Consequences of nutrient enrichment for soil organic matter cycling in grasslands

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Dedication

To my parents, who encouraged my earliest scientific interest in decomposition.

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INTRODUCTION

Investigating the effects of nutrient enrichment for soil carbon decomposition and storage

Human activities have increased the availability of nutrients, such as nitrogen (N), phosphorus (P), and potassium (K), worldwide (Falkowski et al. 2000). Growing demand for food and energy will continue to increase human use and deposition of these nutrients. For example, reactive N is projected to rise an additional 40% by 2050, and contribute to increasing N deposition in the future (Galloway et al. 2004). Since alterations to nutrient cycles influence carbon (C) fixation and decomposition processes, nutrient enrichment affects global C stocks – such as soil C – and carbon dioxide (CO_2) emissions as well. Carbon in soil organic matter (SOM) far outweighs vegetative C in the majority of biomes, especially grasslands (Watson et al. 2000). Consequently, either positive or negative changes to grassland soil C sequestration could feed back to influence the global C cycle. Unfortunately, the effects of nutrient enrichment on SOM cycling remain uncertain (Ciais et al. 2013).

In this dissertation, I present data from three studies that aim to address this gap in knowledge. In each of my studies I examined the effects of nutrient addition on SOM cycling at participatory sites of the Nutrient Network (www.nutnet.org; Borer et al. 2014). The Nutrient Network is a coordinated network of experiments that was established to investigate the effects of multiple nutrient additions and herbivore removal on ecosystem processes in grasslands. Participatory sites are located across the globe and follow standard protocols for sampling and analysis.

In Chapter 1, I focused on the effects of enrichment by multiple nutrients (N, P, and K plus micronutrients) on the total soil C stock at 19 Nutrient Network sites on four continents. Changes to the total soil C stock reflect the net impact of nutrients on both C inputs to and outputs from soil. Consequently, in addition to evaluating the total soil C stock, I examined nutrient effects on soil inputs and evaluated a numerical soil C model to determine the effects of nutrient addition on soil outputs. By

considering nutrient effects on both inputs and outputs, I elucidated the processes driving the total soil C stock response to nutrient enrichment.

The total soil C stock is comprised of several SOM pools with mean residence times (MRTs) that vary from days to centuries (Von Lützow et al. 2006). Distinct biological, chemical, and physical factors determine the variation in SOM pool MRT (Dungait et al. 2012; Cotrufo et al. 2013), and nutrient addition can affect each of these factors differently. Consequently, in Chapter 2 I investigated the effects of nutrient addition, specifically N, on the decomposition rate and size of multiple SOM pools at five Nutrient Network sites in the U.S. Central Great Plains region. By measuring the effects of N enrichment on the decomposition dynamics of both rapidly and slowly cycling soil C pools, my research informs both the short- and long-term effects of N enrichment for soil C sequestration.

Lastly, in Chapter 3 I examined the biological and chemical mechanisms that drive the SOM decomposition response to N enrichment. Results from empirical studies show that N addition tends to decrease the decomposition of SOM and microbial respiration of CO_2. Although predictions from theoretical models support these observations, the mechanisms that drive this response are unknown. I evaluated N addition effects on microbial carbon use, enzyme activity, and biomass at three Nutrient Network site in the U.S. Central Great Plains to address this uncertainty.

Overall, my dissertation furthers the basic scientific understanding of coupled C and nutrient cycling in grassland soils, which are a significant global reservoir of organic C. Furthermore, by investigating the effects of nutrient addition at multiple grassland sites, my research elucidates how the effects of nutrient enrichment may vary with geographic location.

CHAPTER 1

Soil carbon response to nutrient enrichment in a global grassland experiment

Despite worldwide increases in nutrient availability, the consequences of nutrient enrichment for global carbon (C) stocks, including in soils, are uncertain. We examined the effect of multiple nutrient addition treatments, including nitrogen (N), phosphorus (P), and potassium (K) plus micronutrients, on total soil C stocks at 19 experimental grassland sites worldwide. After only three years of nutrient addition, the total soil C concentration in surficial soils that received N, P, and K plus micronutrients increased by 12.7 % on average compared to control plots. Plots that received N alone, K plus micronutrients alone, and N and K plus micronutrients together, also increased relative to control plots (~ 9 % on average), although the differences were not significant. Results from a simple numerical soil C model that accounted for the effects of nutrient additions on plant C inputs suggested that P addition should have stimulated decomposition, given that soil C stocks in plots that received P remained unchanged while inputs of plant C increased significantly to these plots. In the future, anthropogenic increases in nutrients may increase C sequestration in grassland soils, although the effects will depend on nutrient identity.

INTRODUCTION

Human alteration of nutrient cycles is globally ubiquitous: fossil fuel combustion, fertilization, mining, and land use have increased the availability of nutrients, such as nitrogen (N), phosphorus (P), potassium (K), as well as micronutrients (Falkowski et al. 2000). Nutrient and carbon (C) cycles are tightly coupled through biological systems. However, the extent to which increasing nutrients may feed back to influence the global C cycle remains uncertain (Ciais et al. 2013). Soils are a significant reservoir of organic C and consequently, understanding the effects of nutrient enrichment on soils is particularly important.

The majority of studies examining the effects of nutrient enrichment on soil C have focused on N. Meta-analyses of N addition studies find that N enrichment generally, but not consistently, increases total soil organic C (SOC) stocks (Janssens et al. 2010; Liu and Greaver 2010; Lu et al. 2011). The effects of enrichment with non-N nutrients are poorly studied compared to N, and are not well understood, nor are interactive effects of enrichment with multiple nutrients (e.g., N plus P addition). For example, Fornara and colleagues (2013) found that N increased the total SOC stock when added alone in a long-term temperate grassland nutrient addition study, and P and K addition had no effects on total SOC. Furthermore, when N was added in combination with P, K, and magnesium (Mg), the stimulatory effects of N on soil C sequestration disappeared and the total SOC stock of the multi-nutrient addition plots were *no different* than control plots. By contrast, in a long-term fertilization experiment in the Arctic, N and P added together *decreased* total SOC stock (Mack et al. 2004). Clearly, the effects of non-N nutrients and multiple nutrient additions on soil C still need to be elucidated. Addressing this gap in knowledge is the focus of this study.

One reason that addition of N and non-N nutrients, as well as their combinations, might impact soil C stocks differently is that they could have distinct effects on the processes that determine the size of the total soil C stock, including both the rate of C *input* to and *output* from soil (Figure 1.1). Soil C inputs include plant litter both above- and belowground and root rhizodeposition (e.g., root exudates and sloughed roots cells). On the other hand, processes that result in soil C losses (outputs) include microbial

decomposition of SOC to carbon dioxide by saprotrophic soil microbes, as well as leaching of dissolved organic C and erosion of particulate organic C.

Evaluating what is known and unknown about the effects of nutrient addition on both inputs and outputs informs our hypotheses about the effects of nutrient addition on the total SOC stock (Figure 1.1). On the input side, it is well established that N limitation of plant biomass is widespread (LeBauer and Treseder 2008), and that N addition increases plant aboveground biomass and litter production (Liu and Greaver 2010; Lu et al. 2011). Addition of P can also increase plant aboveground biomass and litter production (Wright et al. 2011). Moreover, the simultaneous addition of N plus P may increase biomass more than when either is added alone (Elser et al. 2007; Xia and Wan 2008), such that co-limitation by N and P might be more widespread across terrestrial ecosystems than previously thought (Elser et al. 2007; Harpole et al. 2011). The effects of nutrients beyond N and P on plant aboveground biomass have not been studied as extensively, but there is some evidence to suggest that their addition may also increase biomass when added alone (e.g., Tripler et al. 2006) and in combination with N and P (e.g., Harpole and Tilman 2007).

Although plant allocation shifts towards greater aboveground relative to belowground growth in response to nutrient addition (Lu et al. 2011; Poorter et al. 2012; Yuan and Chen 2012), nutrient addition can still lead to absolute increases in fine root production. For example, a meta-analysis by Yuan and Chen (2012) showed that fine root production increased by 27 and 21%, respectively, when N and P were added alone; and by 40% when N and P were added simultaneously. Root inputs beyond root litter, on the other hand, are especially difficult to measure and few studies have evaluated the effects of nutrient addition on these root processes. In one such study, however, Phillips et al. (2009) found that mass-specific root exudation rates were higher at low N than at high N. Conversely, Adair and colleagues (2009) found that N addition increased total belowground C allocation (which includes root exudates); the effect was driven by the overall increase in total root biomass stock and N did not change total belowground C allocation per unit biomass of roots. Overall, the effects of nutrients on root rhizodeposition remain uncertain (Jones et al.

2004), as do the effects of non-N or P nutrients on root inputs more generally. More research is needed to evaluate and quantify the effects of nutrient addition on root C inputs.

Specific nutrient effects on plant inputs will depend on geographic context. Different nutrients will be limiting in different locations due to variation in soil nutrient supply and availability. For example, along a chronosequence of soil development – and consequently a gradient of N and P supply – in Hawaii, Vitousek and Farrington (1997) found that N limited plant primary production at the youngest site (lowest available N, highest available P), P limited production at the oldest site (highest available N, lowest available P), and production was co-limited by N and P at the middle age site.

On the output side, N addition tends to decrease the loss of C via soil microbial respiration (Janssens et al. 2010; Liu and Greaver 2010; but see Lu et al. 2011) and litter decomposition (Janssens et al. 2010), especially for more low "quality" (e.g., high lignin and recalcitrant content) soil (Chapter 2) and litter (Berg and Matzner 1997; Knorr et al. 2005; Hobbie et al. 2012). On the other hand, P addition tends to increase C loss via soil microbial respiration (Cleveland et al. 2002; Cleveland et al. 2006; Bradford et al. 2008b; Reed et al. 2010; Fanin et al. 2012; Fanin et al. 2015) and litter decomposition (Hobbie and Vitousek 2000; Powers and Salute 2011; but see Chen et al. 2013), although P addition studies are predominately limited to the tropics where older landscape age is associated with decreased P availability (but see Bradford et al. 2008b). Interestingly, the inhibitory effects of N on C loss by decomposers may be eliminated when N is added along with P: N plus P addition can increase soil respiration more than P addition alone (Reed et al. 2010; Fanin et al. 2012), although this effect is not universal (Cleveland et al. 2006; Bradford et al. 2008b).

There is sparse information for evaluating the effects of non-N or P nutrients on soil C loss via soil or litter decomposition. In general, the studies that do exist point toward increasing decomposition following the addition of K (Kaspari et al. 2008) and micronutrients (Kaspari et al. 2008; Powers and Salute 2011; Kaspari et al. 2014). Further, other researchers have found positive relationships between decomposition rate and the concentration of micronutrients in litter that limit the activity of specific microbial enzymes (e.g., manganese (Mn); Berg et al. 2006). This is unsurprising given that soil and litter

decomposer communities are diverse, require multiple enzymes to degrade the heterogeneous organic material, and, consequently, may be limited by a large number of macro- and micro-nutrients (Reed et al. 2010; Powers and Salute 2011). Finally, it is important to note that most non-N addition studies of decomposition have been carried out in the tropics where P and other mineral-derived nutrients are in low supply (but see Bradford et al. 2008b). Consequently, we cannot evaluate to what extent nutrient limitation of decomposition may depend on supply, which will vary with geographic location.

