when their need for osmolytic functioning diminishes, simple sugars would have a higher energy return than osmolytes containing N (McGill, 2007). If osmolytes are preferentially mineralized rather than returned to the extracellular environment and the energetics of this mineralization plays a role in dictating osmolyte choice, we would expect to see patterns of higher C mineralization rates per unit MBC or per unit MBN after a soil is rewet, as seen in KUFS and KNZ LI soils.

Contrary to our prediction, N demand did not vary by treatment in soils from the western, more arid end of the precipitation gradient. We expected to see differences between LI and SI

treatments in HYS and SVR soils even if these differences might be smaller than the treatment induced differences in N demand in KNZ and KUFS soils. To better understand how soil moisture regime was influencing general microbial N demands in our soils, we compared N demand on day 78 (end of last dry down period) to an index reflecting the gradient in the soil moisture stress imposed on soils in this study. The index assesses the stress the microbes were exposed to by using the soils' native MAP, as modified by the CV in soil moisture experienced during the incubation. As overall soil moisture stress increased, moving from western SI soils to eastern LI soils, N demands decreased (Fig. 7a). In other words, compared to the total amount of N that was readily available, soil organisms under higher levels of stress incorporated a lower proportion of N into their biomass. In addition, respiration rates per unit MBN in LI soils on day 72 (when soils were at their driest) were lower than in SI soils. When all soils were re-wet to the same SWC on day 73, LI soils exhibited higher respiration rates per unit MBN compared to SI soils, but these specific activity rates declined such that, on day 78, LI respiration per unit MBN had returned to levels equal to those in SI soils. These responses in LI soils suggest that as the level of stress increases, relative to what the microbial community is adapted to, microorganisms became more efficient with their N use with osmotic upshock. This also supports our conclusion that LI microbes were preferentially using N-free osmolytes.

*Shifts in microbial community N cycling*

Nitrogen transformations in soils are highly dependent on soil moisture level, but little is known about the effects of soil moisture variability on these processes. Using $NH_4^+$ and $NO_3^-$ pool sizes and the $^{15}N$ label as a tracer, we were able to observe time-ordered progression of the N cycle and find evidence for temporal decoupling of nitrification and denitrification, but not

diminishment, with soil drying-rewetting stress. In this and our previous study, we found greater concentrations of $NO_3^-$ relative to $NH_4^+$ when soils were at their driest point, suggestive of increased nitrification relative to denitrification as soils dried. Upon re-wetting, soils in the current study exhibited immediate declines in $NO_3^-$ and increases in $N_2O$ efflux. As the soils dried for the final time, $NO_3^-$ increased in all soils, and in KUFS and KNZ soils this was coupled with decreases in $NH_4^+$ ($NH_4^+$ was consistently undetectable in HYS and SVR soils). In addition, in the KUFS and KNZ soils, the $^{15}N$ label moved from $NH_4^+$ to $NO_3^-$ between days 73 and 78 (Figs. 4b and 5b). Together these data imply that nitrifiers are, as we hypothesized, well-suited to cope with varying levels of soil moisture stress. Their activity in this study apparently was not limited by stress due to variability in soil moisture. Indeed, for HYS and KNZ soils, in our most stressful soil moisture treatment (LI), we found larger $NO_3^-$ pools and greater $^{15}N$ incorporation into $NO_3^-$ compared to the lower stress treatment (SI).

When soils were re-wet and conditions were more favorable for anaerobic metabolism, the $NO_3^-$ that accumulated during soil dry down was likely used in large part for denitrification. We expected denitrifiers to be more sensitive and therefore less likely to maintain their function under higher levels of soil moisture stress. However, the immediate decrease in $NO_3^-$ after soils were wet, which we associate with denitrification due to the enhanced $N_2O$ fluxes observed concurrent with moisture application, was most pronounced in soils from opposite ends of our soil moisture stress gradient, KUFS LI and SVR SI soils. We speculate that contrary to our hypothesis, the microorganisms in these soils responsible for N transformations were not sensitive to different soil moisture stress regimes. We predicted that under increased levels of soil moisture stress, denitrifiers, particularly those from the eastern end of the gradient, would have to acclimate, but perhaps in these and other systems with highly variable soil moisture,

denitrifiers have instead adapted to high soil moisture stress through the use of efficient osmoregulatory systems. Whether or not denitrifiers are susceptible to soil moisture stresses as induced in this study, we observed increases in nitrification relative to N mineralization that could lead to increased N losses from these systems through $NO_3^-$ leaching or gaseous loss through denitrification upon re-wetting (Fierer and Schimel, 2002; Austin et al., 2004). Other studies have suggested that nitrifiers and denitrifiers are sensitive to overall soil moisture levels (Voroney, 2007; Fierer and Schimel, 2002; Stark and Firestone, 1995) but we did not find that to be the case for soil moisture variability; however we did find evidence for temporal decoupling of nitrification and denitrification. As wetting-drying cycles become more pronounced with climate change in the Great Plains region, this de-coupling could have large implications for ecosystem N cycling.

In addition to the treatment differences described above, we also saw differences in the magnitude of nitrification across our soil moisture stress gradient (Fig. 7b,c). We calculated the change in the proportion of $^{15}N$ incorporated in $NH_4^+$ and $NO_3^-$ pools as the soils dried between days 73 and 78. As the level of soil moisture stress increased relative to a soils' native stress regime, the magnitude of these changes, representing the nitrification process, also increased leading to a net accumulation of $NO_3^-$. In our previous soil wetting and drying study we also observed long-lasting legacy effects of wetting-drying treatments on soil accumulation of $NO_3^-$, particularly in the KUFS and KNZ soils under high stress (Tiemann and Billings, 2011). Fierer and Schimel (2002) also found increases in soil $NO_3^-$ after applying wetting-drying stress and an increase in nitrification potential in frequently stressed grassland soils. These results imply that as climate potentially changes across the Great Plains, leading to increased soil moisture stress in the form of more intense soil drying-rewetting patterns, nitrification rates will increase the most

in areas that also exhibit the highest rates of net N mineralization (Burke et al., 1997). This could promote N loss from these systems through $NO_3^-$ leaching or enhanced denitrification upon soil wetting (Fierer and Schimel, 2002).

*Conclusions*

The responses we observed to wetting-drying treatments in specific respiration, relative nitrification vs. denitrification, and substrate flow through C and N processes were of sufficient magnitude to suggest, as others have, that changes in soil microbial resource demand with changes in moisture regime can have ecosystem-level consequences on the cycling of these elements (Schimel et al., 2007). Using bulk density data from KUFS and KNZ soils, where treatment effects were most prominent and where relative changes in precipitation regimes are predicted to be greatest, we estimated the change in fluxes of C and N into microbial biomass in the 24 h immediately following glycine additions. We compared changes in these flux estimates into the microbial biomass pool in LI vs. SI soils. These fluxes of C and N represent the aggregation of C and N uptake and release, including osmolyte flow and nitrification and denitrification. We estimate that LI soils experienced increases in microbial C and N demands of 16 g C $m^{-2}$ and 0.6 g N $m^{-2}$, respectively. Estimates of ANPP (Lauenroth et al., 2000) and annual average net N mineralization (Burke et al., 1997) near our sites indicate that these shifts with treatment in fluxes of C and N through the microbial biomass during this one dry-rewet event are equivalent to 3% of the annual net primary production and 6% of annual net N mineralized. Schimel et al. (2007) estimate that microbial N demand for osmolyte protection in grasslands can represent a significant flux of ecosystem N. Though we cannot assume that incubation results are directly applicable in the field, we use our incubation data to refine the idea presented by Schimel et al. (2002), relating quantified changes with moisture stress in both C and N demand

by soil microorganisms to known ecosystem parameters. Our work highlights how changing precipitation regimes with climate change in the Great Plains may have unanticipated consequences in belowground ecosystem C and N cycling, and that these influences may be most prominent on the eastern end of the gradient, where ANPP, soil C and net N mineralization rates are highest.

In this study we found evidence for shifts in microbial osmolyte preference and fate under different levels of soil moisture stress. Microorganisms likely employ a suite of protective solutes, selected on the basis of availability and energetics (Xia and Williams, 2010). However, the exact mechanisms involved in osmolyte selection are unknown. Our study indicates that in soils where microorganisms are adapted to relatively low moisture deficits and variability, enhancement of moisture stress and induced osmolyte demand can promote lower N demand and relatively greater C mineralization. These data are consistent with microorganisms in these soils preferentially using N-free compounds as osmolytes, and subsequently mineralizing them when moisture stress is relieved. In addition to changes in N demands, wetting and drying stress may also alter the potential for nitrification and denitrification and thus N availability. The evidence we present here to support our hypothesized mechanisms for a shift in osmolyte preference is indirect, but highlights a gap in our understanding of the microbial response to drying-rewetting cycles in soils. Osmolyte use during one drought period can account for a significant proportion of the total N and C cycling though these ecosystems on an annual basis, therefore, shifts in osmolyte preference and mineralization rates, regardless of the mechanisms, could have large implications for both C and N budgets in the grassland systems from which these soils originated.

**Figure 1.** Soil water content (SWC) across the 78 day incubation in a) SVR, b) HYS, c) KNZ and d) KUFS soils. Error bars represent ± one standard error of the mean (n = 3). Symbols without error bars represent days in which water was added and soil moisture was estimated. The horizontal dotted line represents 100 % water holding capacity for each soil.

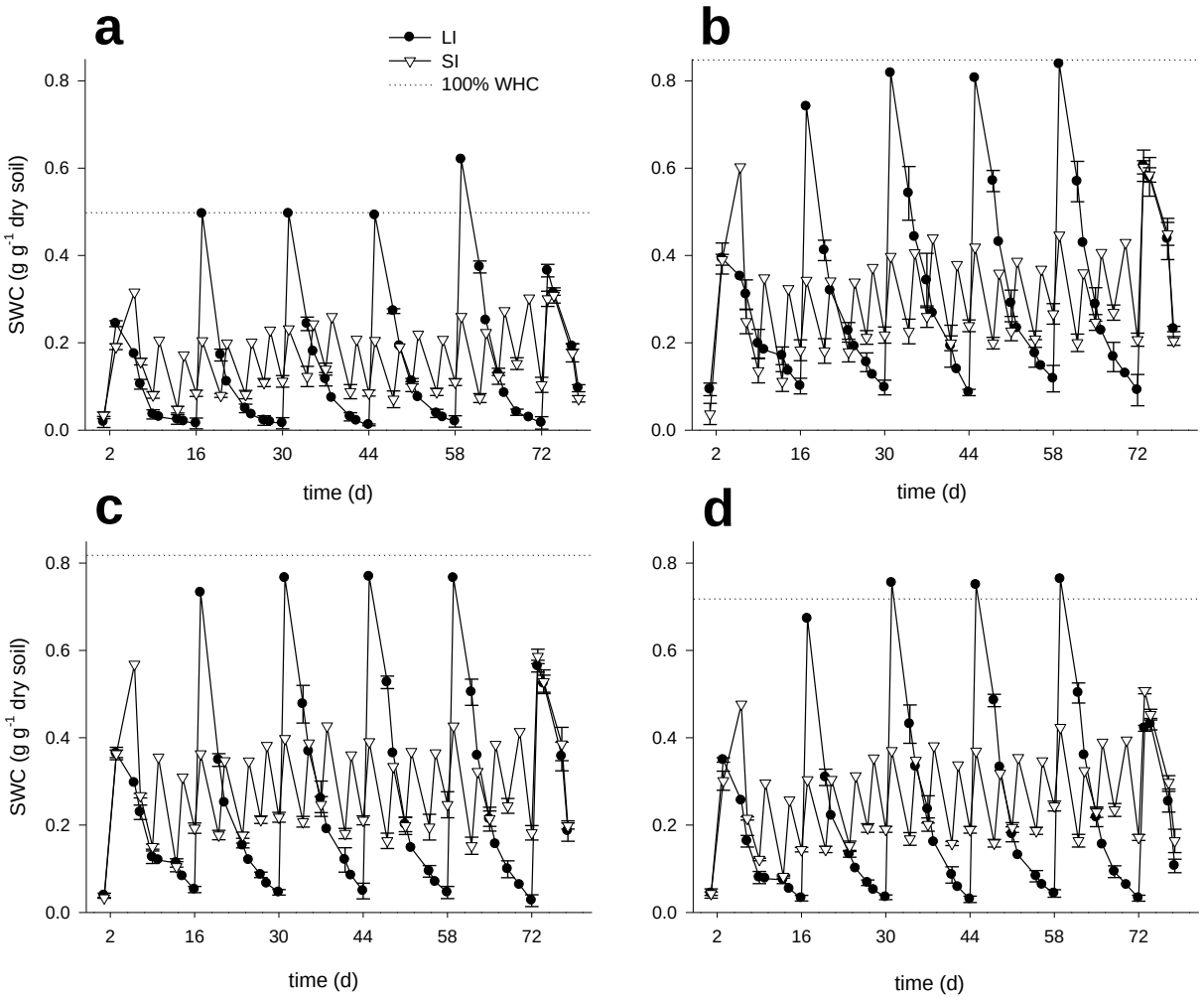


**Figure 2.** Size of SVR soils extractable nitrogen pools (a) and the proportion of the added $^{15}N$ recovered in each of these pools (b) through time for long interval (LI) and short interval (SI) soil moisture treatments. Water plus $^{15}N$ labeled glycine were added to dry soils on day 73. Error bars represent ± one standard error of the mean (n = 3).