In addition to affecting microbial decomposition of OM, nutrient addition can also influence the loss of soil C by affecting mechanisms that alter chemical and physical protection of OM, and hence its availability for microbial decomposition (Schimel and Schaeffer 2012). Specifically, nutrient addition effects on bacterial, fungal, and plant biomass, root rhizodeposition, OM chemistry, and soil pH could impact OM occlusion in soil aggregates and association with mineral surfaces (King 2011; Dungait et al. 2012; Cotrufo et al. 2013; Bardgett et al. 2014). Additionally, the observed negative effect of base cations, particularly Mg, on decomposition (Lukumbuzya et al. 1994; Powers and Salute 2011) could be due to their direct participation in soil C stabilization processes, such as cation bridges, that reduce C accessibility for microbes (Muneer and Oades 1989). To date, nutrient effects on OM decomposition via these mechanisms have received little attention, but could influence the longer-term fates of soil C stocks.

Finally, we know little about how nutrient addition may affect soil C losses via leaching and erosion. Factors occurring at the scale of the soil pedon and landscape control rates of soil erosion, and therefore C loss via erosion (Yoo et al. 2006). Of these factors – climate, slope gradient, and vegetative cover – only the last can reasonably be expected to change in response to nutrient addition. For example, nutrient addition that leads to a substantial increase in plant biomass could decrease soil erosion, and therefore loss of particulate C. For soil C losses via leaching, existing evidence suggests that N addition tends to increase DOC (Liu and Greaver 2010; Lu et al. 2011), and consequently C loss via leaching. However, the effects of non-N nutrients on leaching are unknown.

Overall, the increases in total soil C in response to N found in meta-analyses are congruent with observed positive effects of N addition on plant biomass and negative (or inhibitory) effects of N addition

on microbial decomposition of soil organic matter. The picture is less clear about how non-N nutrients and multiple nutrient additions will impact inputs and decomposition (and, consequently, the total soil C stock).

Our research objective was to investigate the effects of enrichment by multiple nutrients on soil C sequestration. Given the known effects of N, P, K, and micronutrient addition on soil C inputs and outputs, reviewed above, we hypothesized that: 1) soil C stocks would *increase* in response to addition by N alone because of the positive effects of N addition on plant C inputs and the inhibitory effects of N addition on C losses via decomposition, 2) soil C stocks would *stay the same* in response to addition of non-N nutrients due to the positive effects of nutrient addition on plant C inputs and the positive effects of non-N nutrient addition on soil C loss via decomposition, and 3) soil C stocks would *stay the same* in response to the addition of N and non-N nutrients due to the positive effects of nutrient addition on plant C inputs and the positive effects of simultaneous addition of N and non-N nutrients on soil C loss via decomposition. To address these hypotheses, we sampled soil C from participatory sites of the Nutrient Network (www.nutnet.org) that had received at least three years of nutrient addition treatments. In order to evaluate the effects of nutrient addition on soil C *inputs* and *outputs*, we also constructed a numerical soil C model to estimate nutrient effects on soil decomposition rates given observed input and soil C responses from the field data.

MATERIALS AND METHODS

Experimental set-up

To evaluate the experimental effects of nutrient addition on soil C, we sampled soils from participatory sites of the Nutrient Network. The Nutrient Network is a coordinated research network designed to experimentally evaluate the effects of nutrient addition and herbivory on ecosystem processes in grasslands worldwide (Borer et al. 2014). Participatory sites are located across a range of climate and soil types (see *Results*), but follow identical methods for experimental set-up, sampling, and analysis. Consequently, Nutrient Network sites provide a unique opportunity to examine how soil C responses to nutrient addition vary, or are generalizable, across grassland ecosystems.

Nutrient Network experimental set-up, sampling and sample analyses are described in detail in Borer et al. (2014). Briefly, each site consists of 5 x 5 m plots, with treatments replicated across at least three blocks (completely randomized block design). The experimental nutrient addition treatments – N, P, and K plus micronutrients – are applied at the plot level in full factorial for a total of eight treatment combinations per block. At some sites, the control and +NPK plots are also crossed with a fencing treatment; we focus on the unfenced nutrient addition plots only here. N, P, and K are applied annually (10 g m^{-2} yr^{-1}) as time-released urea [$(NH_2)_2CO$], triple-super phosphate [$Ca(H_2PO_4)_2$], and potassium sulfate [K_2SO_4], respectively. In the K addition plots, 100 g m^{-2} of a micronutrient mix (Micromax Micronutrient; Everris (previously Scotts brand), Dublin, Ohio, USA) was applied during the first year of treatment addition. The micronutrient mix included calcium (Ca; 6.0%), magnesium (Mg; 3.0%), sulfur (S; 12.0%), boron (B; 0.1%), copper (Cu; 1.0%), iron (Fe; 17.0%), manganese (Mn; 2.5%), molybdenum (Mo; .05%), and zinc (Zn; 1.0%) applied as iron sulfate [$FeSO_4 + 1H_2O$], calcium magnesium carbonate (dolomite) [$CaMg(CO_3)_2$], manganese sulfate [$MnSO_4$], copper sulfate penthydrate [$CuSO_4 + 5H_2O$], zinc sulfate anhydrous [$ZnSO_4$], sodium borate [$Na_2B_4O_7$], and sodium molybdate [$Na_2MoO_4 + 2H_2O$] (Table 1.1). We refer to the treatments as "N", "P", and "K" throughout, even though they included the addition of other nutrients (i.e., Ca in addition to P in the case of the P treatment, and K plus micronutrients in the K treatment).

Soil sampling and analysis

After at least three years of experimental nutrient addition, at least two 2.5 cm diameter and 10 cm deep soil cores were collected from random locations in each plot. Surficial plant litter was removed from the top of each core and the cores were homogenized, air dried, and analyzed for total % C and % N by combustion (Costech ESC 4010 Elemental Analyzer, Valencia, California, USA); soil pH (1:1 soil:water slurry method, A&L Analytical Laboratory, Memphis, Tennessee, USA); and P, K, and micronutrient concentration by Mehlich-3 extraction (A&L Analytical Laboratory, Memphis, Tennessee, USA). The objective of the Mehlich-3 extraction is to measure "available" soil P, K, and micronutrients. Mehlich-3

nutrient concentrations co-vary with other measures of available or exchangeable nutrients, although soil properties determine the strength of the relationship (Burt et al. 2002; Zheng and Zhang 2012). Consequently, we refer to Mehlich-3 soil nutrient concentration as "extractable" throughout and consider this measurement to be suggestive of soil exchangeable or available nutrient concentration. Soil texture was assayed in control plots only (A&L Analytical Laboratory, Memphis, Tennessee, USA). Finally, in order to examine the effects of nutrient addition on soil *organic* C, we excluded sites with alkaline soil (soil pH > 7) where inorganic C contributes to total C. Furthermore, we excluded sites with less than three blocks. Since not all Nutrient Network sites have contributed post-treatment soils data, 19 sites were included in the analyses (Figure 1.2 and Appendix 1 – Table S1.1).

Other covariates

We supplemented our soils data with measurements of aboveground live plant biomass and root biomass standing stock sampled at each plot the same year soil samples were collected. Plant biomass sampling methods are detailed in Borer et al. (2014). Root biomass sampling methods were modified from Robertson et al. (1999) and are detailed in Cleland et al. (unpublished manuscript). Briefly, during soil sampling (described above) an additional intact core (2.5 cm diameter, 10 cm deep) was sampled and submerged in water in the lab. Clean roots were captured using sieves and tweezers and dried for > 48 hrs at 40 °C. Additionally, site mean annual precipitation (MAP) site mean annual temperature (MAT), and site aridity (rainfall (or MAP) minus potential evapotranspiration) were extracted from the WorldClim database (Hijmans et al. 2005).

Data analysis

All analyses were performed using R (R version 3.0.1; R Foundation for Statistical Computing 2013). We evaluated the effects of factorial nutrient addition and ecosystem covariates (e.g., plant, soil, and climate variables) on total soil C concentration using a mixed-effects statistical model (lme4 package) and model averaging (Grueber et al. 2011). Model averaging is a model selection approach that identifies the

set of sub-models that fit the data best and are equivalent from the largest possible model that includes all possible predictors (described below). In our implementation of this approach, we designated sub-models as equivalent if the difference in the Akaike Information Criterion (AIC; a measure of model fit) among them was < 4 (dredge function in the MuMIn package). Next, we evaluated the importance and significance of each of the predictor variables that were included in this top set of models. Reported p-values are from the full model-averaged coefficients (e.g., across all possible models, including the ones where the parameter was absent), which is a better estimate of which factors have the strongest effects on the response variable (Grueber et al. 2011). In the final "averaged" model (i.e., the set of sub-models that fit the data best and were equivalent), the variance explained by the fixed effects only (marginal R^2; see below for fixed and random effects) and the variance explained by both fixed and random effects (conditional R^2) was calculated using the r.squaredGLMM function (MuMIn package; Nakagawa and Schielzeth 2013).

Model averaging selected equivalent sub-models from the largest possible model that included all possible predictors. In this largest possible model, the factorial nutrient addition (N x P x K), ecosystem covariates (e.g., plant, soil, and climate variables), and their interactions were included as fixed effects; site was included as a random effect. Ecosystem covariates were selected from the suite of available ecosystem covariates that could influence the soil C response to nutrient addition by changing inputs into and outputs from the soil C pool: rain minus potential evapotranspiration (a measure of aridity), plant aboveground biomass and plant root biomass, soil pH, and soil texture (% silt and clay particles). We tested for multicollinearity among these predictors by calculating the variance inflation factor (VIF) of each predictor. Since all predictor VIFs were < 1.14, all predictors were included in the largest possible model. All predictor variables were rescaled using the standardize function (arm package), which adjusts the variables to have mean of zero and standard deviation of .5 (Gelman 2008). The response, total soil C concentration, was ln-transformed to meet the normality assumptions of linear regression.

Nutrient by covariate interactions were not significant and, consequently, were omitted from the final "averaged" model; however, there was substantial variation in soil C concentration by site (see *Results*). In order to account for this site-by-site variation in soil C and more clearly understand the nutrient

effects, we constructed a second statistical model in which we explored the magnitude of the average nutrient effects on soil C by examining the proportion change in soil C concentration in each nutrient addition plot relative to the control plot in each block (proportion change in soil C = (treatment plot soil C – control plot soil C)/control plot soil C). We evaluated the effects of factorial nutrient addition on proportion change in soil C concentration using a mixed-effects model (nlme package): N x P x K were fixed effects and site was a random effect. We used the anova function to evaluate the significance of the fixed effects. When there were significant nutrient treatment interactions, we used the lsmeans package to perform post-hoc comparisons between control and nutrient addition treatments. Marginal and conditional R^2 were calculated using the MuMI package.

Finally, we constructed additional statistical models to analyze the effects of factorial nutrient addition on plot-level variables that may influence soil C responses to nutrient enrichment. The plot-level response variables we analyzed in these models included: plant aboveground biomass, plant root biomass, soil pH, and extractable soil micro- and macro-nutrient concentration (P, K, Ca, Mg, S, Na, Zn, Mn, Fe, Cu, and B). Response variables were ln-transformed to meet the assumption of normality. N x P x K were fixed effects and site was a random effect in all models (nlme package). When there was a significant nutrient treatment interaction, we used the lsmeans package to perform post-hoc comparisons (Bonferroni corrected for multiple comparisons). The variance explained by the fixed effects only (marginal R^2) and the variance explained by both fixed and random effects (conditional R^2) were calculated using the MuMIn package.

Numerical soil C model

In order to explore the mechanisms that could lead to the observed changes in soil C in response to nutrient addition, we constructed a simple numerical soil C model. We used the model to solve for decay rate (k), given the observed changes in soil C inputs and the total soil C stock in response to nutrient addition treatments in each plot.

The numerical model assumed:

$$C_t = C_{t-1} + \frac{dC}{dt}$$

where C_t is the total soil C stock at time t (g C m^{-2}), C_{t-1} is the total soil C stock the previous year (g C m^{-2}), and dC/dt is the change in the total soil C between the current and previous year (g C m^{-2} yr^{-1}). dC/dt was equal to the difference between inputs and outputs, or:

$$\frac{dC}{dt} = I - kC_t$$

where I is total plant inputs (g C m^{-2}) from plant aboveground biomass and plant roots and k is the decay rate of the total soil C stock (yr^{-1}). To evaluate the soil C stocks after several years, the previous two equations were combined as follows:

$$C_t = C_0 (1-k)^t + \sum_{i=1}^{i=t} I (1-k)^{i-1}$$

where C_o is the size of the initial soil C stock (g C m^{-2}). Since we evaluated soil C stocks after receiving a minimum of three years of nutrient addition, we rearranged the equation to solve for C_{t3}, or the total soil C stock at year 3:

$$C_{t3} = C_{t0} (1-k)^3 + I (1-k)^2 + I (1-k) + I$$

We then rearranged the year 3 equation as a cubic polynomial:

$$0 = (3I + C_{to} - C_{t3}) + (-3C_{to} - 3I)k + (3C_{to} + I)k^2 + (C_{to})k^3$$

and solved for k using the polynomial solver function (polynom package). This function returned three possible k values from the three polynomial roots. We checked each of these solutions by solving for C_{t3} using the year 3 equation and known values of C_{t3} and I. In all cases, the checked solutions showed that polynomial roots one and three were spurious and polynomial root two was accurate and is reported here.

We solved for k at the plot level and used plot-level soil C stock and plant biomass observations to parameterize the model. All sites where plot-level bulk density measurements were available were included in the analysis (i.e., all sites except Cedar Creek (USA) and Elliot Chaparral (USA)). We made C_{t0} equal to

the observed control plot soil C stock in each block. We assumed control plots were at steady state (dC/dt = 0) and set $C_{t3} = C_{t0}$. In the nutrient addition plots (+N, +P, +K, +NP, +NK, +PK, +NPK), we set C_{t3} equal to the observed soil C stock of each plot (g C m^{-2}). I was equal to the sum of plant aboveground biomass and root biomass inputs to the soil C pool:

$$I = A + B$$

where A equals plant aboveground biomass inputs (which we assumed to be equal to the observed plant aboveground biomass standing stock; g C m^{-2} yr^{-1}) and B equals plant root biomass inputs (observed plant root biomass standing stock in the top 10 cm (g C m^{-2}) x plant root turnover rate (yr^{-1})). We made the simplifying assumption that all of the aboveground biomass enters the soil C pool each year; this ignores litter incorporation rates, C losses from litter oxidation aboveground, and other litter loss pathways. We also assumed that no biomass was removed during the growing season, e.g., by herbivory. To estimate belowground inputs from root biomass measurements, we assumed that the plant root turnover rate was .52 yr^{-1}, the average value for temperate grasslands reported in a meta-analysis by Gill and Jackson (2000).

We examined the relationship between the modeled k values and site-level variables (e.g., soil, plant, and climatic factors), in order to elucidate how k varied across our study sites and whether the nutrient effect on decomposition rate interacted with site-level characteristics. We constructed mixed-effects statistical models to test for nutrient treatment effects (and their interaction with site-level variables) on modeled decay rate. Since there were no significant site-level variables or interactions the final model included N x P x K as fixed effects and site was a random effect (nlme package). We used the anova function to evaluate the significance of the fixed effects. Marginal and conditional R^2 were calculated using the MuMI package.

Finally, we examined numerical model sensitivity by constructing three alternate numerical models that varied one parameter assumption each: 1) root biomass inputs, 2) aboveground biomass inputs, and 3) root biomass turnover rates in response to nutrient addition. (See Appendix 1 – Supplement S1.1 for more details on model sensitivity analyses and results.)

RESULTS

Effects of factorial nutrient addition and ecosystem covariates on total soil C

Soil C concentration, as well as other ecosystem covariates, varied significantly among the 19 Nutrient Network experimental sites included in this study. Among the study sites, average soil C concentration ranged from 10.17 mg C g^{-1} soil (Cedar Creek, USA) to 192.81 mg C g^{-1} soil (Lancaster, UK). Plant aboveground biomass ranged from 42.7 g m^{-2} (Lookout, USA) to 1609 g m^{-2} (Trelease, USA), while plant root biomass ranged from 59.68 g m^{-2} (Sierra Foothills,USA) to 1675.18 g m^{-2} (Cowichan, CA). The 19 sites also spanned a range of climates and soil types. MAP varied from an average of 300 mm (Mt. Caroline, AU) to 1898 mm (Lookout, USA), while MAT ranged from 4.8 °C (Lookout, USA) to 18.1 °C (Ukulinga, ZA). Soil texture varied from 0.8 (Lookout, USA) to 44.63 (Ukulinga, ZA) average % clay and from 18.28 (Ukulinga, ZA) to 88.75 (Cedar Creek, Minnesota) average % sand. Average soil pH varied from 4.7 (Bogong, AU) to 7.2 (Cedar Point, USA).

Model averaging identified the set of equivalent sub-models that best explained total soil C concentration in response to nutrient addition and ecosystem covariates (Table 1.2). In the final averaged model, plant aboveground biomass was significantly positively associated with total soil C concentration (plant aboveground biomass main effect: $p = 0.03$; Figure 1.3). This relationship strengthened when we excluded three sites with the highest concentrations of soil C. Moisture availability (i.e., rainfall minus potential evaporation) also significantly increased total soil C concentration (rainfall minus potential evapotranspiration main effect: $p < 0.0001$; Figure 1.4). Root biomass, soil pH, and % silt and clay particles were not significant predictors of soil C concentration. Lastly, there was a significant N x P x K interactive effect on total soil C concentration (N x P x K interaction: $p = 0.03$). Overall, the factorial nutrient treatment and ecosystem covariate effects accounted for 55% variation in total soil C concentration (marginal $R^2 = 0.55$), while site identity accounted for an additional 40% of the variation in total soil C concentration (conditional $R^2 = 0.95$).

Effects of factorial nutrient addition on proportional change in total soil C

The effects of factorial nutrient addition on the absolute and proportional change in total soil C concentration were similar (Figure 1.5; Table 1.3; Appendix 1 – Table S1.2). Given the significant variation among sites, examining the proportional change allowed us to more clearly explore the magnitude of nutrient treatment effects on soil C concentration, although the model had minimal predictive power (marginal R^2 = .03, conditional R^2 = .08). There was a significant N x P x K interactive effect on the proportional change in soil C concentration (N x P x K interaction: $p = 0.04$), a significant main effect of K (K main effect: $p = 0.04$), and a marginally significant main effect of N (N main effect: $p = 0.05$). Nitrogen and K increased soil C concentration relative to control plots by 8.8 and 9.1 %, respectively, when added alone and by 9.0 % when added together. The positive effects of N and K on soil C concentration disappeared when each was added along with P, except for when all three nutrients were applied together. The +NPK treatment increased soil C concentration by 12.7 % relative to the control. Of the treatments that increased soil C concentration relative to the control, only the +NPK treatment was significantly different from the control (pairwise post-hoc comparison (control vs. +NPK): $p = 0.01$), all others were only marginally significantly different compared to the control treatment (post-hoc comparisons: $p = 0.07$ (control vs. +N); $p = 0.05$ (control vs. +K); $p = 0.06$ (control vs. +NK)).

Effects of factorial nutrient addition on plant and soil variables

In addition to the effects of factorial nutrient addition on total soil C concentration, the nutrient treatments significantly influenced a number of plant and soil variables that could change the rate of inputs to or outputs from the soil C pool (and therefore may influence the soil C response to nutrient addition; see Appendix 1 – Table S1.4 for ANOVA tables). In the Nutrient Network plots included in this study, plant aboveground biomass increased 18 and 26% on average in response to N and P addition, respectively (N main effect: $p = 0.007$; P main effect: $p < 0.0001$) but did not respond to K addition (Appendix 1 – Table S1.3**)**. The effects of N and P addition on plant biomass were additive: plant aboveground biomass increased more when N and P were added together (+NP or +NPK), compared to when added singly

(Figure 1.6). Plant root biomass did not respond to nutrient additions; although there was a significant N x P x K interaction (N x P x K interaction: $p = 0.02$), post-hoc comparisons revealed that no treatments were significantly different from one another. Soil pH decreased slightly, but statistically significantly, in response to N addition (N main effect: $p < 0.0001$; ambient N average = 5.71, added N average = 5.55) and K addition (K main effect: $p = 0.04$; ambient K average = 5.66, added K average = 5.61; Figure 1.7**)**.

The nutrient treatments also significantly influenced the concentration of extractable soil nutrients (see Appendix 1 – Table S1.4 for ANOVA tables). The P treatment had a significant positive effect on soil P (P main effect: $p < 0.0001$) and increased soil P concentration ~ 3x on average. The K treatment had a significant positive effect on soil extractable K concentration (K main effect: $p < 0.0001$) and increased soil K concentration by 60 % on average. The N treatment significantly decreased extractable soil Ca and Mg concentration (N main effects: $p = 0.003$ and $p = 0.03$) by 2 % and 4 % respectively, while the P treatment increased extractable soil Ca concentration (P main effect: $p < 0.0001$) by 5 % on average. The K treatment also significantly positively increased the concentrations of extractable soil B (K main effect: $p < 0.0001$) by 21 %, soil Cu (K main effect: $p < 0.0001$) by 72 %, and soil Mn (K main effect: $p = 0.01$) by 3 % on average. There were significant main effects of N (N main effect: $p = 0.04$) and K (K main effect: $p < 0.0001$) on extractable soil S concentration, increasing soil S by 4 and 40 %, respectively. Phosphorus and K had significant main effects on extractable soil Fe (P main effect: $p = 0.001$; K main effect: $p = 0.02$), increasing soil Fe by 5 and 6 %, respectively. There was a significant N x P x K interactive effect on extractable soil Zn (N x P x K interaction: $p = 0.02$). Soil Zn concentration in the +NK treatment was significantly higher than soil Zn concentration in the +P treatment (post-hoc comparison: $p = 0.001$); all other treatments were not statistically significantly different. Finally, there were no significant effects of nutrient addition on extractable soil Na.