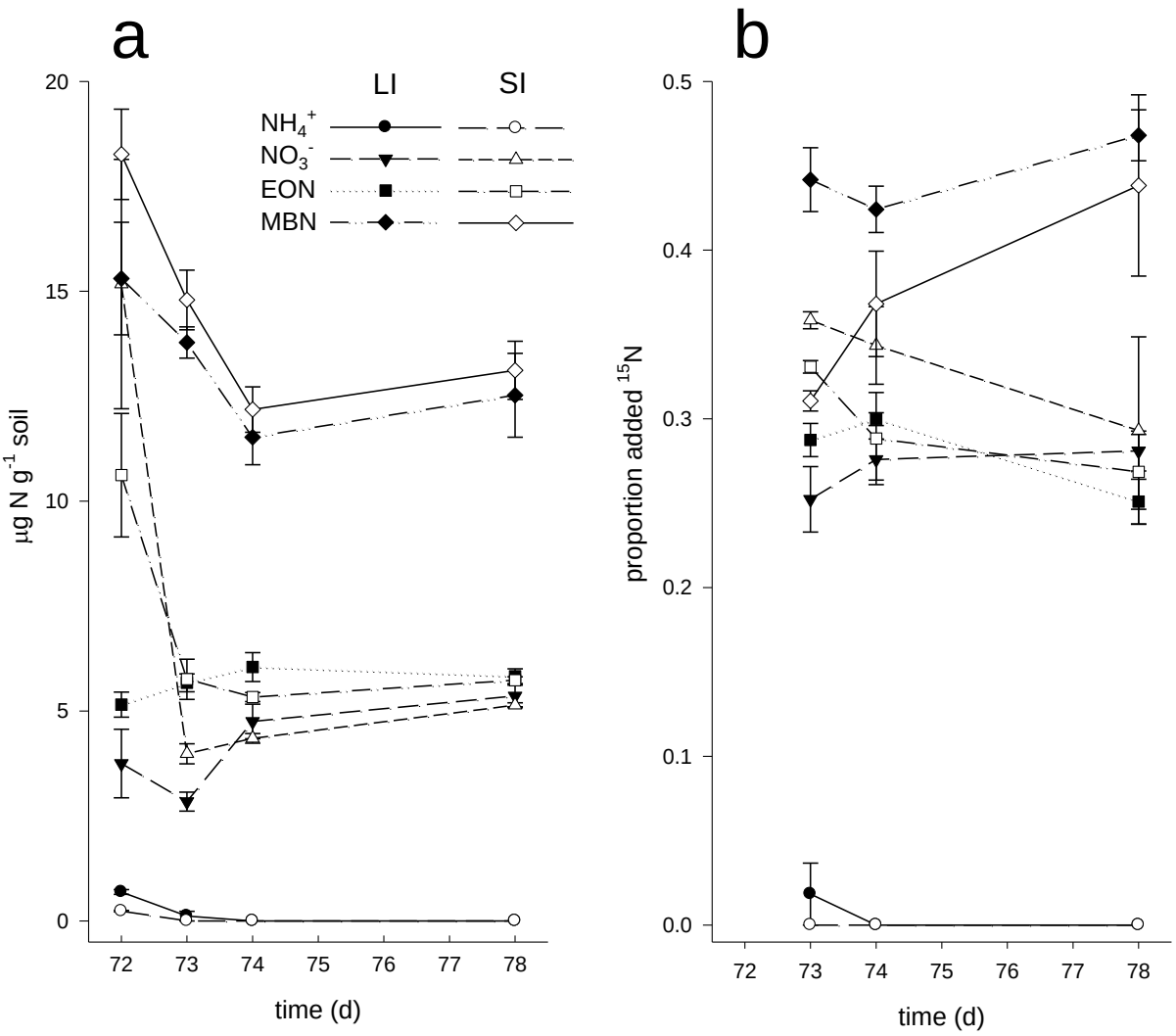


**Figure 3.** Size of HYS soils extractable nitrogen pools (a) and the proportion of the added $^{15}N$ recovered in each of these pools (b) through time for long interval (LI) and short interval (SI) soil moisture treatments. Water plus $^{15}N$ labeled glycine were added to dry soils on day 73. Error bars represent ± one standard error of the mean (n = 3).

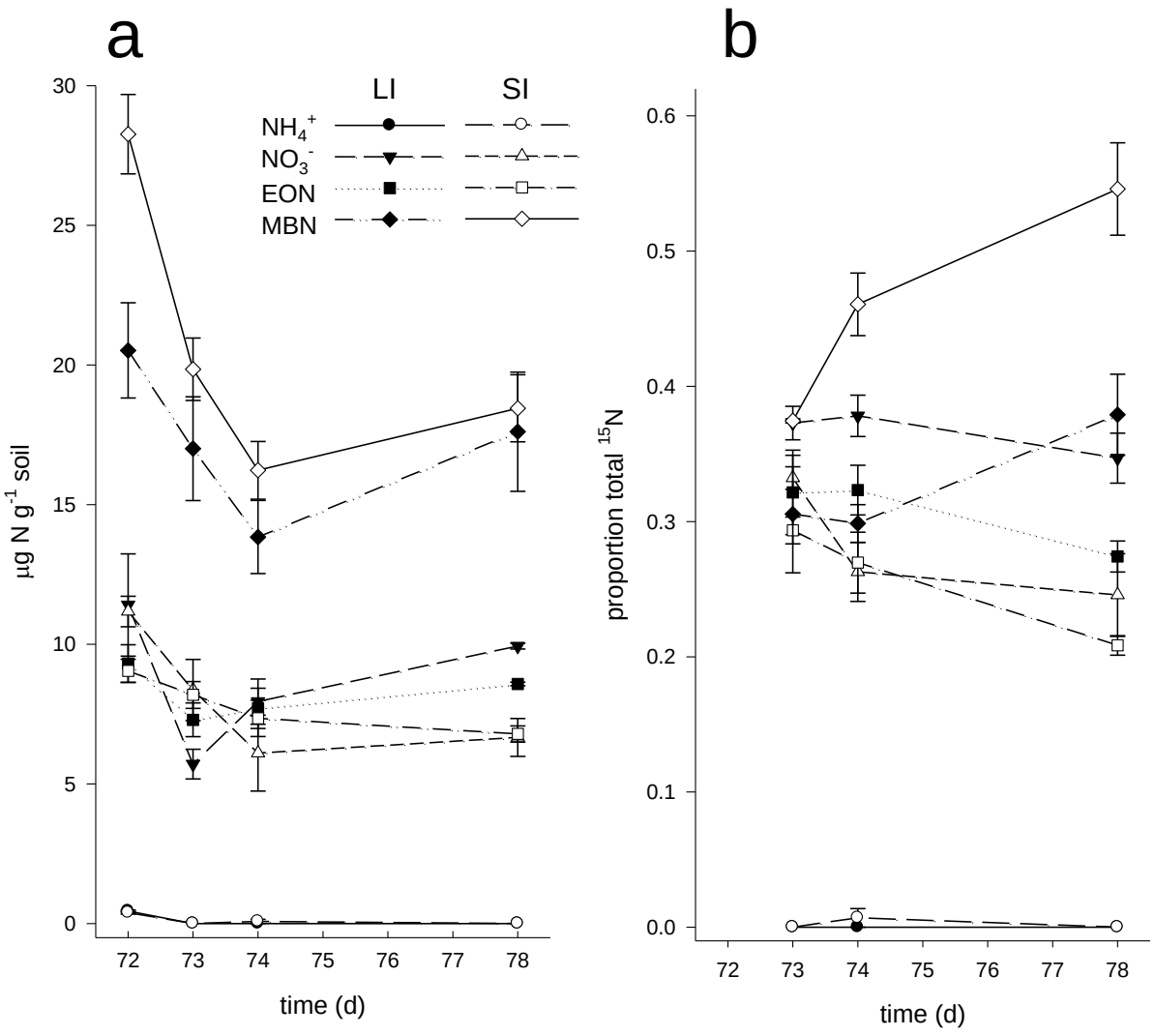


**Figure 4.** Size of KNZ soils extractable nitrogen pools (a) and the proportion of the added $^{15}N$ recovered in each of these pools (b) through time for long interval (LI) and short interval (SI) soil moisture treatments. Water plus $^{15}N$ labeled glycine were added to dry soils on day 73. Error bars represent ± one standard error of the mean (n = 3).

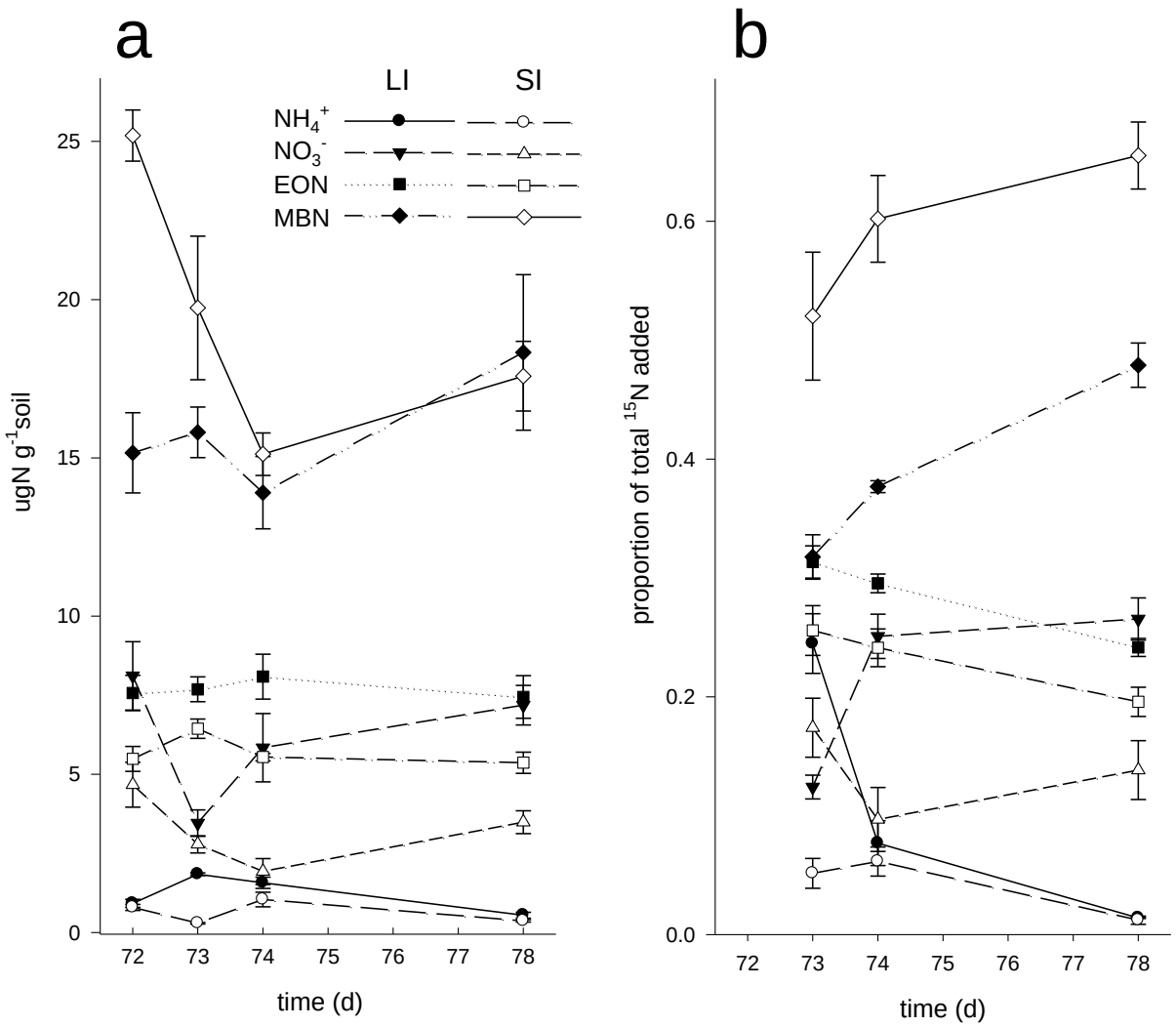


**Figure 5.** Size of KUFS soils extractable nitrogen pools (a) and the proportion of the added $^{15}N$ recovered in each of these pools (b) through time for long interval (LI) and short interval (SI) soil moisture treatments. Water plus $^{15}N$ labeled glycine were added to dry soils on day 73. Error bars represent ± one standard error of the mean (n = 3).