Effects of factorial nutrient addition and ecosystem covariates on modeled decay rate (*k*)

Across all plots, modeled decay rates ranged from -0.22 to 0.66 (mean $k = 0.16$, median $k = 0.12$). Modeled decay rates tended to increase as total plant inputs (from aboveground and belowground biomass)

increased (Appendix 1 – Figure S1.1). Additionally, there was a humped shaped relationship between modeled decay rate and site climate: *k* was smallest at temperature and moisture extremes and largest at median values of temperature and moisture (e.g., see Appendix 1 – Figure S1.2 which depicts the relationship between modeled decay rate and MAT).

Phosphorus addition significantly increased the modeled decay rate across sites on average (P main effect: $p = 0.0002$; Table 1.4; Figure 1.8; see Appendix 1 – Table S1.5 for data table). Site identity accounted for a large amount of variation in modeled decay rate (~ 50 %); however, the effects of P addition did not vary systematically across sites (e.g., see Appendix 1 – Figures S1.3-4). Finally, the stimulatory effects of P addition on modeled decay rate were robust across the model variations we tested during model sensitivity analyses, especially for the +P, +NP, and +PK plots (see Appendix 1 – Supplement S1.1 and Figures S1.5-7 for model sensitivity results).

DISCUSSION

Across 19 grassland sites, biotic and climatic factors were strong predictors of soil C concentration. Furthermore, on average we found that nutrient additions of N, P, and K plus micronutrients had distinct effects on total soil C concentration, although we did not elucidate site-specific responses to nutrient additions. Given the treatments that did not affect soil C concentration (i.e., +P, +NP, and +PK treatments), the results from our numerical soil C model suggested that P had a stimulating effect on microbial decomposition (*k*): under P addition, increasing inputs (plant biomass) must have been offset by increasing outputs (decomposition), to result in no change in the soil C pool. On the other hand, increasing soil C concentration under N and K addition (+N, +K, +NK, +NPK) may be explained by the increasing inputs (plant biomass) in response to nutrient addition, along with small (positive or negative) changes to the decay rate.

Ecosystem covariates were strongly related to soil C stocks

In our overall statistical model, plant aboveground biomass and site aridity (rainfall minus potential evapotranspiration) had significant positive relationships with soil C concentration. These results are unsurprising given the established relationships between soil C and climatic and vegetation factors, especially for surficial soils (Jenny 1941; Schmidt et al. 2011). For example, in a global analysis of SOC stocks, Jobbágy and Jackson (2000) found that climate and soil texture were strong predictors of soil C up to 1 m depth: SOC was positively correlated with MAP and clay content, and negatively with MAT and sand content. At > 1 m depth these relationships changed. Soil texture increased in importance and climatic factors declined or were no longer significant. At the surface, where plant inputs and biological activity are the greatest, we expect biology (microbial and plant biomass) and factors that influence biological activity, such as climate, to play a key role in regulating soil C stock size (Schimel and Schaeffer 2012).

At our study sites, the relationship between plant aboveground biomass and soil C might be even stronger than our data suggest: plant biomass might be under predicted at the three sites included in this dataset that contain the highest concentrations of soil C (Bunchgrass (US). Lancaster (UK), and Lookout (US)). At these three sites, data from the Nutrient Network's herbivore exclosure plots show that aboveground biomass is 9, 110, and 200 % more in fenced plots compared to control plots, respectively, which suggests there is herbivore removal of plant biomass from the nutrient factorial plots examined in this study. In response to grazing, researchers have found that plants increase the translocation of labile C to roots and increase root exudation (Bardgett et al. 1998). These processes could increase C inputs to the soil C stock, despite herbivore removal of plant C, and may explain the higher soil C concentration despite lower than predicted plant aboveground biomass. Alternately, increased C inputs from roots could increase microbial decomposition of SOM (e.g., by "priming" decomposition); whether the increased microbial activity would still lead to increased soil C stocks is unknown.

Additionally, the higher soil C concentration at Bunchgrass, Lancaster, and Lookout could reflect the importance of climate effects on decomposition in these systems. Despite moderate plant biomass inputs (compared to the other sites in this study), soil C stocks may be much larger than average at these

three sites because decomposition is inhibited by the cold and wet conditions. Bunchgrass, Lancaster, and Lookout were the sites with the highest available moisture (high rainfall minus potential evapotranspiration).

Interestingly, in our statistical model, only 55% of the variation was explained by the inclusion of ecosystem covariates and the factorial nutrient addition factors. An additional 40% of the variation was accounted for by the random effect, site identity. This gap in variance explained demonstrates that there are still significant site-by-site differences that were not captured in the available ecosystem covariates. These could have included site history, including disturbance, land-use change, grazing, and other management strategies, all of which are known to influence soil C stocks (Conant et al. 2001; Guo and Gifford 2002). Additionally, error associated with estimating aboveground and belowground net primary production, differences in plant chemistry, and/or soil mineralogy could have contributed to the unexplained variation among sites.

Nutrient effects on soil C stocks and decay rates

We hypothesized that nutrient addition would have no effect on the total soil C pool due to increased plant biomass inputs and increased decay rates in all nutrient treatments except for when N was added alone, where, we expected soil C to *increase* in response to increasing inputs and *slower* decay rates. In contrast to our expectations, the +NPK treatment was the only treatment where soil C concentration increased significantly compared to the control. The +N, +K, and +NK treatments also increased relative to the control, but the difference from the control was not statistically significant. Among these treatments, plant aboveground biomass inputs in the +N, +NK, and +NPK treatments significantly increased in response to nutrient addition, and likely explain the soil C responses. As detailed above, we observed a positive relationship between soil C concentration and plant aboveground biomass at most sites, likely due to the strong influence of biological and climatic factors on soil C cycling in these surficial soils (Jobbágy and Jackson 2000; Schimel and Schaeffer 2012). Consequently, the positive biomass response to nutrient addition is likely driving the change in soil C concentration observed in these treatments.

Why then was there no effect of P addition, which also significantly increased plant aboveground biomass, on total soil C concentration? The estimated decay rates (*k*) from our numerical soil C model suggest that P addition increased the rate of soil C decomposition. We found that treatments that included P (+P, +NP, +PK, and +NPK) had the largest modeled decay rates. The positive effect of P addition on decomposition is consistent with previous studies that have also found stimulating effects of P on soil and litter decomposition (e.g., Hobbie and Vitousek 2000; Cleveland et al. 2002; Cleveland et al. 2006; Bradford et al. 2008b; Reed et al. 2010; Powers and Salute 2011; Fanin et al. 2012; Fanin et al. 2015). Phosphorus addition could increase decomposition by increasing microbial production of ATP (and consequently energy for decomposition) and/or P-rich enzymes (Bradford et al. 2008b). Notably, most of the previous studies of P effects on decomposition were conducted in the tropics where low P supply is expected to limit biological activity. It is surprising that P had such a strong positive effect on *k* in our numerical soil C model since most of our study sites are in the temperate region.

In our numerical soil C model we also found that N addition had a slight negative effect on *k* when added alone. The negative effects of N addition on modeled decay rate were even stronger when we used a "lower bound estimate" of plant inputs to soil (see model variation 2 in Appendix 1 – Supplement S1.1 and Figure S1.6). These results supported our prediction: we expected N to *decrease* the rate of soil C decay. Several researchers report that N inhibits the decomposition rate of soil organic matter, due to a variety of microbial-mediated mechanisms (e.g., microbial community shifts (Ramirez et al. 2012), inhibition of oxidative enzymes (Zak et al. 2008), decreased microbial growth efficiency (Schimel and Weintraub 2003), as well as factors that cause microbial biomass to decrease in response to N (Treseder 2008)). Consequently, the slight increase in soil C concentration may be due to slower microbial decay rates, in addition to an increase in plant inputs under N enrichment.

Finally, in the +K treatment, the soil C concentration increased (moderate significant difference), despite small increases in aboveground biomass. This suggests that decay rates decreased in this treatment, although this was not supported substantially by our numerical soil C model results: although average decay rate was lower in the +K treatment relative to the control, the effect was not robust across model

variations (see Appendix 1 – Supplement S1.1) and the standard error intervals around the mean overlapped with the control treatment. Alternately, the stimulating effect of the K treatment on the total soil C concentration could have been due to significant increases in biomass inputs that were not appropriately parameterized in our model. Although N and P often stimulate root biomass inputs (Yuan and Chen 2012), the effects of K and micronutrients on root production and turnover are largely unknown.

Conclusions

The increase in soil C concentration in response to enrichment by multiple nutrients (N, P, and K plus micronutrients) suggests that grassland soil C stocks will increase with continued anthropogenic enrichment of multiple nutrients. In surficial grassland soils, such as those studied here, the positive effects of increased nutrients on plant biomass could be the primary factor that drives the increase in soil C. Unoccluded soil C, such as plant litter, does not contribute to soil C sequestration on the decadal or longer time scale unless it is stabilized against microbial decomposition in soil aggregates or on mineral surfaces (Dungait et al. 2012). Consequently, the long-term fate of this increased soil C stock in response to nutrient addition will depend site-specific capacity for C occlusion (e.g., the availability of free soil mineral surfaces), as well as the effects of nutrient addition on the mechanisms that stabilize C against microbial decomposition. Furthermore, our results demonstrate that not all nutrients are created equal: plant biomass and decomposition may respond to different nutrients differently, which means nutrient identity will matter for soil C stock responses to nutrient addition.

Table 1.1 – Nutrients added in each experimental nutrient treatment.

Nutrients added	Nitrogen (N) treatment [a]	Phosphorus (P) treatment [a]	Potassium (K) treatment [a]
Nitrogen (N)	X (Urea)		
Phosphorus (P)		X (Triple-super phosphate)	
Potassium (K)			X (Potassium sulfate)
Carbon (C)	X (Urea)		X (Calcium carbonate, magnesium carbonate)
Calcium (Ca)		X (Triple-super phosphate)	X (Calcium carbonate)
Sulfur (S)			X (Potassium sulfate, ferrous sulfate, manganese sulfate, zinc sulfate, copper sulfate)
Magnesium (Mg)			X (Magnesium carbonate)
Iron (Fe)			X (Ferrous sulfate)
Manganese (Mn)			X (Manganese sulfate)
Zinc (Zn)			X (Zinc sulfate)
Copper (Cu)			X (Copper sulfate)
Sodium (Na)			X (Sodium borate, sodium molybdate)
Boron (B)			X (Sodium borate)
Molybdenum (Mo)			X (Sodium molybdate)

[a] Form of the nutrient added indicated in parentheses.

Table 1.2 – Effects of nutrient addition and ecosystem covariates on total soil carbon concentration from model averaging. [a]

Effect	Estimate	Standard Error	Adjusted SE	z value	p-value [b]	Importance	Number of containing models
Intercept	3.43	0.13	0.13	26.43	**< 0.0001**	1	9
Plant aboveground biomass	0.11	0.05	0.05	2.22	**0.03**	0.97	9
Plant root biomass	0.03	0.04	0.04	0.76	0.4	0.53	9
Rain minus potential evapotranspir-ation	1.34	0.27	0.27	5.04	**< 0.0001**	1	8
Soil pH	0.02	0.04	0.04	0.59	0.6	0.42	9
Soil % silt and clay	-0.13	0.12	0.12	1.04	0.3	0.67	9
K	0.03	0.02	0.02	1.66	0.1	1	9
N	0.03	0.02	0.02	1.24	0.2	1	9
P	-0.04	0.02	0.02	1.89	0.06	1	9
K x N	0.04	0.04	0.04	0.90	0.4	1	5
K x P	0.02	0.04	0.04	0.46	0.6	1	5
N x P	0.02	0.04	0.04	0.43	0.7	1	4

K x N x P	0.18	0.08	0.08	2.19	**0.03**	1	9
Marginal R^2: 0.55 [c]							
Conditional R^2: 0.95 [d]							

[a] Results from the top equivalent models (AIC difference < 4) of soil carbon concentration (g C g^{-1} soil) identified using model averaging.

[b] P-value based on full model-averaged coefficients.

[c] Marginal R^2 represents the variance that is explained by fixed effects only.

[d] Conditional R^2 represents the variance that is explained by both fixed and random effects.

Table 1.3 – Effects of nutrient addition on proportion change in total soil carbon concentration: ANOVA table.

Effect	df	F-value	p-value
Intercept	1, 389	11.0	**0.001**
N	1, 389	3.82	0.05
P	1, 389	1.12	0.3
K	1, 389	4.39	**0.04**
N:P	1, 389	0.03	0.9
N:K	1, 389	0.08	0.8
P:K	1, 389	0.00	0.9
N:P:K	1, 389	4.39	**0.04**
Marginal R^2: 0.03 [a]			
Conditional R^2: 0.08 [b]			

[a] Marginal R^2 represents the variance that is explained by fixed effects only.

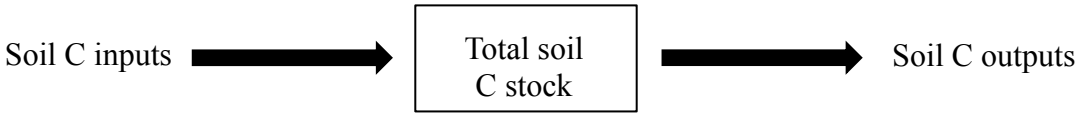
[b] Conditional R^2 represents the variance that is explained by both fixed and random effects.

Table 1.4 – Effects of nutrient addition on modeled decay rate (*k*): ANOVA table.

Effect	df	F-value	p-value
Intercept	1, 351	30.46	**< 0.001**
N	1, 351	0.12	0.7
P	1, 351	14.22	**0.0002**
K	1, 351	0.43	0.5
N:P	1, 351	0.03	0.9
N:K	1, 351	0.03	0.9
P:K	1, 351	0.07	0.8
N:P:K	1, 351	1.96	0.2
Marginal R^2: 0.02 [a]			
Conditional R^2: 0.52 [b]			

[a] Marginal R^2 represents the variance that is explained by fixed effects only.

[b] Conditional R^2 represents the variance that is explained by both fixed and random effects.



Soil C inputs	N	P	Other	N + P	N + P + Other
Plant litter (aboveground)	+	+	+?	+	+?
Plant litter (belowground)	+	+	?	+	?
Root rhizodeposition	?	?	?	?	?

Soil C outputs	N	P	Other	N + P	N + P + Other
Decomposition of SOC to CO_2	- *	+	+	+	+
Leaching of DOC	+	?	?	?	?
Erosion of POC	?	?	?	?	?

Figure 1.1 – Conceptual diagram of the hypothesized (and unknown) effects of nutrient addition on soil carbon inputs and outputs. Figure codes: N = nitrogen; P = phosphorus; Other = "other" (non-N and non-P) nutrients; SOC = soil organic C; DOC = dissolved organic C; POC = particular organic C; + = nutrient addition increases the input or output rate; - = nutrient addition decreases the input or output rate; ? = unknown effect of nutrient addition on the input or

output rate. * Nitrogen addition tends to decrease SOC microbial decomposition rate, although the negative effect may be limited to low "quality" (e.g., high lignin and recalcitrant content) organic matter (Knorr et al. 2005).

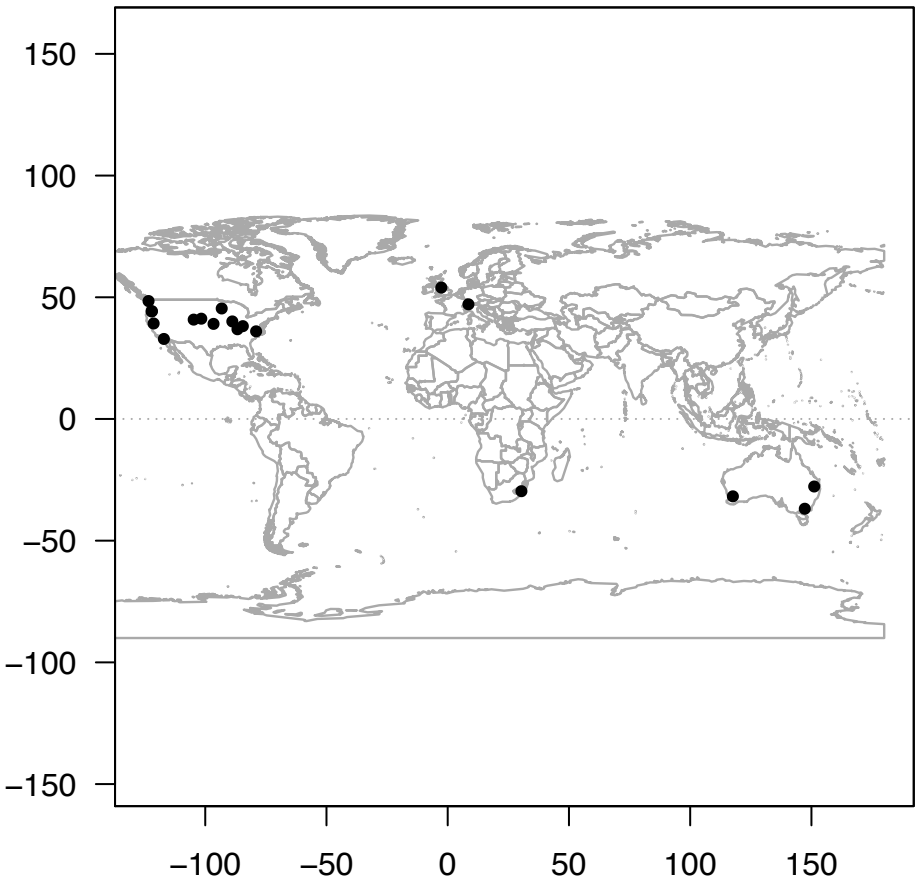


Figure 1.2 – Nutrient Network experimental sites included in this study.

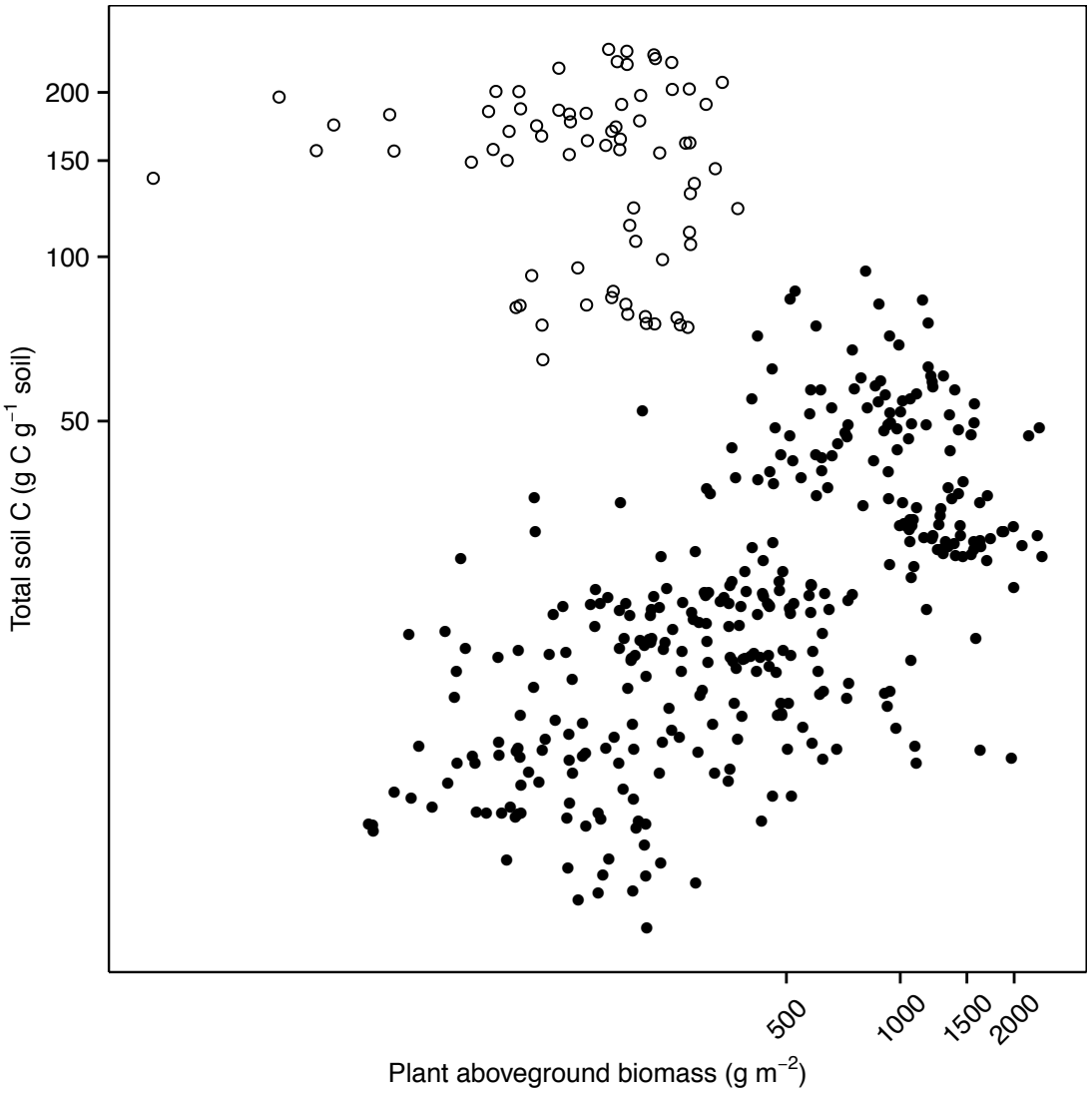


Figure 1.3 – Relationship between plant aboveground biomass and total soil carbon concentration (top 10 cm). Symbol codes: open circles = three sites with high soil C concentration (Bunchgrass (US). Lancaster (UK), and Lookout (US)); closed circles = all other experimental sites.