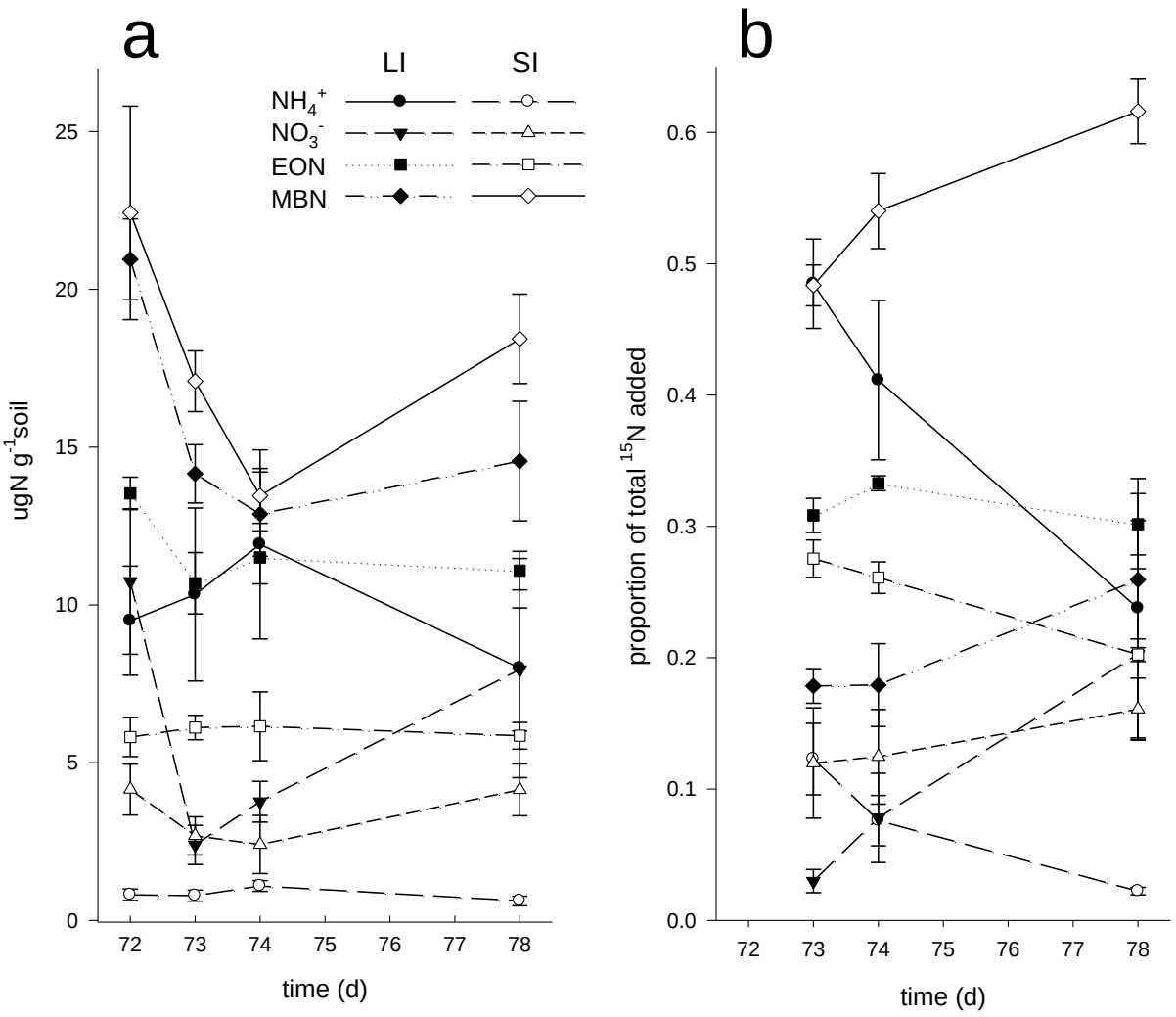


**Figure 6.** Index of microbial nitrogen demand calculated as microbial biomass N divided by total N available in LI and SI a) SVR, b) HYS, c) KNZ and d) KUFS soils. Error bars represent ± one standard error of the mean (n = 3).

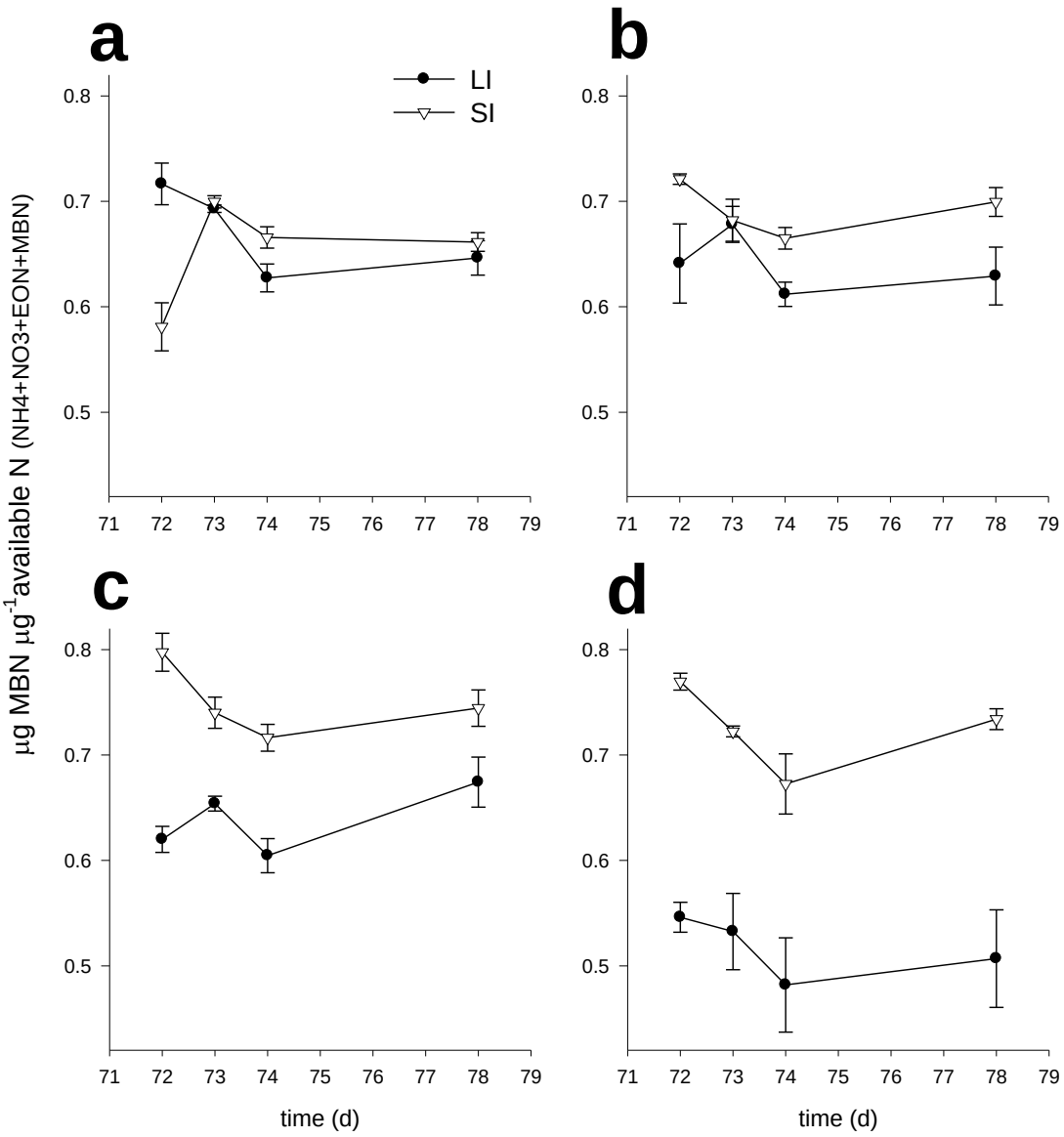
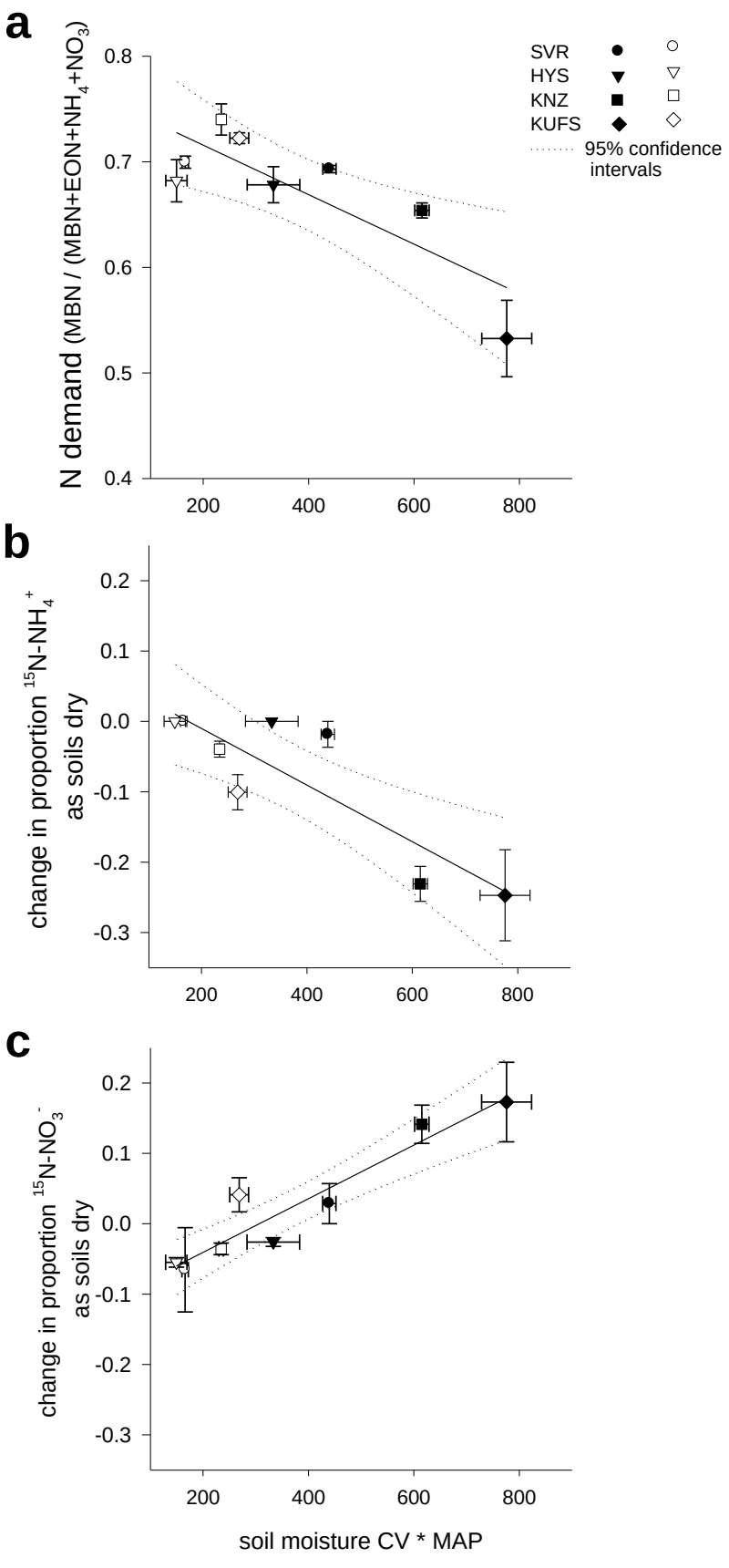


**Figure 7.** Relationship between indices of soil moisture stress and N demand for LI and SI soils from SVR, HYS, KNZ and KUFS day 78 (a), and the change in the proportion of $^{15}N$ incorporated in $NH_4^+$ (b) or $NO_3^-$ (c) pools as the soils dried between days 73 and 78. The index of soil moisture stress was calculated by dividing MAP at each site by the soil moisture CV experienced during the incubation for each treatment. Error bars represent ± one standard error of the mean (n = 3).


a
b
c
SVR
HYS
KNZ
KUFS
95% confidence intervals
N demand (MBN / (MBN+EON+NH4+NO3)
change in proportion 15N-NH4+ as soils dry
change in proportion 15N-NO3- as soils dry
soil moisture CV * MAP
0.8
0.7
0.6
0.5
0.4
0.2
0.1
0.0
-0.1
-0.2
-0.3
200
400
600
800

# CHAPTER 4: Resistance, resilience and redundancy in microbial community structure influence soil nitrogen cycling across a precipitation gradient

*Abstract*

Microbial communities responsible for mediating ecosystem processes are not always resistant to increases in environmental stress and disturbance. As these communities are altered by novel environments introduced through global change, the processes that they mediate can also be affected. In this study we examined two functional groups important to N cycling, nitrifiers and denitrifiers. We reciprocally transplanted soil cores along a precipitation gradient to determine how resistant, resilient or redundant these microbial functional groups, and processes they mediate, would be when exposed to altered soil moisture regimes. We sampled cores after 1.5 and 2.5 y of *in situ* incubation and measured functional gene abundance (*amo*A, *nir*K, *nir*S and *nos*Z) as well and nitrification (NP) and denitrification (PDEA) potentials. Across soils of all origins, we found increases in *amo*A abundance associated with decreases in NP as soil moisture stress and disturbance was increased. After 1.5 y we saw some functional redundancy and resistance to changes in denitrifier functional groups. After 2.5 y, resistance had dissipated, and denitrification gene abundance and PDEA declined as soils were exposed to increased soils moisture stress and disturbance. Our results indicate that in these grasslands, NP may result from expression of genes other than *amo*A, but that *nir*S and *nos*Z are closlely linked to PDEA. Reciprocal transplant of soils provides useful insights, but determining appropriate incubation time and quantifying the extent of environmental changes imposed on transplanted soils remain challenges to be overcome when using this approach.

# INTRODUCTION

Microbial communities mediate global biogeochemical cycles and are an integral part of the cycling of greenhouse gases (Singh et al., 2010). In recent years microbial ecologists have capitalized on molecular approaches to determine the structure of these diverse microbial communities and the factors that most influence these structures, and how that, in turn, may influence the biogeochemical processes these communities mediate. Two important factors controlling microbial community structure are environmental stress and disturbance (Allison and Martiny, 2008; Schimel et al., 2007). Environmental stress can be defined as a chronic condition that forces an alteration in physiology detrimental to growth, while disturbance can be defined as a discrete event that causes mortality and disrupts community or population structure (Schimel et al., 2007; Grime, 1977; White and Pickett, 1985). Environmental stress can be caused by multiple factors, including low soil moisture availability, nutrient limitation and heavy metal contamination (Schimel et al., 2007; Pace and Cole, 1994; Pennanen et al., 1996). Examples of disturbance include freeze-thaw cycles, intensive land use such as tilling, and increased soil moisture variability in the form of drying-wetting events (Allison and Martiny, 2008; Schimel et al., 2007; Degens et al., 2001). Stress and disturbances can have large impacts on both microbial community structure and function (Allison and Martiny, 2008; Schimel et al., 2007), but the degree to which changes in structure are linked to changes in biogeochemical function is relatively unknown.

Microbial communities frequently must cope with environmental stress and disturbance, and on relatively short time scales, are likely resilient, dealing with environmental stress or minor disturbances by altering their physiological state through the regulation of resource allocation, growth, and activity rates (Schimel et al., 2007; Allison and Martiny, 2008).