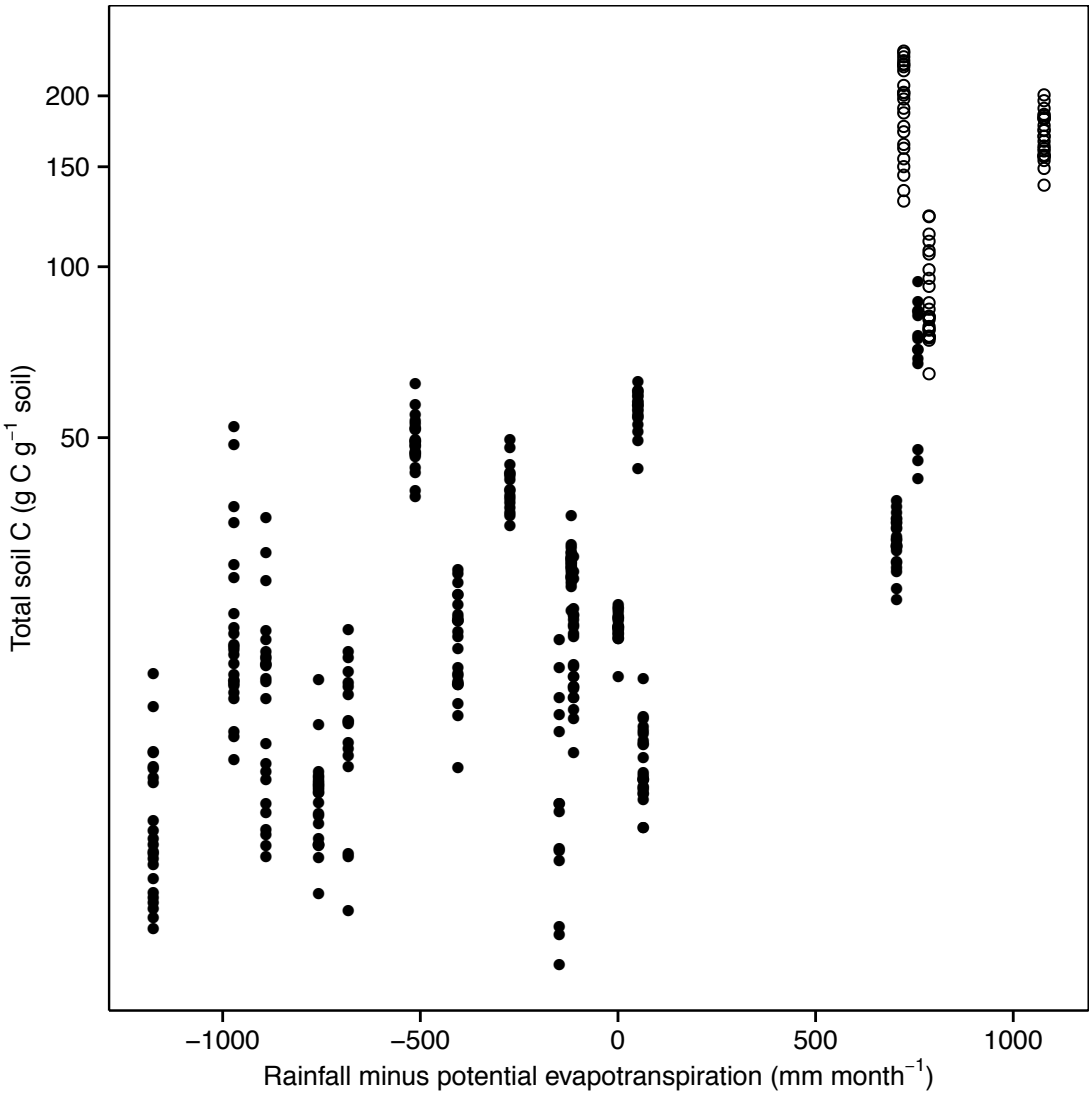


Figure 1.4 – Relationship between aridity (rainfall minus potential evapotranspiration) and total soil carbon concentration (top 10 cm). Symbol codes: open circles = three sites with high soil C concentration (Bunchgrass (US). Lancaster (UK), and Lookout (US)); closed circles = all other experimental sites.

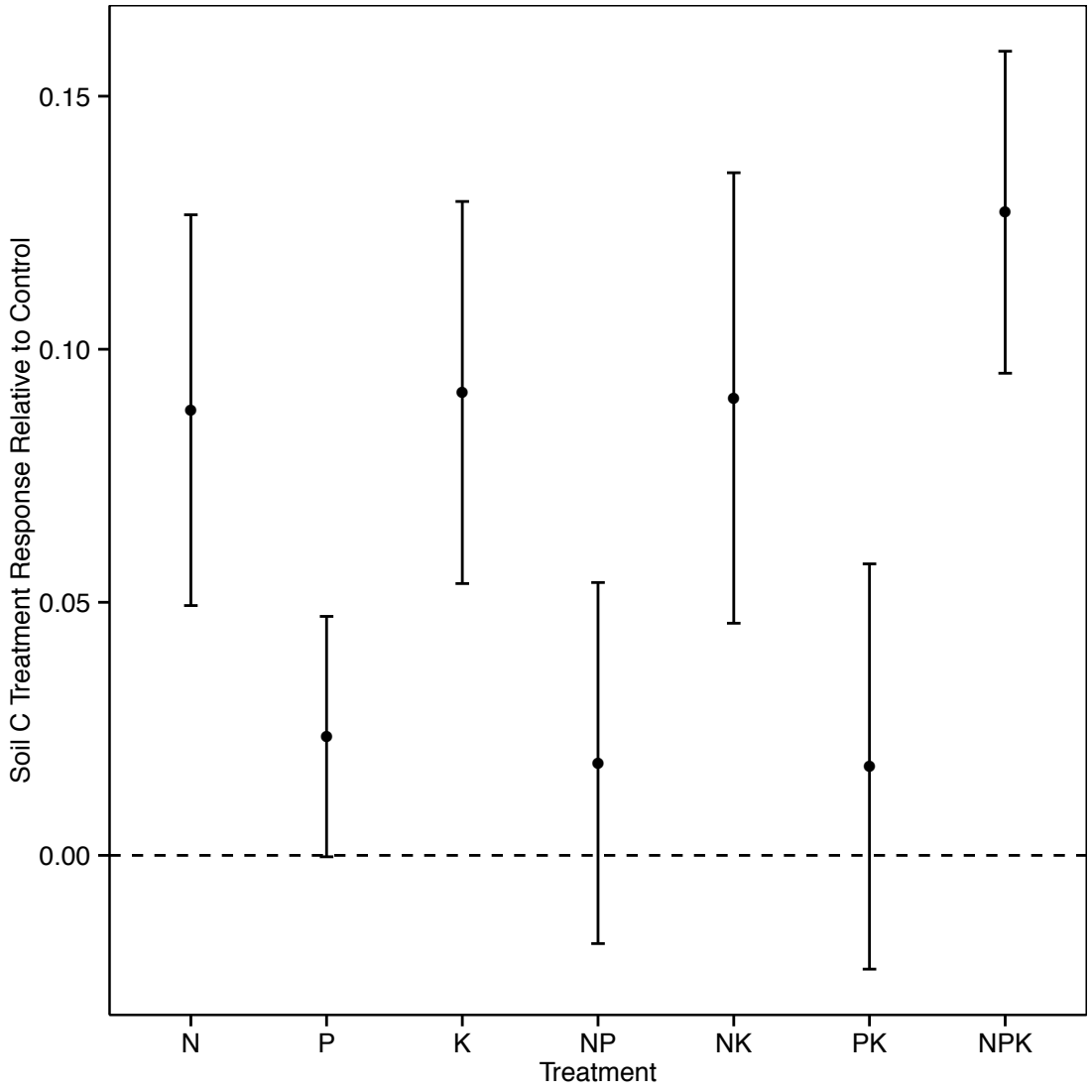


Figure 1.5 – Effects of nutrient addition on proportion change in total soil carbon concentration (top 10 cm). Proportion change in soil carbon (C) = ((treatment plot soil C – control plot soil C)/control plot soil C). Figure shows mean +/- one standard error. Treatment codes: N = nitrogen addition plots; P = phosphorus addition plots; K = potassium plus micronutrient addition plots; NP = nitrogen and phosphorus addition plots; NK = nitrogen and potassium plus micronutrient addition plots; PK = phosphorus and potassium plus micronutrient addition plots; NPK = nitrogen, phosphorus, and potassium plus micronutrient addition plots.

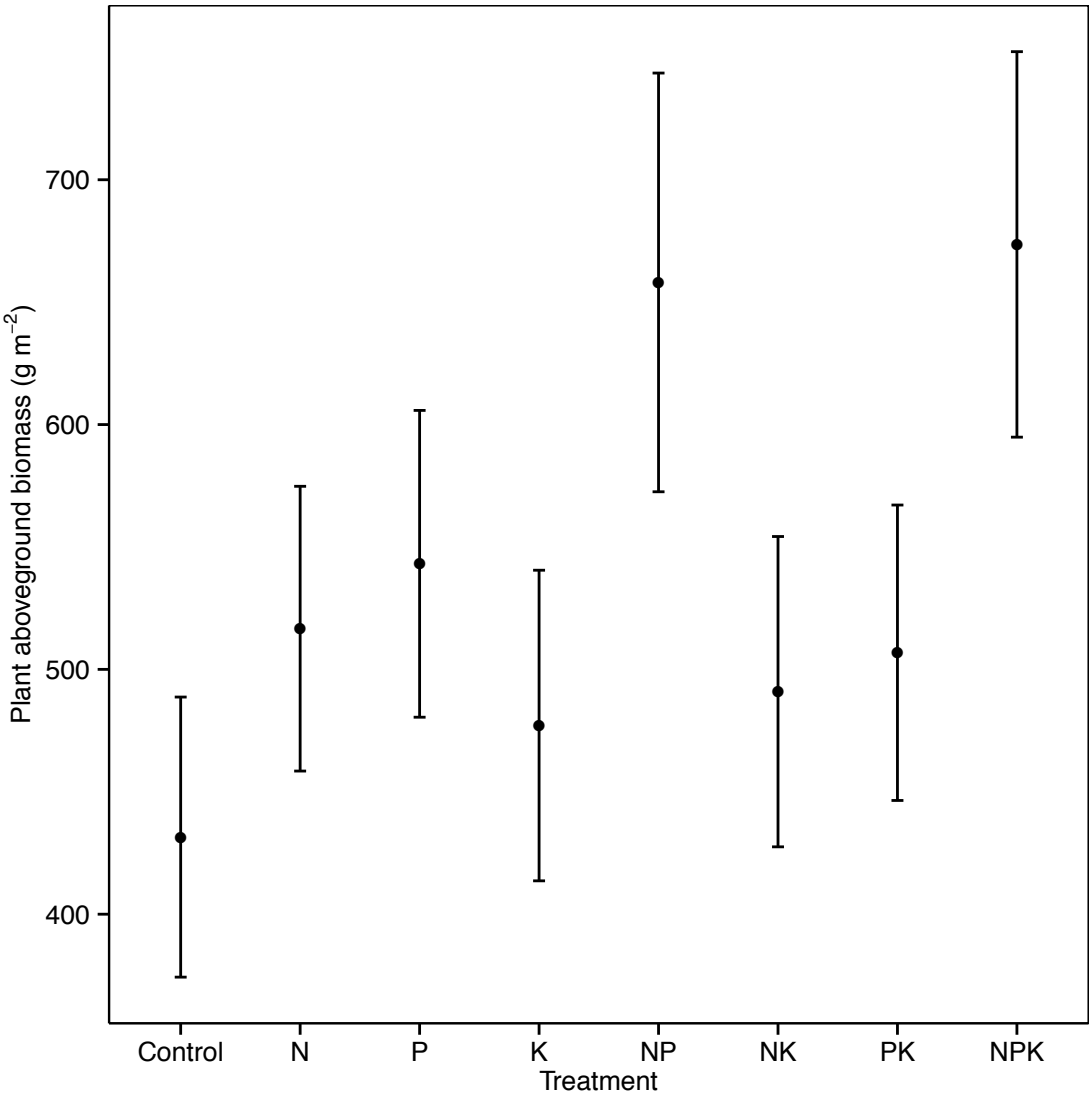


Figure 1.6 – Effects of nutrient addition on plant aboveground biomass. Figure shows mean +/- one standard error. Treatment codes: Control = control plots; N = nitrogen addition plots; P = phosphorus addition plots; K = potassium plus micronutrient addition plots; NP = nitrogen and phosphorus addition plots; NK = nitrogen and potassium plus micronutrient addition plots; PK = phosphorus and potassium plus micronutrient addition plots; NPK = nitrogen, phosphorus, and potassium plus micronutrient addition plots.

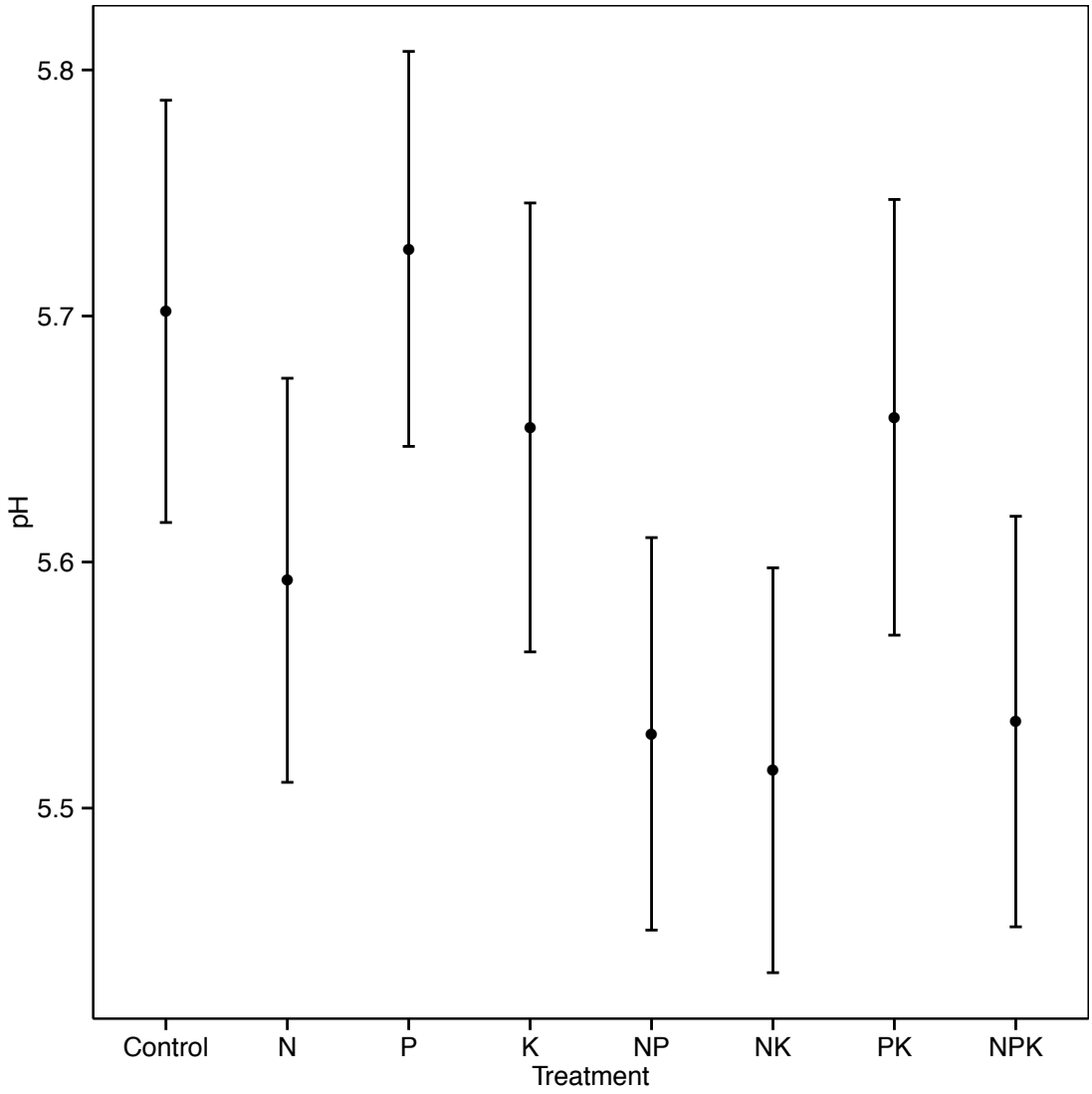


Figure 1.7 – Effects of nutrient addition on soil pH. Figure shows mean +/- one standard error. Treatment codes: Control = control plots; N = nitrogen addition plots; P = phosphorus addition plots; K = potassium plus micronutrient addition plots; NP = nitrogen and phosphorus addition plots; NK = nitrogen and potassium plus micronutrient addition plots; PK = phosphorus and potassium plus micronutrient addition plots; NPK = nitrogen, phosphorus, and potassium plus micronutrient addition plots.

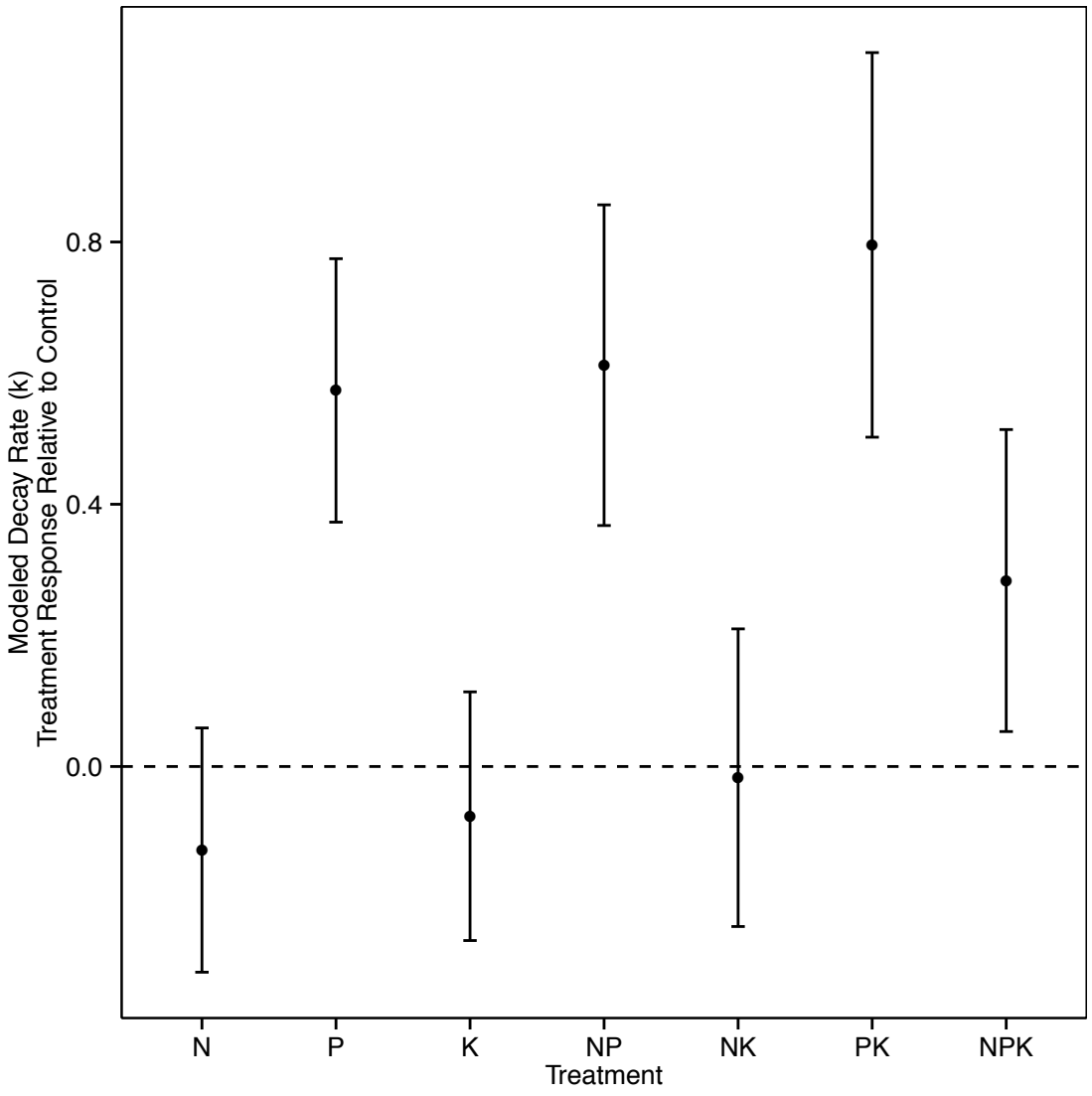


Figure 1.8 – Effects of nutrient addition on proportion change in modeled decay rate. Decay rates (*k*, yr^{-1}) were computed from a numerical soil C model of observed plant inputs and soil C stocks in each plot. Figure shows mean (plus/minus standard error) modeled decay rates (*k*) in each treatment relative to the control plot modeled decay rate of each block. Treatment codes: N = nitrogen addition plots; P = phosphorus addition plots; K = potassium plus micronutrient addition plots; NP = nitrogen and phosphorus addition plots; NK = nitrogen and potassium plus micronutrient addition plots; PK = phosphorus and

potassium plus micronutrient addition plots; NPK = nitrogen, phosphorus, and potassium plus micronutrient addition plots.