However, prolonged environmental stress or disturbance can permanently alter function through shifts in community structure (Schimel et al., 2007; Allison and Martiny, 2008). A recent review of microbial community responses to changes in their environment suggests that many communities are not resistant to disturbance, and that structurally distinct post-disturbance communities are not always functionally redundant (Allison and Martiny, 2008). This lack of resilience can lead to long-term changes in ecosystem processes. Though microbial community composition is a potential driver of ecosystem response to perturbation, how specific phylogenetic or functional groups respond to specific environmental stresses or disturbances remains unclear. With more specific information about linkages between the structure of microbial communities and process rates, we can increase our ability to predict ecosystem responses to anthropogenic global change.

In this study we examine how environmental stress and disturbance in the form of soil moisture availability and variability affect the function and structure of two important microbial functional groups, nitrifiers and denitrifiers, in North American grasslands. These grassland systems are well suited for exploring microbial response to soil moisture stress and disturbance because large fluctuations in soil moisture levels are common in this region, and microbial activities are most likely limited by organic C and N substrates only when soil moisture is not limiting (Austin et al., 2004; Epstein et al., 2002). Precipitation across this region controls aboveground net primary productivity (ANPP), rates of photosynthesis, soil respiration, decomposition rates, microbial biomass, litterfall quality and nutrient availability and can be more influential than temperature or soil type in driving important ecosystem-level processes (McCulley et al., 2005; Murphy et al., 2002; Epstein et al., 2002; Burke et al., 1997). There is a precipitation gradient across these grasslands, running from east to west that not only controls

the characteristics listed above, but also creates large variability in native soil moisture regimes. General circulation models predict future increases in drought severity and precipitation variability across North American grasslands, with both the magnitude and interval between precipitation events increasing (Easterling et al., 2000). Thus, in addition to the natural range of soil moisture stress and disturbance presented by these grasslands, ideal for our study, these native soil moisture regimes are likely to change in the future, making it important to understand how microbes in these systems will respond.

We chose nitrifiers and denitrifiers as the functional groups with which to test hypotheses about microbial responses to stress and disturbance because they mediate two important ecosystem level processes within the nitrogen (N) cycle, the genes associated with these organisms' functions are well characterized, and methods to quantify the biogeochemical fluxes they mediate are well-established (Phillipot and Harlin, 2005; Wallenstein and Vilgalys, 2005). In addition, effects of soil moisture on nitrification and denitrification have been well documented (Robertson and Groffman, 2007; Stark and Firestone, 1995). Because representatives of each of these functional groups are culturable, we are relatively knowledgeable about their physiologies. We know that nitrifiers, or ammonia oxidizing bacteria, tend to be slow growing and are poor competitors for critical resources such as $NH_4^+$, but are able to survive during periods of very low soil moisture (Stark and Firestone, 1995; Robertson and Groffman, 2007). Nitrifiers can adapt to changes in soil moisture regimes, but respond relatively slowly to sudden inputs of substrates during pulse events (Bock and Wagner, 2006; Fierer and Schimel, 2002; Stark and Firestone, 1995; Robertson and Groffman, 2007). These traits make nitrifiers well suited to survive during prolonged soil moisture stress with relatively low levels of disturbance. In contrast, denitrifiers are generally fast growers and appear to be

sensitive to environmental stress (Cavigelli and Robertson, 2000; Wallenstein et al., 2006), but may be better suited to respond positively during a disturbance. Denitrifiers are a versatile and evolutionarily disparate group of heterotrophs that shift from employing $O_2$ to $NO_3^-$ as an electron acceptor when $O_2$ becomes limiting, due either to high soil moisture levels or high rates of heterotrophic respiration (Shapleigh, 2006; Zumft, 1997). Because soil moisture has such a large influence on the process of denitrification, increases in soil moisture stress likely affects this group adversely. Disturbance in the form of rewetting a dry soil should create soil moisture conditions well suited for denitrification to proceed, and with such a wide variety of physiological traits, as well as rapid growth rates, some portion of the denitrifier functional group should be able to survive between wetting events to take full advantage of those conditions. While nitrifiers and denitrifiers have been well characterized in lab studies and in their responses to soil moisture availability, little is known about how these functional groups respond to chronic soil moisture stress and disturbance in terms of both community structure and process rates (Borken and Matzner, 2009).

In order to simultaneously test effects of both environmental stress and disturbance on microbial community structure and ecosystem function we reciprocally transplanted intact soil cores across the precipitation gradient in North America between four grassland sites (Reed and Martiny, 2007). We measured microbial responses to different soil moisture regimes after 1.5 and 2.5 years of *in situ* incubation to assess their resistance, resilience, and functional redundancy. For the purposes of this study, we defined community resistance as no change in community structure (i.e. nitrifier or denitrifier gene abundance) or function (i.e. denitrification or nitrification rates) as soils were exposed to new soil moisture regimes (Fig. 1a). We defined community resilience as significant changes in function and structure initially, with a later return

to previous levels (Fig. 1b). This definition assumes that such changes would be evident on the timescales represented by our 1.5 and 2.5 y sampling scheme. We hypothesized that nitrifiers would be either resistant or resilient to when faced with increasing soil moisture stress or disturbance because of their ability to adapt to high stress environments. We defined redundancy as changes in community structure with no concurrent changes in function (Fig. 1c). Finally, we consider communities sensitive to environmental stress and disturbance if we see changes in both function and structure after 2.5 years (Fig. 1d). We hypothesized that denitrifier functional groups would be somewhat sensitive to increases in soil moisture stress and disturbance because of their reliance on water for substrate diffusion and anaerobic conditions. We also hypothesized that denitrifiers may be resilient because of their relatively high evolutionary diversity (Shapleigh, 2006). The reciprocal transplant of soils permits us to assess the degree of coupling between N transformation rates and changes in the relative abundances of functional genes associated with these functions under changing precipitation regimes, while merging both field and manipulative approaches. Such approaches are particularly important in light of the challenges of interpreting laboratory incubations of soils and the alterations in precipitation patterns with predicted climate change.

## METHODS

### *Study Sites*

We chose four locations along a precipitation gradient in the Great Plains of North America. The sites have been historically grazed and burned except for the westernmost site, which is grazed but not burned. In early spring of 2008, fencing was erected at all sites to exclude cattle for the duration of the study. Moving from west to east, the first site, located at the Nature

Conservancy's Smoky Valley Ranch (SVR, W 100°58'55" N 38°51'50"), is a short grass prairie with silt loams (fine-silty, mixed, superactive, mesic, Aridic haplustolls) that receives mean annual precipitation (MAP) of 504 ± 11.6 mm (n = 118; High Plains Regional Climate Center (HPRCC)). The next site is the Kansas State University Western Kansas Agricultural Research Center near Hays, KS (HYS, W 99°17'46" N 38°50'13"). It is a mixed grass prairie with silt loams (fine-silty, mixed, superactive, mesic, Cumulic haplustolls) with MAP of 579 ± 13.5 mm (n = 118; HPRCC). Next, the Konza Prairie LTER (KNZ, W 96°33'18" N 39°5'2"), a tallgrass prairie, is a mix of silty loams (smectitic mesic Typic natrusolls) and silty clay loams (fine mixed superactive mesic Pachic argiustolls) with MAP of 838 ± 19.0 mm (n = 117; HPRCC). Finally, the easternmost site, part of the Kansas Field Station and Ecological Reserves (KUFS, W 95°14'35" N 38°10'21"), is a tallgrass prairie with gravelly silt loams (smectitic, thermic, Typic paleudolls) and MAP of 996 ± 21.8 mm (n = 104; HPRCC). Weather stations equipped with HOBO data loggers and air temperature, soil temperature and soil moisture sensors were installed at each site in April 2008 (Onset Computer Corporation, Bourne, MA), but we were only able to obtain a full data set for each site during the 2010 growing season.

*Soil Core Transplant and Harvest*

On April 13, 2008 we inserted 60, 10 cm diameter by 11 cm long PVC cores to a depth of 10 cm at 1 m intervals at KUFS. The cores, with intact soils, were immediately removed and a piece of 1 x 1 cm steel mesh was attached to the bottom with wire. We chose 15 of these cores at random and returned them to holes made by the previous extractions to serve as the KUFS controls. The remaining 45 cores were randomly distributed in sets of 15 to KNZ, HYS, and SVR, where they were placed in holes left by subsequent core extractions. The reciprocal

transplant of cores from all sites was accomplished in like manner over the course of three days. Sub-sets of these soil cores were harvested twice, on September 28-30, 2009 (after ~1.5 y) and October 1-3, 2010 (after ~2.5 y). We harvested cores in sets of 24 from each site, which included six control cores (those representing soils incubated *in situ* at their native site) plus six replicate cores originating from the other three sites. This sampling scheme generated a total of 96 cores each year. Intact soil cores were stored in coolers for no longer than 12 hours before they were returned to the University of Kansas and placed in storage at 4° C. All soil cores were processed within 72 h of collection, at which time time aboveground vegetation and roots > 2 mm in diameter were removed, and soil from each core was homogenized by hand. After processing, sub-samples of each soil were dried at 60°C for >48 h to determine gravimetric water content and frozen at -20° C for ensuing DNA extractions.

*Nitrification and Denitrification Potentials*

Nitrification potential (NP) was determined following Belser and Mays (1980) and Hart et al. (1994) with minor modifications. Briefly, 10 g soil was added to 100 ml of nitrification potential solution in 236 ml jars. The jars were capped with lids fitted with septa and the air was evacuated and replaced with 100% $O_2$ to 1 atm to prevent denitrification. Jars were incubated for 24 hours at 24°C on an orbital shaker, during which time we extracted 10 ml soil slurry samples at intervals of 5 to 6 h. After each sampling, the jars were recapped, evacuated and flushed with $O_2$ before being returned to the shaker. We added a flocculent solution to the slurry sub-samples, centrifuged them, and filtered the resulting supernatant through Whatman #4 filter papers. Filtered supernatants were analyzed for nitrite concentration using the cadmium reduction

method on a Lachat QuikChem 8000 auto-analyzer (Hach Co., Loveland, CO, USA). The rate of nitrification was calculated as the linear increase in nitrite concentration through time.

We assessed potential denitrification enzyme activity (PDEA) following Tiedje (1994). We used 5 g of soil placed in 127 ml Mason jars, to which we added 20 ml of PDEA media before sealing with lids fitted with septa. We evacuated then flushed each jar with pure $N_2$ gas to 1 atm three times before adding 10 ml of $C_2H_2$. We placed the jars on a shaker for 0.5 h before taking a 14 ml gas sample from each jar by syringe and injecting that sample into an evacuated gas vial. After an additional 1 h on the shaker, a second gas sample was obtained in the same manner. We analyzed $N_2O$ concentrations with an electron capture detector on a Varian gas chromatograph (Varian, Walnut Creek, CA, USA). The difference in $N_2O$ concentration between time one and two was used to calculate potential rates of denitrification.

*Nitrification and Denitrification Functional Genes*

To asses changes in microbial community structure associated with the processes of nitrification or denitrification, we quantified the abundance of ammonia monooxygenase (*amo*A), nitrite reductase (*nir*K and *nir*S) and nitrous oxide reductase (*nos*Z) using primers described in the literature (Table 1). We extracted DNA from soils previously frozen at -20° C using the MoBio PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA). We also extracted DNA from pure cultures of *Nitrosospira multiformis*, *Alcaligenes faecalis* and *Pseudomonas fluorescens* with the MoBio UltraClean Microbial DNA Isolation Kit. The soil and culture DNA was tested for purity and quantified spectrophotometrically on a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE, USA). Quantitative real-time PCR (qPCR) was performed on an Applied Biosystems 7500 Fast Real-time PCR System (Foster City, CA, USA)

with a reaction mix containing 12.5 µl ABsolute QPCR SYBR Green Mix (Abgene Ltd, Epsom, Surrey, UK), 7.5 µl of a 10 µM primer mix (Invitrogen Corporation, Carlsbad, CA), and 5 µl of 0.5 ng $\mu l^{-1}$ DNA for a final volume of 25 µl. Reaction conditions were 15 min at 95° C followed by 40 cycles of 30 s at 95° C, 30 to45 s at the appropriate annealing temperature (Table 2) and 30 s at 72° C. We used one plate per soil, per primer pair, containing three replicates of each DNA sample as well as three negative controls. Standard curves were generated using genomic DNA from pure cultured species, and consisted of five standards in a dilution series ranging from 30 to 300,000 gene copies. For all assays, standard curves had $R^2 > 0.99$. Gene copy numbers per ng DNA were calculated assuming a mass of $1.096E^{-21}$ g per base pair and one gene copy per genome. We obtained *N. multiformis* and *P. fluorescens* genome sizes from the NCBI database (3.18 and 7.07 Mb respectively), and estimated *A. faecalis* genome size from the closest related species with a known genome size, *Burkholdia mallei* (3.5 Mb, Wallenstein and Vilgalys, 2005). Melting curve analysis was performed after each qPCR assay to assess product quality and specificity.

*Statistical Analyses*

Though we present minimum and maximum values and averages for measures of NP, PDEA, *amo*A, *nir*K, *nir*S, and *nos*Z (Table 2), statistical assessment using these values was hindered because several indigenous soil characteristics, such as texture, pH, and C content, varied across our study sites. Therefore, to compare soils originating from all four of our study sites in the same analyses, we normalized the data by using control soils at each site, collected each year, as a reference to transform gene abundance and activity levels into measures of relative change. For example, measures of function (NP and PDEA) and gene abundance in SVR

soils incubated at KUFS, KNZ or HYS, were divided by the same measure made in the control soils (SVR soil at SVR). We were then able to analyze each year's measures of NP, PDEA, *amo*A, *nir*K, *nir*S, and *nos*Z by 2-way ANOVA with factors of site (location core was moved to) and soil (location of soil origin) using PERMANOVA (Anderson, 2001). We considered the 'site' factor to represent effects of environmental stress and disturbance and the 'soil' factor to represent effects of the soil origin and the original microbial community. We used Bray-Curtis similarity matrices with 999 unrestricted permutations of the raw data for PERMANOVA analyses and Monte Carlo asymptotic *P*-values (Anderson, 2001; McArdle and Anderson, 2001). When there was a significant site*soil interaction effect, we ran pairwise tests between pairs of sites for each soil type separately. If the interaction was not significant, we ran pairwise tests on site or soil factors when these were significant effects.

## RESULTS

### *Precipitation and soil moisture*

We retrieved precipitation data for 2008, 2009 and 2010 using the HPRCC online data base from weather stations within 12 km of the HYS, KNZ and KUFS sites. For SVR we used an average of two weather stations that were 19 and 27 km from the study site. At SVR, precipitation totals in 2008 were similar (516 mm), in 2009 higher than (707 mm) and in 2010 lower (441 mm) than the historical MAP of 504 mm. At HYS, 2008 precipitation totaled 833 mm while in 2009 and 2010, precipitation totals of 552 mm and 583 mm, respectively, were very close to historical MAP of 579 mm. We observed some variation in precipitation totals at KNZ between 2008 (917 mm), 2009 (926 mm) and 2010 (656 mm), but all years were within one standard deviation of the MAP of 838 mm. The KUFS site received higher precipitation than its

MAP of 996 mm in 2008 (1278 mm), 2009 (1249 mm) and 2010 (1118 mm). In order to gauge differences in precipitation and soil moisture variability between sites, we plotted precipitation and SWC in the top 5 cm during the growing season, April through September, 2010 (Fig. 2). We concentrated on growing season variation because 63-76% of annual precipitation at our study sites falls during this time period.

*Nitrification and denitrification potentials*

Across the precipitation gradient, west to east, we saw general declines in NP based on incubation site. Nitrification potentials in 2009 were affected by site of incubation ($P = 0.001$) and soil origin ($P = 0.001$) with a significant interaction between site and soil ($P = 0.001$; Fig 3a). As we present the results for NP we direct the reader to the appropriate symbol comparisons to assist in this and future figure interpretation. Pairwise comparisons of soils originally from SVR in 2009 (Fig. 3a, triangle symbols compared across X-axis) had significantly higher NP at their native location, SVR, compared to at HYS ($P = 0.028$), KNZ ($P = 0.001$), or KUFS ($P = 0.001$). We also found higher NP in SVR soils at HYS compared to SVR soils at KNZ ($P = 0.006$) or KUFS ($P = 0.003$). The soils originating from HYS (Fig. 3a, square symbols compared across x-axis) showed higher NP in 2009 at SVR compared to at HYS ($P = 0.006$), at KNZ ($P = 0.001$) or at KUFS ($P = 0.003$) and higher NP at HYS compared to at KNZ ($P = 0.007$). Also in 2009, KNZ soils (Fig. 3a, circle symbols compared across x-axis) at KNZ had lower NP than KNZ soils at SVR ($P = 0.006$), at HYS ($P = 0.029$), or at KUFS ($P = 0.042$). Nitrification potentials in KUFS soils in 2009 were not different between sites (Fig. 3a, diamond symbols compared across x-axis). In contrast to 2009 NP results, in 2010 only site of incubation had a significant effect on NP such that all soils located at KUFS (Fig. 3b, average of triangle, square,

circle and diamond symbols at each site of incubation, compared across x-axis) had on average lower NP than all soils located at HYS ($P$ = 0.001) or at SVR ($P$ = 0.046). All soils at KNZ in 2010 also had lower NP than all soils at HYS ($P$ = 0.047; Fig. 3b).

Denitrification potential in 2009 was variably affected by incubation location depending on soil site of origin, but by 2010 there were general increases in PDEA from west-east across the precipitation gradient. In 2009 we found significant effects of site of incubation ($P$ = 0.001), soil origin ($P$ = 0.001) and an interaction between site and soil ($P$ = 0.034) on PDEA (Fig 3c). Within the interaction, in 2009 soils originating from SVR, had greater PDEA when incubated at KNZ than at SVR ($P$ = 0.039; Fig 3c, triangles). PDEA was lower in HYS soils incubated at HYS than at SVR ($P$ = 0.013) or at KUFS ($P$ = 0.005) and lower at KNZ than at SVR ($P$ = 0.010) or at KUFS ($P$ = 0.004; Fig. 3c, squares). In 2009 KNZ soils incubated at KNZ had lower PDEA than at HYS ($P$ = 0.030) or at KUFS ($P$ = 0.001) while KNZ soils exhibited greater PDEA incubated at KUFS than at SVR ($P$ = 0.002) or at HYS ($P$ = 0.038; Fig. 3c, circles). Denitrification potentials in KUFS soils in 2009 were not significantly different between sites of incubation (Fig. 3c, diamonds). In 2010, both site of incubation ($P$ = 0.001) and soil ($P$ = 0.001) had significant effects on PDEA with no significant interaction (Fig. 3d). By site of incubation, there was lower PDEA at SVR compared to HYS ($P$ = 0.003), KNZ ($P$ = 0.001) or KUFS ($P$ = 0.001), lower PDEA at HYS compared to KUFS ($P$ = 0.002), and lower PDEA at KNZ than at KUFS ($P$ = 0.004). By soil, we found overall higher PDEA in SVR soils compared to HYS ($P$ = 0.002), KNZ ($P$ = 0.001) and KUFS ($P$ = 0.001) soils and higher PDEA in HYS soils compared to KNZ ($P$ = 0.033) and KUFS ($P$ = 0.002) soils.

*Functional gene abundance*

In contrast to NP, we found generally increasing *amo*A gene abundance from west-east across the precipitation gradient. Using the normalized data, in 2009 we found significant effects of site of incubation ($P = 0.001$) and soil ($P = 0.027$) on *amo*A gene abundance (Fig. 4a). Averaged across all soils, at SVR there was lower *amo*A abundance than at HYS ($P = 0.006$), at KNZ ($P = 0.005$) or at KUFS ($P = 0.002$; Fig 4a). Comparing between soils of the same origin, regardless of incubation site, we found higher *amo*A abundance in soils originating from SVR (Fig. 4a, all triangles) compared to soils originating from KUFS (Fig. 3a, all diamonds; $P = 0.007$). There were similar incubation site ($P = 0.002$) and soil origin ($P = 0.001$) effects on *amo*A in 2010. Averaged across soils of different origins, *amo*A abundance was lower at SVR than at HYS ($P = 0.004$), at KNZ ($P = 0.002$) or at KUFS ($P = 0.003$; Fig. 4b). In soils originating from SVR (Fig. 4b, average of all triangles), there was greater abundance of *amo*A compared to soils originally from HYS (all squares; $P = 0.001$) or from KNZ (all circles; $P = 0.001$). We also saw greater *amo*A abundance in soils originally from KUFS (Fig. 4b, all diamonds) compared to soils of HYS origin (all squares; $P = 0.033$).

Across both years, we saw relatively few changes in *nir*K abundance regardless of soil origin or incubation site. In 2009, *nir*K abundance was affected by a significant site*soil interaction ($P = 0.026$; Fig 5a). Within the interaction, *nir*K abundance was not different by incubation site in soils originally from SVR (Fig. 5a, triangles). Soils of HYS origin incubated at HYS had lower *nir*K abundance than when incubated at KUFS ($P = 0.043$; Fig. 5a, squares), soils of KNZ origin incubated at KNZ had lower abundance than when incubated at KUFS ($P = 0.016$; Fig. 5a, circles), and soils of KUFS origin incubated at SVR had lower abundance than when incubated at KNZ ($P = 0.040$; Fig 5a, diamonds). In 2010 there were also significant differences in *nir*K abundance by site ($P = 0.001$) and soil ($P = 0.001$) with a significant

interaction ($P$ = 0.009; Fig. 5b). As in 2009, *nir*K abundance in soils originally from SVR did not differ between sites (Fig. 5b, triangles). Soils originally from HYS however, had greater *nir*K abundance when incubated at SVR or HYS than at KNZ ($P$ = 0.006; $P$ = 0.012; Fig. 5b, squares) or KUFS ($P$ = 0.018; $P$ = 0.032; Fig 5b, squares). In soils originally from KNZ (Fig. 5b, circles), there were fewer *nir*K copies when incubated at SVR than at HYS ($P$ = 0.003), at KNZ ($P$ = 0.005) or at KUFS ($P$ = 0.001), and similarly, KUFS origin soils (Fig. 5b, diamonds) had fewer copies when incubated at SVR than at KNZ ($P$ = 0.011) or at KUFS ($P$ = 0.008).

The abundance of *nir*S generally increased across the precipitation gradient (west to east). In 2009, *nir*S varied by incubation site ($P$ = 0.001) and soil origin ($P$ = 0.001) with a site*soil interaction ($P$ = 0.001; Fig 5c). For soils of SVR origin, there were no differences between sites of incubation (Fig. 5c, triangles). Soils initially from HYS (Fig. 5c, squares) had lower *nir*S abundance when incubated at SVR and HYS than at KNZ ($P$ = 0.001; $P$ = 0.001) or at KUFS ($P$ = 0.001; $P$ = 0.001) and lower abundance when incubated at SVR than at HYS ($P$ = 0.011). Moving soils of KNZ origin to SVR in 2009 resulted in lower nirK abundance compared to the move to KUFS ($P$ = 0.021; Fig. 5c, circles), and moving KUFS soils (Fig. 5c, diamonds) to SVR resulted in lower abundance relative to KUFS soils at HYS ($P$ = 0.018), at KNZ ($P$ = 0.002) and at KUFS ($P$ = 0.011). In 2010 there was no interaction, but *nir*S differed between incubation sites ($P$ = 0.001) and by soil origin ($P$ = 0.002; Fig 5d). We found lower *nir*S abundance averaged across soils of differing origin at SVR and HYS compared to KNZ ($P$ = 0.001; $P$ = 0.001) and KUFS ($P$ = 0.001; $P$ = 0.002). Differences due to soil origin were divided between soils of western versus eastern origin. Soils originating in the west, from SVR and HYS (Fig. 5d, averages of all triangles or all squares), had greater *nir*S abundance than soils originating in the east, from either KNZ (average of all circles vs. all triangles, $P$ = 0.017; versus

all squares, $P = 0.001$) or KUFS (average of all diamonds vs. all triangles, $P = 0.030$; vs. all squares, $P = 0.002$).

As with *nir*S, *nos*Z abundance generally increased west-east across the precipitation gradient. In 2009 *nos*Z abundance varied across sites ($P = 0.001$) and soils ($P = 0.001$) but with different magnitudes depending on soil origin (site*soil, $P = 0.004$; Fig 5e). Moving SVR soils to any other location did not result in different *nos*Z abundances (Fig. 5e, triangles). Moving HYS soils (Fig. 5e, squares) east to KNZ or KUFS resulted in higher *nos*Z abundance ($P = 0.007$; $P = 0.002$). Soils of HYS origin also had higher *nos*Z abundance incubated at KUFS than at SVR (Fig. 5e, squares, $P = 0.011$). In soils of KNZ origin, *nos*Z abundance was lower at SVR or HYS incubation sites than at KUFS (Fig. 5e, circles; $P = 0.030$; $P = 0.029$). Similarly, soils from KUFS (Fig. 5e, diamonds) had lower *nos*Z abundance when moved to SVR or HYS compared to being moved to KNZ ($P = 0.026$; $P = 0.017$) or staying at KUFS ($P = 0.012$; $P = 0.006$). We found similar trends in *nos*Z in 2010 with changes by site ($P = 0.001$) and soil ($P = 0.001$) depending on soil origin (site*soil, $P = 0.046$; Fig 5f). Within the interaction, soils of SVR origin had greater *nos*Z abundance after incubation at KNZ than at HYS (Fig. 5f, triangles; $P = 0.026$). We found increased *nos*Z in soils originating from both HYS and KNZ as those soils were moved west to east; HYS soils incubated at SVR or HYS had fewer copies than at KNZ ($P = 0.001$; $P = 0.028$) or at KUFS ($P = 0.003$; $P = 0.021$; Fig. 5f, squares) and soils of KNZ origin incubated at SVR or HYS also had fewer copies than at KNZ ($P = 0.002$; $P = 0.012$) or at KUFS ($P = 0.001$; $P = 0.003$ Fig. 5f, circles).

## DISCUSSION

In this study we used reciprocally transplanted soils to explore the resistance, resilience, and functional redundancy of two important N-cycling processes and the microbial communities that mediate them with altered precipitation regimes. We compared process rates with appropriate functional gene abundances across sites and through time to determine if and how process rates and community composition are linked (Fig. 1). The study was conducted over a relatively long time scale, because on shorter time scales grassland microbial communities appear well-adapted to cope with changes in precipitation patterns (Iovieno and Baath, 2008; Fierer et al., 2003), reflective of the large inter- and intra-annual precipitation variability inherent to grassland systems like the Great Plains (Seastedt and Knapp, 1993; Lauenroth and Burke, 1995). Given the variation in results observed when comparing 2009 and 2010 data, our study also indicates the importance of selecting appropriate time scales for sampling when attempting to link structural characteristics of microbial communities to their biogeochemical functioning. Our data further highlights the challenges of discerning differences between microbial variation due to the functional and structural characteristics we measure versus that due to inter-annual climate variability.

*Nitrification potential across the precipitation gradient*

Nitrifying bacteria in a multitude of ecosystems demonstrate an ability to adapt to prolonged soil moisture stress with little or no change in nitrification potential after that stress is relieved (Gleeson et al., 2008; Robertson and Groffman, 2007; Fierer and Schimel, 2002; Davidson, 1992; Allison and Prosser, 1991). In the current study, we found that neither NP nor the functional group responsible for nitrification, represented by *amo*A abundance, were resistant to the changes in environmental stress and disturbance imposed by our reciprocal transplants (Fig 1d). In 2009, after 1.5 y of field incubation, we observed increasing abundances of *amo*A

we as moved from west to east across the precipitation gradient, regardless of the soil origin (Fig. 4). In 2010, we observed declines in NP as we move from west to east (Fig. 3). These results indicate that over this time scale (~2.5 y) these microbial communities are not resilient (Fig 1b), and that the abundance of nitrifying bacteria is controlled by soil moisture regime. Environmental control of microbial community structure is not surprising, given the previously described links between nitrification rates and soil moisture (Gleeson et al., 2008; Robertson and Groffman, 2007; Fierer and Schimel, 2002; Davidson, 1992; Allison and Prosser, 1991). However, the decoupling of a*mo*A abundance and nitrification potential – reflected by increasing *amo*A abundance and decreasing nitrification potential – suggests that this functional gene may not be the best indicator for nitrifier functional groups when relative gene abundances, and not gene expression, are measured. This decoupling may reflect functional redundancy in nitrification potential due to ammonia oxidizing archaea (Francis et al., 2007). Ammonia oxidizing archaea in particular can be important drivers of nitrification and have been found in greater abundance than ammonia oxidizing bacteria in many of the soils surveyed thus far (Leininger et al., 2006). Ammonia oxidizing archaea appear to be less influenced by environmental parameters such as precipitation (Schwartz and Adair, 2008), perhaps making the archaeal *amo*A gene a better indicator for the changes in the nitrifier functional group that would result in altered rates of nitrification.

Another possible explanation for the decoupling we observed between *amo*A and NP is nitrifier denitrification. In 2010 we observed increases in *amo*A across sites driven primarily by high *amo*A abundance in soils of SVR origin (Fig. 4b, triangles). These dramatic increases in *amo*A abundance in SVR soils were not linked to NP, but may be related to the large increase in PDEA also observed in 2010 SVR soils across sites (Fig. 3d, triangles). Nitrifier denitrification

increasingly has been receiving recognition as an important process in N cycling (Kool et al., 2011; Shaw et al., 2006; Wrage et al., 2005). Nitrifier denitrification, which occurs under high $O_2$ environments, unlike denitrification, can account for up to 40% of total denitrification (Wrage et al., 2005). If this is the case in these Great Plains soils, it may explain why *amo*A abundance appears to be decoupled from NP. During the NP assay we measure the increase in $NO_2^-$ as an indicator for nitrification, but if nitrifier denitrification is occurring, concentrations of $NO_2^-$ would decline during the assay, causing underestimation of nitrification potential. We have no direct evidence to support this speculation, but it highlights the need for more comprehensive analyses of nitrification and denitrification pathways under varying environmental conditions. Overall, we conclude that nitrifiers are sensitive to changes in soil moisture regimes and that both community composition and function are controlled by the environment, which in this case is likely a reflection of altered soil moisture stress. However, the changes in structure and function we observed are decoupled and further research, including surveys of archaeal nitrification and nitrifier denitrification, are needed to understand how nitrification and community structure are linked.

*Denitrification potential across the precipitation gradient*

Denitrifiers are a diverse functional group, using multiple enzymes to complete the process of denitrification when $O_2$ becomes limiting (Shapleigh, 2006; Zumft, 1997). Because of their metabolic as well as evolutionary diversity, as a functional group, denitrifiers would seem well-suited to thrive in rapidly changing environments such as soils with high soil moisture variability. However, due to the relatively high energy costs associated with generation and maintenance of denitrification genes and the constitutive production of denitrification enzymes,

much of the competitive advantage that denitrifiers exhibit in their flexibility of acceptable electron acceptors, compared to strictly aerobic heterotrophs, may be lost when $O_2$ is present (Shapleigh, 2006). Thus, when soil microbial communities accustomed to relatively moist environments are subjected to chronic low soil moisture levels with relatively plentiful soil $O_2$, denitrifiers in that soil presumably would suffer a decline in competitive advantage.

Consistent with this idea, in 2009, we observed reductions in PDEA when KNZ and HYS soils were moved from relatively low to high soil moisture stress environments. In conjunction with the changes observed in PDEA in KNZ and HYS soils, these shifts were associated with concurrent decreases in *nir*S and *nos*Z gene abundance moving west to east across the gradient, suggesting that in these soils, denitrifier functional groups were not resistant (Fig. 1a) to environmental stress, nor were functionally redundant groups active (Fig. 1c). Soils from the extreme ends of our precipitation gradient exhibited contrasting patterns to the trends observed in HYS and KNZ soils. SVR soil denitrifiers seemed to be completely resistant (Fig. 1a) to changes in soil moisture regimes, exhibiting no differences in PDEA, or *nir*S, *nir*K or *nos*Z gene abundance when moved to any other site. KUFS soils, in contrast, exhibited declines in *nos*Z and *nir*S gene abundances, but no change in PDEA, suggesting that functional redundancy (Fig. 1c) is an important feature for these processes in KUFS soils. Interestingly, we saw only a few small shifts in *nir*K abundance across all soils and sites. Reasons for this trend, as distinct from *nir*S trends, remain unclear.

The variability in results associated with denitrification after 1.5 y of transplant implementation is likely a function of the broad evolutionary diversity that is found in denitrifying bacteria (Phillippot, 2005) as compared to the narrower taxonomic group of nitrifying bacteria (Bock and Wagner, 2006). Previous studies have reported variability in

denitrifier community structure or function dependent on the origin of those communities (Sharma et al., 2005; Wolsing and Prieme, 2004; Nogales et al., 2002; Prieme et al., 2002). We suggest that the gradient in soil moisture stress to which soils in the current study were exposed resulted in different community structures through selection of different sets of denitrifier taxa, specific to each soil's origin. The inherent difference between these communities may be driving the variability in responses to reciprocal transplant found after the first year and a half of incubation.

After an additional year of being transplanted, denitrifiers in all soils exhibited sensitivity to soil moisture stress and disturbance (Fig. 1d). There was a consistent pattern of increasing PDEA with decreasing soil moisture stress across all soils, with corresponding increases in *nir*S and *nos*Z abundance. Soil communities that exhibited different structure at the onset of our experiment, some of which were resistant to change over the first 1.5 y (KUFS and SVR soil), converged in *nir*S and *nos*Z abundance, presumably as the environment 'selected' taxa at each site, which in turn altered process rates. In 2010, we again saw few changes in the abundance of *nir*K across sites. It is unclear why there was this disparity between *nir*K and *nir*S results as these nitrite reductases appear to be functionally redundant and have no obvious phylogenetic distribution (Shapleigh, 2006; Sharma et al., 2005). In fact, these two genes may be found in different members of the same genus, although they are never found together in a single bacterium (Shapleigh, 2006). Based on raw abundance data, in 2009 *nir*K was more prevalent than *nir*S in soils originating from SVR and HYS soils while *nir*S was more prevalent than *nir*K in soils originating from KNZ and KUFS. By 2010, *nir*K appeared to be the dominant nitrite reductase with higher abundance than *nir*S in all soils. This shift to *nir*K dominance in 2010 coincided with the loss of resistance and functional redundancy seen in 2009 which may indicate

a difference in stress tolerance between denitrifiers that possess *nir*K rather than *nir*S, or a difference in the availability of copper versus iron, the enzymatic co-factors for *nir*K and *nir*S respectively (Shapleigh, 2006; Sharma et al., 2005). As with our assays of nitrifier community structure, we have not included archaeal or fungal denitrifiers, both of which may be important contributors to this process (Crenshaw et al., 2008; Laughlin and Stevens, 2002; Phillippot, 2002). Although we saw similar trends in PDEA and functional gene abundance, future studies that quantify the contribution of bacteria, as well as archaea and fungi, to denitrification processes should help provide a clearer picture of the links between microbial community structure and denitrification.

*Conclusions*

In Figure 6 we present a schematic diagram representing the influence of soil moisture stress and disturbance on nitrifier and denitrifier functional groups. We propose that contrary to our hypothesis, nitrifiers in these soils exhibited sensitivity to environmental change. Sensitivity to soil moisture stress and disturbance was evident in both function (NP) and structure (*amo*A), but not in the same manner. We cannot confidently predict the directionality of any future changes in nitrification in Great Plains soils, given that our measures of function indicate an increase and measures of structure a decrease in nitrifier activity as soil moisture stress and disturbance is increased.

Denitrifier functional groups responded differently to changes in soil moisture regimes, depending on their origin. In addition to changes in denitrifier function and gene abundance, and contrary to our hypotheses, we saw some resistance to soil moisture regime change after 1.5 y. This could also be resilience on a shorter time scale, but we have no way of assessing this. After

2.5 y, as we predicted, all denitrifier functional groups appeared to be sensitive to environmental change, converging on the same relationship between decreasing PDEA and functional gene abundance as soil moisture stress and disturbance increased. If nitrifier and denitrifier functional groups were to respond to environmental perturbations in like manner, then these two important N cycling processes would remain coupled. If however these processes were to change in opposite directions as seen in this study, with increases in nitrification linked to decreases in denitrification, then the N cycle could be de-coupled. This has important implications for N cycling in these systems. If nitrification increases while denitrification is decreasing, then soil $NO_3^-$ concentrations could increase, particularly during periods of drought, to then be lost through leaching at the next precipitation event.

Reciprocal transfer of soils permits simultaneous assessment of the effects of community composition and environmental factors and their interactions on soil processes, however this method presents challenges. For example, the appropriate time scale for *in situ* incubation can be difficult to determine. The time scale of this study likely played an important role in driving our conclusions. Other reciprocal transplant studies however, using similar time scales, have reported little to no connection between process and microbial community structure (Balser and Firestone, 2005; Boyle et al., 2006). In addition, inherent inter-annual climate variability can make comparisons between sampling points difficult. Differences in precipitation patterns between 2009 and 2010 likely influenced our data, however, in general the differences in precipitation totals between years was lower than differences between sites. Working at four sites across a gradient of precipitation regimes helped us to overcome the annual climate differences so that we could make connections between changing structure and function. In other studies, where reciprocal transplants are carried out between two locations, researchers were limited in their

ability to link changing microbial function and community composition (Balser and Firestone, 2005; Waldrop and Firestone, 2006; Boyle et al., 2006). In assessing multiple sites along an environmental gradient we were able to find trends that would not have been evident if only two sites had been used. Reciprocal transplant of soils can provide valuable information about how microbial communities respond to changes in their environment, but the difficult challenge is determining the appropriate length of time and the extent of the environmental gradient used for the transplants. These *in situ* studies can give us insight into what forms of environmental change are important drivers of changes in microbial community structure and when these structural changes are indicators of changing function.

**Table 1.** Selected primers and source references for amplification of functional genes.

| gene | primer | sequence | Annealing temperature (°C) | Reference |
|---|---|---|---|---|
| *amo*A | amoA-1F | GGGGTTTCTACTGGTGGT | 56 | Geets et al., 2007 |
| | amoA-2R | CCCCTCKGSAAAGCCTTCTTC | | |
| *nir*K | nirK876 | ATYGGCGGVAYGGCGA | 58 | Henry et al., 2004 |
| | nirK1040 | GCCTCGATCAGRTTRTGGTT | | |
| *nir*S | nirS-cd3aF | GTSAACGTSAAGGARACSGG | 50 | Geets et al., 2007 |
| | nirS-R3cd | GASTTCGGRTGSGTCTTGA | | |
| *nos*Z | nosZ-F | CGYTGTTCMTCGACAGCCAG | 52 | Wallenstein and Vilgalys, 2006 |
| | nosZ-R | CATGTGCAGNGCRTGGCAGAA | | |

**Table 2.** Minimum and maximum values followed by the mean ± one standard error for measures of microbial community function and structure. Values are ranges and averages calculated by soil origin, regardless of incubation site.

| | **SVR** | | **HYS** | | **KNZ** | | **KUFS** | |
|---|---|---|---|---|---|---|---|---|
| | *2009* | *2010* | *2009* | *2010* | *2009* | *2010* | *2009* | *2010* |
| Nitrification Potential | 1.1 - 10.1 | 0.6 - 26.6 | 7.6 - 34.0 | 0.4 - 26.3 | 1.0 - 27.6 | 0.8 - 16.6 | 1.0- 8.0 | 0.6 - 27.6 |
| μgN $g^{-1}d^{-1}$ | 3.8 ± 0.5 | 8.2 ± 1.2 | 16.2 ± 1.4 | 11.4 ± 1.3 | 7.2 ± 1.3 | 4.8 ± 0.8 | 4.7 ± 0.3 | 6.0 ± 1.1 |
| Denitrification Potential | 0.2 - 1.9 | 0.1 - 1.5 | 0.6 - 11.9 | 1.2 - 7.8 | 3.5 - 9.9 | 1.8 - 8.2 | 2.5 - 12.8 | 1.5 - 6.9 |
| μgN $g^{-1}d^{-1}$ | 0.7 ± 0.1 | 0.5 ± 0.1 | 7.6 ± 0.6 | 3.0 ± 0.3 | 5.8 ± 0.4 | 4.3 ± 0.3 | 5.7 ± 0.5 | 3.8 ± 0.3 |
| *amo*A | 0.1- 2.1 | 0.2 - 0.4 | 0.4 - 5.4 | 0.1 - 7.2 | 0.2 - 4.9 | 0.05 - 2.4 | 0.1 - 2.6 | 0.01 - 0.3 |
| copies $\mu g^{-1}$ soil | 0.6 ± 0.1 | 0.1 ± 0.02 | 6.4 ± 0.2 | 0.88 ± 0.3 | 1.6 ± 0.3 | 0.4 ± 0.1 | 1.0 ± 0.1 | 0.1 ± 0.02 |
| *nir*K | 162 – 3115 | 909 – 5507 | 337 – 1665 | 745 – 28786 | 5 – 219 | 1743 – 16691 | 4 – 346 | 1641 – 12528 |
| copies $\mu g^{-1}$ soil | 810 ± 137 | 2760 ± 259 | 673 ± 78 | 3851 ± 1151 | 32 ± 11 | 5846 ± 774 | 61 ± 14 | 6236 ± 597 |
| *nir*S | 51 – 505 | 54 – 415 | 111 – 698 | 99 – 969 | 1 – 5155 | 41 – 466 | 58 – 1446 | 24 – 472 |
| copies $\mu g^{-1}$ soil | 212 ± 24 | 186 ± 21 | 270 ± 30 | 335 ± 46 | 1084 ± 232 | 157 ± 23 | 608 ± 71 | 151 ± 21 |
| *nos*Z | 4 – 20 | 335 – 3352 | 16 – 113 | 492 – 3263 | 9 – 139 | 601 – 5725 | 9 – 145 | 571 – 6685 |
| copies $\mu g^{-1}$ soil | 10 ± 1 | 1232 ± 163 | 40 ± 5 | 1287 ± 146 | 67 ± 6 | 1789 ± 219 | 64 ± 8 | 3000 ± 329 |

**Figure 1**. Hypothetical results for the transplant of one microbial community across an environmental gradient from point A to point B. Solid symbols and lines represent a process rate measure and dashed sybols and lines represent a measure of community structure. If the community is resistant (a), there will be no change in structure or function. If the community is resilient (b), there will be shifts in structure and function (t1) followed by a return to previous structure and level of function (t2). If the community is functionally redundant (c) then shifts in community structure will not lead to shifts in function. Finally, a community may be sensitive to disturbance (d) evidenced by shifts in community structure and process rates.

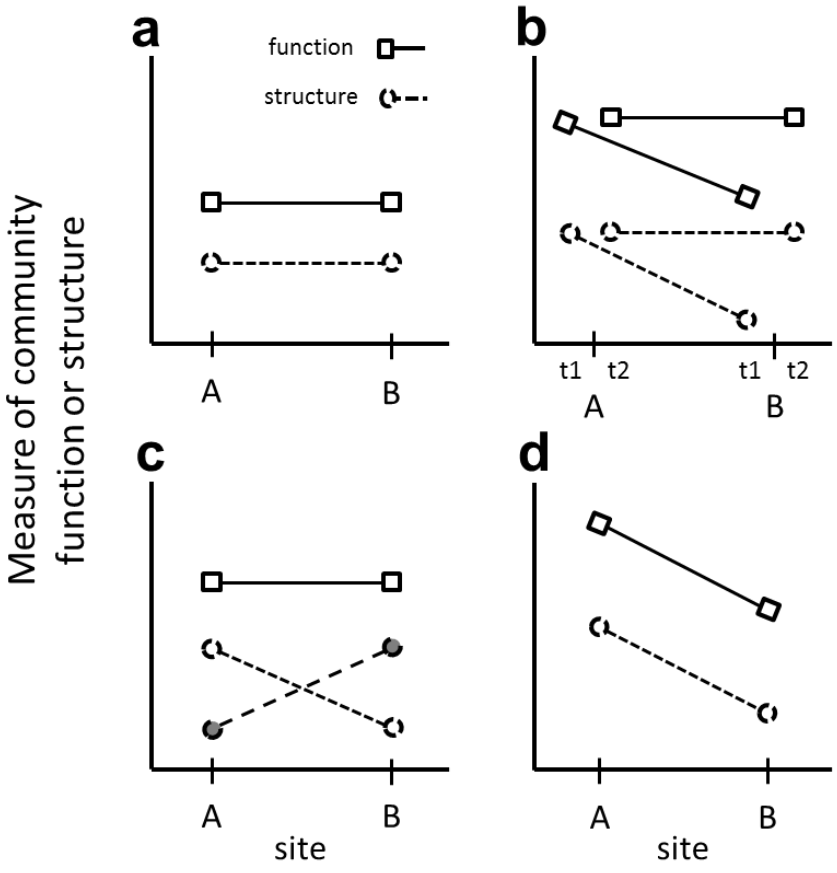


**Figure 2**. Study site precipitation (bars) and soil water content (SWC; circles) for April or May through September 2010 at SVR (a), HYS (b), KNZ (c), and KUFS (d). Coefficient of variation (CV) was calculated for SWC at each site and serves as a proxy for the level of soil moisture disturbance each sites experiences. KUFS SWC data was not collected between May and July 12 due to instrumentation failure.

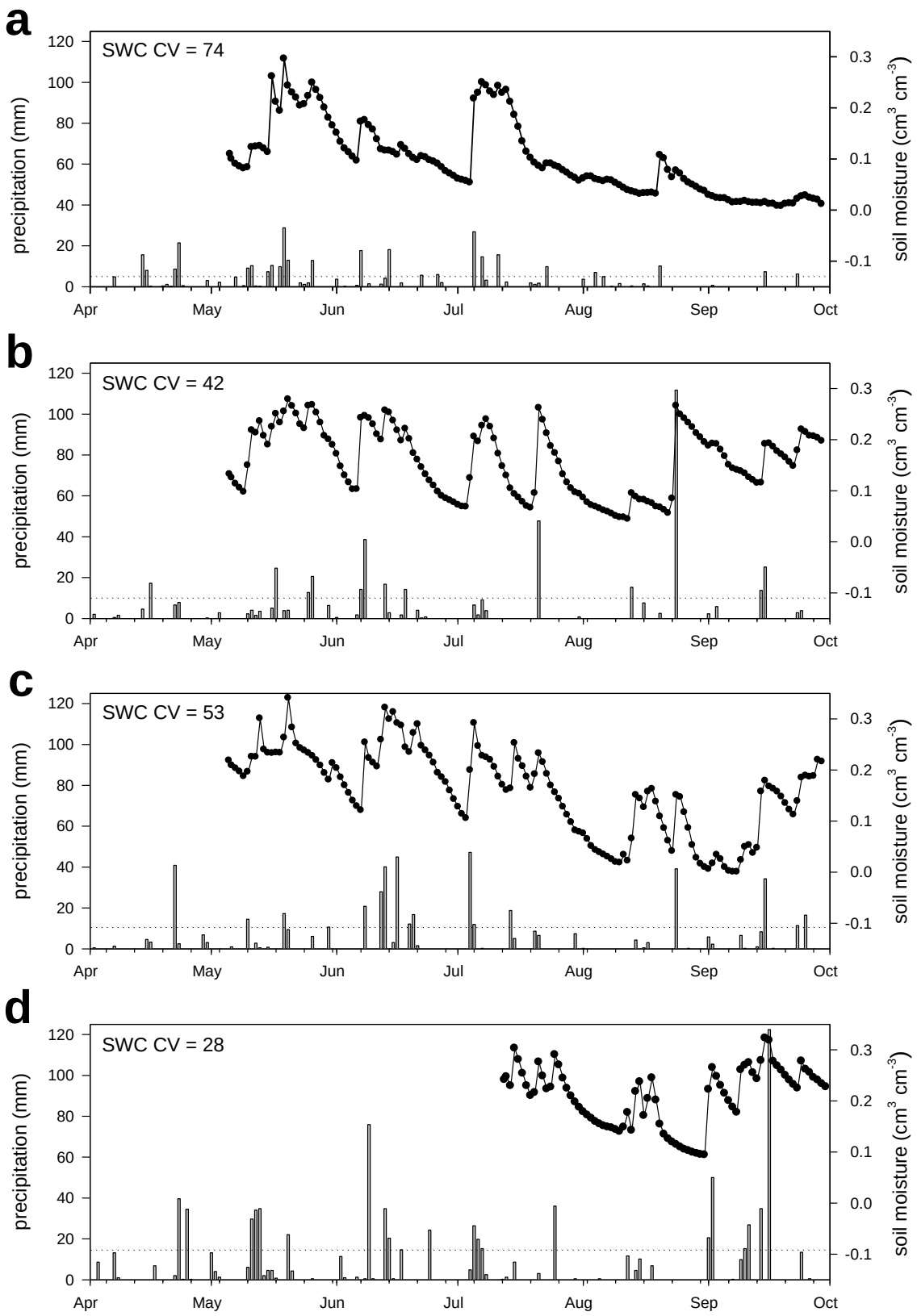

a
SWC CV = 74
b
SWC CV = 42
c
SWC CV = 53
d
SWC CV = 28
precipitation (mm)
soil moisture (cm3 cm-3)
0
20
40
60
80
100
120
-0.1
0.0
0.1
0.2
0.3
Apr
May
Jun
Jul
Aug
Sep
Oct

**Figure 3**. Nitrification potential measured 1.5 y (a) and 2.5 y (b) and denitrification potential measured 1.5 y (c) and 2.5 y (d) after reciprocal transplant of soils across a precipitation gradient. Location of soil incubation are depicted on the X-axis, and move from high to low stress and disturbance from left to right. Different symbols represent soil origins, SVR soils (▼), HYS soils (■), KNZ soils (●) and KUFS soils (◇) (see text for abbreviations). Data are presented as proportions of control soils, represented by the horizontal dotted line, and error bars represent one standard error of the mean.

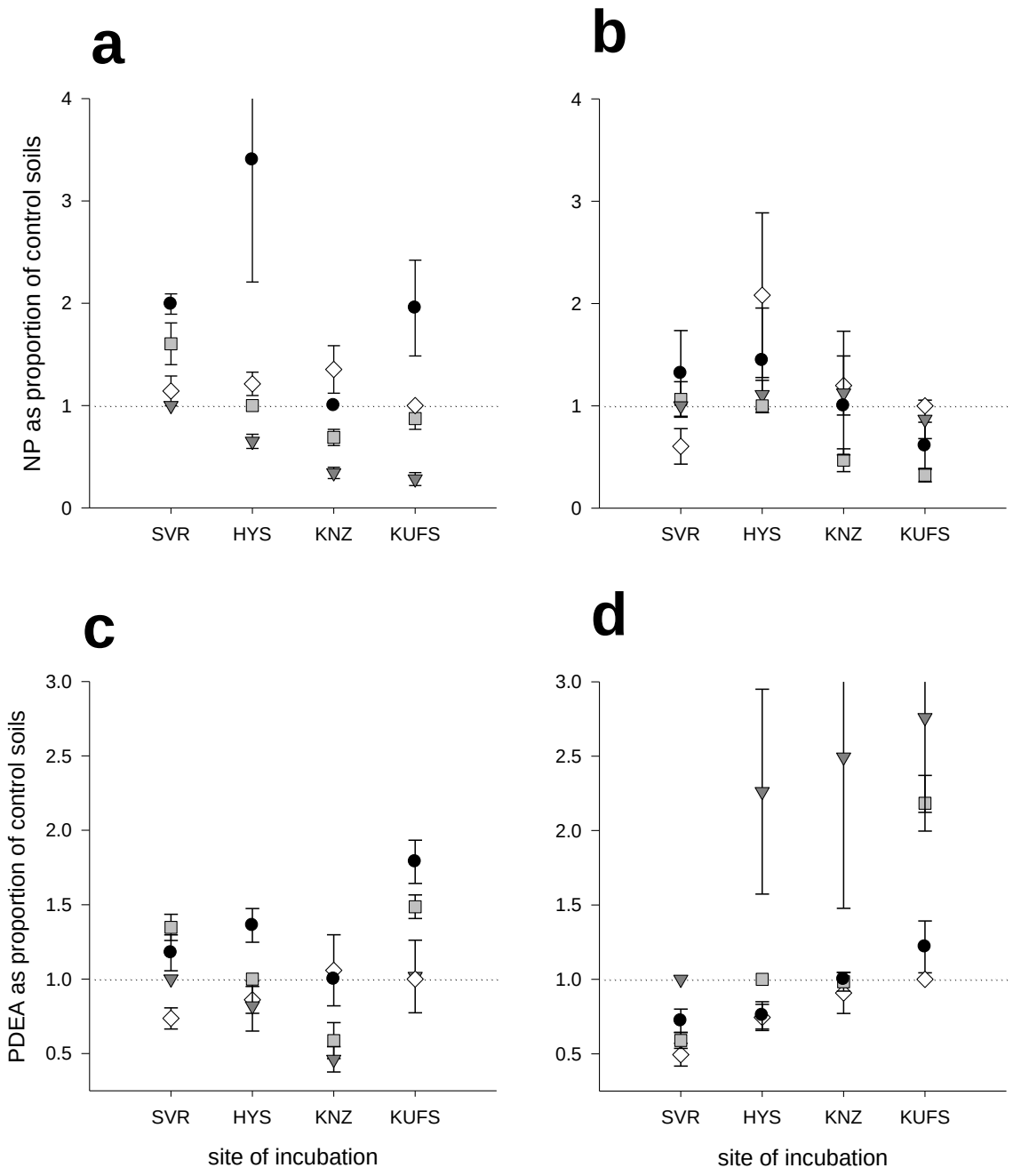

a
b
c
d
NP as proportion of control soils
PDEA as proportion of control soils
0
1
2
3
4
0.5
1.0
1.5
2.0
2.5
3.0
SVR
HYS
KNZ
KUFS
site of incubation

**Figure 4**. *amo*A gene abundance measured 1.5 y (a) and 2.5 y (b) after reciprocal transplant of soils across a precipitation gradient. Sites across the X-axis move from high to low stress and disturbance from left to right. Different symbols represent soil origins, SVR soils (▼), HYS soils (■), KNZ soils (●) and KUFS soils (◇) (see text for abbreviations). Data are presented as proportions of control soils, represented by the horizontal dotted line, and error bars represent one standard error of the mean.

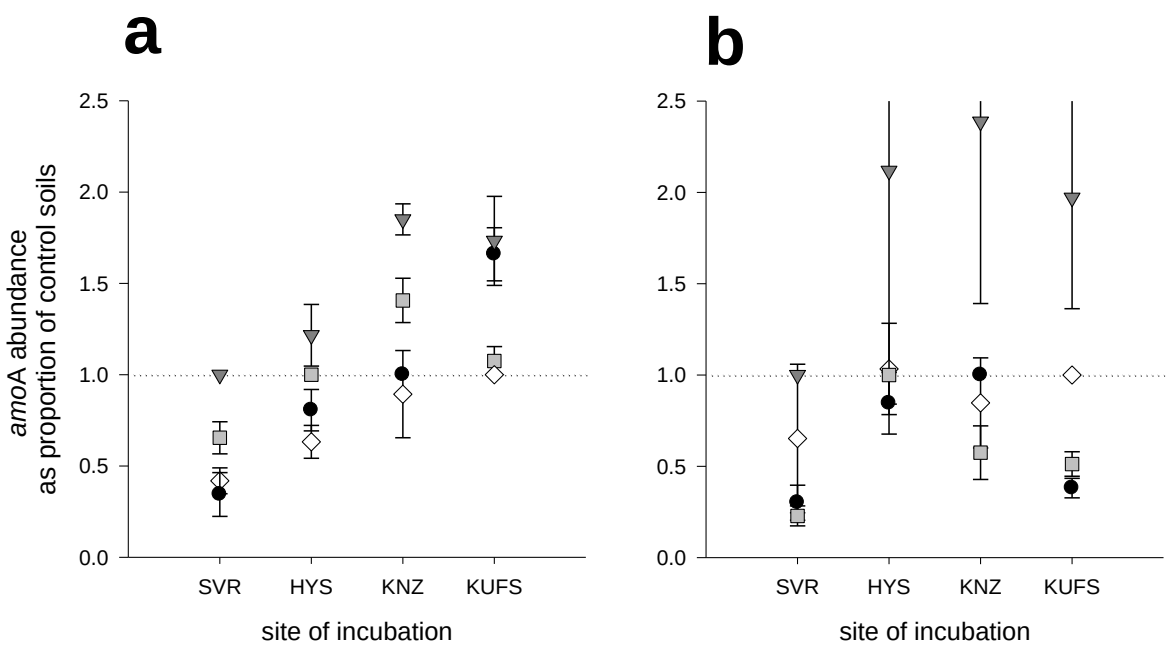


**Figure 5**. *nir*K, *nir*S, and *nos*Z gene abundance measured 1.5 y (a, c, e) and 2.5 y (b, d, f) after reciprocal transplant of soils across a precipitation gradient. Sites across the X-axis move from high to low stress and disturbance from left to right. Different symbols represent soil origins, SVR soils (▼), HYS soils (■), KNZ soils (●) and KUFS soils (◇) (see text for abbreviations). Data are presented as proportions of control soils, represented by the horizontal dotted line, and error bars represent one standard error of the mean.

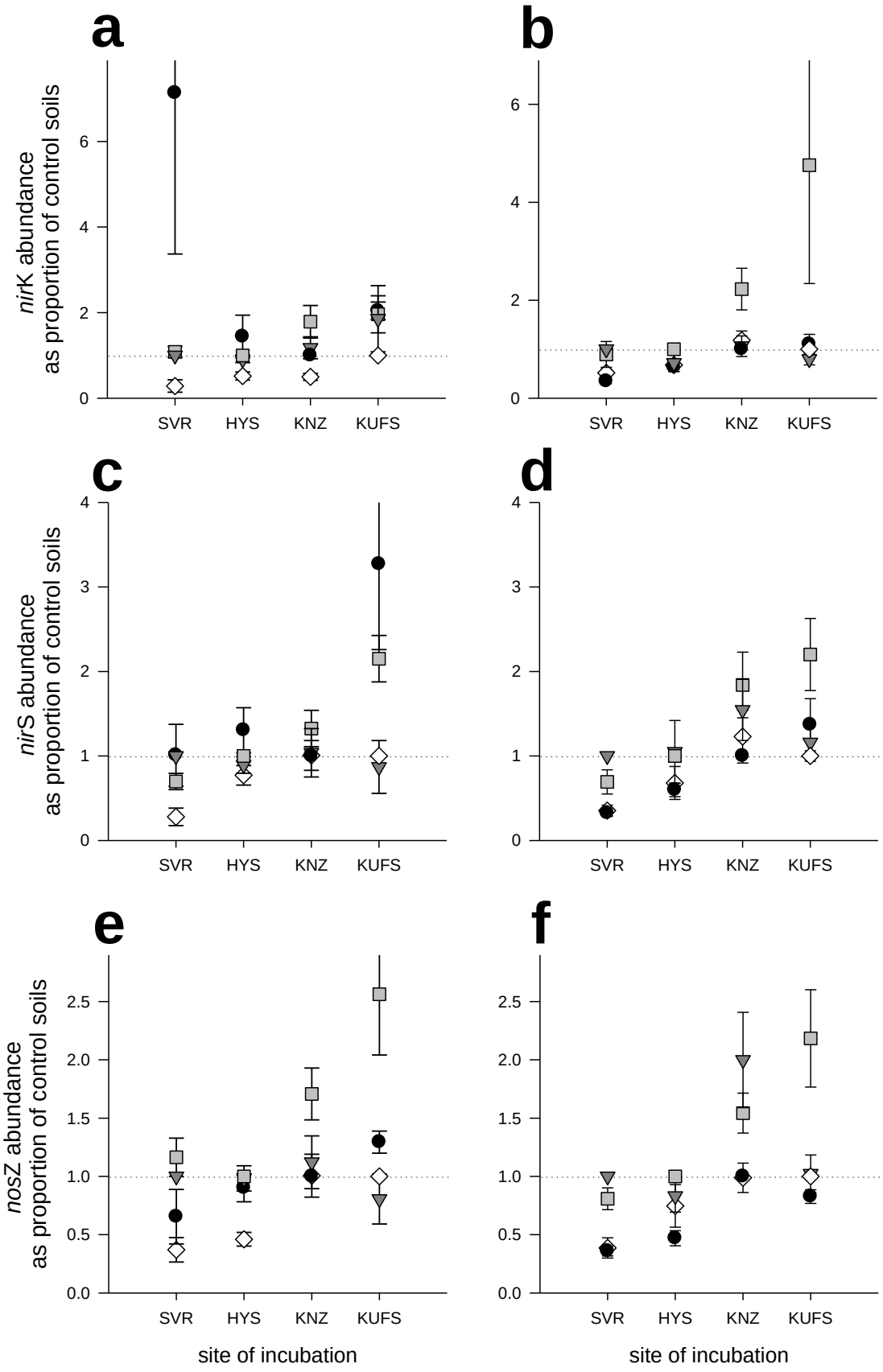
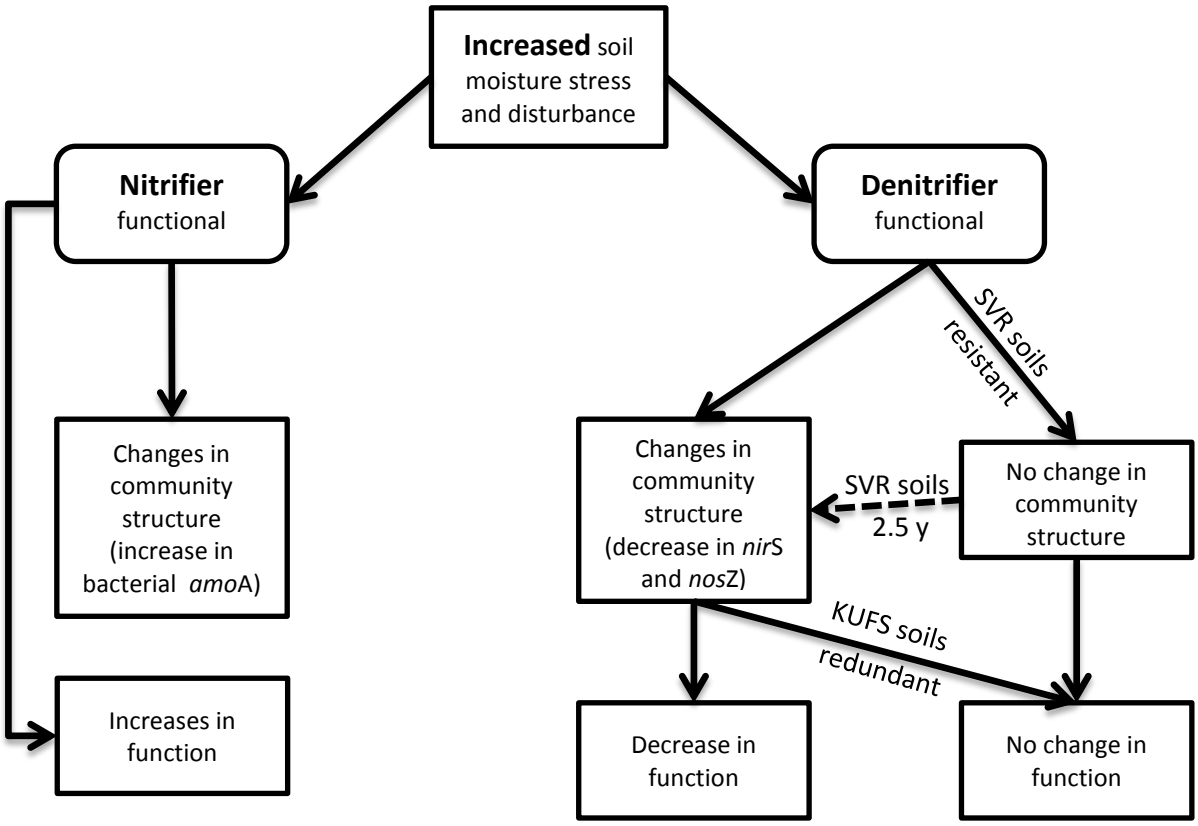

a
b
c
d
e
f
nirK abundance
as proportion of control soils
nirS abundance
as proportion of control soils
nosZ abundance
as proportion of control soils
SVR
HYS
KNZ
KUFS
site of incubation

**Figure 6**. Schematic of the effects of soil moisture stress and disturbance on grassland microbial communities, specifically nitrifier and denitrifier functional groups. Dotted arrow represents time related progression. All unlabeled arrow represent sensitivity to stress and disturbance.



# GENERAL CONLCUSIONS

In this dissertation I have presented evidence for the importance of environmental change in determining microbial community dynamics and the flow of C and N through the soil microbial biomass. As anthropogenic influences on ecosystems, such N addition and global climate change persist, we cannot determine the fate of C and N flowing through these systems unless we understand the extent to which these perturbations are altering microbial community structure and function.

The results from my first chapter highlight the complex interactions between the environment and soil communities. The driver of microbial functional change in the fertilized grassland plots of that study was not the direct effects of increased N availability but the indirect effects of fertilizer application on plant litter quality. In this study I was able to combine measures of decomposition activity with isotopic characterization of soil C and N resources to better understand the changes that have taken place in the microbial communities subjected to increased N availability. I found that even though soil C stocks had increased with fertilization, microbial communities in fertilized plots were relatively C limited, which resulted in increased decomposition activity and efforts to acquire C. This work provided evidence for links between microbial decomposition of soil organic matter and litter quality, providing a mechanism that explains increases in biomass inputs that are greater in magnitude than the associated increases in mineral soil organic matter.

Links between soil moisture availability and microbial processes have been well established, but the relationship between stress in the form of soil moisture variability and microbial processes is poorly understood. The laboratory incubations described in Chapters 2 and 3 provided valuable information about the way in which microbial communities utilize C

and N resources when physiological stress is intensified. I found that C demands can increase while N demands decrease as soil moisture stress increases. Changes in C use efficiency were likely related to increased effort by microbes to obtain organic substrates for use as protective osmolytes, but the lack of evidence for a concurrent increase in N demand was surprising and is contrary to the current paradigm of N-rich osmolyte preference in soil microorganisms. I found differences in the way in which microbial communities from mesic versus semi-arid precipitation regimes responded to soil moisture stress. Soil microorganisms from mesic regions seemed to be more sensitive to increases in soil moisture stress. Nitrogen cycling processes, specifically nitrification and denitrification, also seemed to be sensitive to soil moisture stress, but to different degrees causing decoupling of the N cycle. In addition, the magnitude of change in nitrification potential as soil moisture stress increased was controlled by the origin of the soil communities. Calculations based on results from these studies suggest that increases in drought severity in these systems could induce soil C and N losses from these grassland systems.

In my final chapter I explored the links between microbial community structure and function. Microbial community composition is a potential driver of ecosystem response to perturbation, but the way specific phylogenetic or functional groups respond to specific environmental stresses or disturbances remains unclear. In this study I chose two important microbial functional groups, nitrifiers and denitrifiers, that I had already seen affected by changes in soil moisture stress. Overall, little is known about how these functional groups respond to soil moisture stress and disturbance in terms of both community structure and process rates. The use of four sites along a precipitation gradient in conjunction with a relatively long incubation time for microbial community studies allowed me to find patterns that suggested link between microbial community structure and function.

While this dissertation serves to answer some questions, it also raises many new ones. For example, how large is the role that Archaea play in driving N cycling processes? How robust are links between microbial community structure and function when considered across different functions and ecosystems? Even if a function is altered by changes in community structure, how long is that change sustained? Unlike many problems in macro ecology, microbial ecology seems to lack strong predictive frameworks for analyzing the community level functional and structural shifts that occur. However, to understand how ecosystem processes and biogeochemical cycling will be altered by a changing environment, we must find a way to analyze these microbial community patterns.

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