CHAPTER 2

Nitrogen addition changes grassland soil organic matter decomposition

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Humans have dramatically increased the deposition and availability of nutrients, such as nitrogen (N), worldwide. Soil organic matter (SOM) is a significant global reservoir of carbon (C); however, the effects of N enrichment on this large, heterogeneous C stock are unclear. Nitrogen has variable effects on the biological, chemical, and physical factors that determine SOM pool mean residence time; consequently, we predicted that N enrichment would have distinct effects on SOM pools, including the pool that is readily available for microbial decomposition, as well as the pools that have been stabilized against microbial decomposition via aggregate occlusion and mineral association. We addressed this gap in knowledge by measuring the effects of N addition on different SOM pools at five grassland experiments in the US Central Great Plains that participate in the Nutrient Network and have been fertilized for three or five years. Overall, N addition decreased microbial respiration of unoccluded OM by as much as 29% relative to control plots, and consequently, decreased C loss from this pool. Furthermore, N addition tended to increase soil aggregation and C occlusion in large macro-aggregates. These results suggest that N addition will increase C sequestration by slowing the decomposition of SOM, as well as stabilizing SOM against microbial decomposition in aggregate-occluded pools. However, the effects of N on all pools studied varied among sites, possibly due to site variation in soil texture. Consequently, increased sequestration of soil C in response to N enrichment may not be universal across grasslands.

INTRODUCTION

Since the late 1800s, anthropogenic creation of reactive nitrogen (N) has increased 10-fold (Galloway et al. 2004; Galloway et al. 2008), due to fossil fuel combustion, cultivation of N-fixing crops, and fertilizer production, causing a concomitant rise in atmospheric N deposition. Perturbation of the global N cycle influences global carbon (C) stocks as well. The addition of N can significantly increase net primary production, and total C aboveground stocks (Gough et al. 2000; LeBauer and Treseder 2008; Lee et al. 2010). Soil organic matter (SOM) – a globally significant C reservoir – contains two to five times as much C than aboveground biomass, and two to four times as much C than is present in the atmosphere (Ciais et al. 2013). Consequently, either positive or negative changes to soil C sequestration, in response to N addition, could significantly alter atmospheric carbon dioxide (CO_2) concentrations, and thus have implications for future global climate. However, the influence of added N on soil C remains uncertain.

The effects of N addition on soil C storage differ across studies. Specifically, N can increase (Frey et al. 2014), decrease (Waldrop et al. 2004), or not change (Zeglin et al. 2007) the *total* SOM C stock (for meta-analyses of N addition studies see Liu and Greaver 2010 and Lu et al. 2011). However, the variable effects of N addition on total soil C are not surprising. The total SOM stock is comprised of multiple pools with mean residence times (MRTs) that vary from days to centuries (Von Lützow et al. 2006). Three key factors play a role in determining the variation in pool MRT: recalcitrance of SOM, physical protection of SOM in soil aggregates, and associations between soil minerals and SOM (Dungait et al. 2012; Cotrufo et al. 2013). Nitrogen addition can directly affect each of these key factors; consequently, the effects of N enrichment on each of these pools may differ. Unfortunately, few studies have examined the effects of N addition on multiple SOM pools with distinct MRTs and the results of these studies have not been concordant. Researchers report no change (Reid et al. 2012), increased (Neff et al. 2002), and decreased (Torn et al. 2005) decomposition of the unprotected SOM pool (rapidly cycling C with short MRT) and no change (Kaye et al. 2002), increased (Bradford et al. 2008a), and decreased (Hagedorn et al. 2003) decomposition of aggregate-occluded or mineral-associated SOM pools (slowly cycling C with long MRT)

in response to N addition. Current knowledge, which is riddled with inconsistent patterns and little mechanistic understanding, is still insufficient to predict future soil C sequestration potential in response to N enrichment.

What are the possible effects of N addition on SOM pools with distinct MRTs? Microbial decomposition of physically unoccluded, available SOM is influenced by SOM biochemistry, environmental conditions, and microbial physiology (Schimel and Schaeffer 2012). Consequently, the effects of N addition on the chemistry of the decomposition substrates and/or soil microbes (e.g., enzyme activity, physiology, community composition) will determine how these rapidly cycling C pools (MRT days to years) will respond to N addition (Janssens et al. 2010). Since leaf litter decomposition studies measure the decomposition of unoccluded (e.g., physically accessible) organic matter, results from litter N addition studies can offer insight into how unoccluded *soil* organic matter pools may respond to N. For example, Berg and Matzner (1997) and Hobbie et al. (2012) have found that N addition increased the decomposition rate of the litter fraction comprising more labile substrates that decomposes first (the "fast" decomposing litter fraction), but decreased the decomposition rate of the remaining litter comprising more chemically complex substrates (the "slow" decomposing litter fraction). The contrasting effects of N on "fast" versus "slow" litter fractions are likely due to differing effects of N on the decomposition of the substrates that make up each litter fraction. Specifically, previous litter studies have shown that the addition of N alleviated N limitation of microbes seeking C in relatively labile OM substrates, such as polysaccharides, which would lead to faster decomposition of the "fast" decomposing, labile litter fraction (Berg and Staaf 1980; Talbot and Treseder 2011). By contrast, N addition can inhibit lignin-degrading enzyme activity, leading to slower decomposition of the more chemically complex substrates that dominate the "slow" decomposing litter fraction (Carreiro et al. 2000). These effects could also occur in the unoccluded *soil* organic matter pool, which is also heterogeneous in substrate composition. Consequently, we expect divergent effects of N addition on the decomposition of unoccluded SOM: N addition may increase the decomposition of unoccluded labile SOM, and decrease the decomposition of unoccluded chemically complex SOM.

By contrast, in pools comprised of slowly cycling C (MRT years to centuries), SOM is stabilized against microbial decomposition via physical protection (occlusion) within soil aggregates (Jastrow et al. 1996). Along with soil particles, biological components such as roots, fungal hyphae, and microbial biomass and necromass, all contribute to aggregate formation (Oades and Waters 1991; Jastrow et al. 1998; Six et al. 2004; Wilson et al. 2009; King 2011; Gupta and Germida 2015). Nitrogen addition can decrease plant investment in belowground nutrient acquisition (such as root biomass and mycorrhizae), leading to less belowground biomass (Feng et al. 2010; Janssens et al. 2010; Li et al. 2015). Consequently, we expect aggregate-binding "agents" and aggregate-occluded SOM to decrease in response to N addition.

Finally, the very slowest cycling C pools (MRT centuries to millennia) are stabilized against microbial decomposition via adsorption onto soil mineral surfaces (Torn et al. 1997). Nitrogen addition can lead to acidification and increased solubility of polyvalent, hydrolyzing cations, such as Al^{3+} and Fe^{3+} in soils (Bouwman et al. 2002; Mueller et al. 2012). In turn, these pH-mediated effects can increase mineral surface reactivity and the abundance of exchangeable cations, respectively, resulting in increased SOM binding to mineral surfaces (Oades 1988; Baldock and Skjemstad 2000). Consequently, we expect that N addition will increase mineral-associated SOM through changes to mineral surface reactivity.

The objective of this study was to determine the effects of N enrichment on the decomposition of multiple SOM pools using empirical measurements of key SOM pools and fluxes. Specifically, we examined the effects of N enrichment on: 1) rapidly cycling C in the unoccluded SOM pools, 2) slowly cycling C in the aggregate-occluded SOM pools, and 3) very slowly cycling C in the mineral-associated SOM pools. Understanding the effects of N addition on soil C cycling is particularly important in grassland ecosystems as they comprise a significant proportion of the earth's land area (~24%) and soil C (~28%) and contain more soil C per unit area than the global average (Watson et al. 2000). Consequently, this study focused on examining the effects of N addition at multiple grasslands sites.

We tested the following hypotheses for the effects of N addition on distinct SOM pools: *Hypothesis 1 (H1)*: Nitrogen addition will increase the decomposition (and decrease the amount) of relatively labile unoccluded SOM by alleviating microbial nutrient limitation, and decrease the

decomposition (and increase the amount) of more chemically complex unoccluded SOM by inhibiting its decomposition. *Hypothesis 2 (H2)*: Nitrogen addition will decrease the size of the aggregate-occluded SOM pool through decreased root and mycorrhizal biomass and associated soil aggregation. *Hypothesis 3 (H3)*: Nitrogen addition will increase the size of the mineral-associated SOM pool through pH-mediated increases to mineral surface reactivity.

MATERIALS AND METHODS

Study sites

Soils were sampled from nutrient addition plots at five participatory sites of the Nutrient Network (www.nutnet.org; Table 2.1). The Nutrient Network is a collaborative, global network of experiments established to investigate the effects of multiple nutrient additions, including N, on ecosystem processes in grasslands. Participatory sites are located across the globe and follow standard protocols for sampling and analysis (Borer et al. 2014). We focused on the nutrient addition plots at five Nutrient Network sites located in the U.S. Central Great Plains region (Table 2.1): Cedar Creek Ecosystem Science Reserve (East Bethel, Minnesota; 45.40°, -93.20°), Cedar Point Biological Station (Ogallala, Nebraska; 41.2°, -101.63°); Chichaqua Bottoms Greenbelt (Maxwell**,** Iowa; 41.79°, -93.39°); Konza Prairie Biological Station (Manhattan, Kansas; 39.07°, -95.58°); and Shortgrass Steppe (Nunn, Colorado; 40.82°, -104.77°). At each of these sites, nitrogen (N), phosphorus (P), and potassium plus micronutrients (K), have been added in a full factorial manner since 2008 (Colorado, Kansas, Minnesota, Nebraska) or 2010 (Iowa). N, P, and K were applied annually (10 g m^{-2} yr^{-1}); a micronutrient mix, including B, Cu, Fe, Mg, Mn, Mo, S, and Zn, was applied once at the start of the experiment in the K plots only. The treatments are replicated across three blocks in Colorado, Kansas, and Nebraska, across five blocks in Minnesota, and across six blocks in Iowa. More details on the experimental set-up and nutrient sources are available in Borer et al. (2014).

Our focus was on the effects of N on SOM decomposition and stabilization. To maximize the statistical power for detecting N effects, we sampled the full factorial of nutrient treatments for most

analyses. For the more labor-intensive analyses, we analyzed the control and N addition plots only (see Appendix 2 – Tables S2.1-2 for a summary of sampled plots included in each analysis).

Soil sampling

Soil samples were collected in July and August of 2012. Three cores (5 cm diameter and 10 cm deep) were sampled from each plot and composited across the full factorial of nutrient treatments; a fourth core was sampled from the control and N addition treatments for root analyses. Samples were kept on ice or in the refrigerator for a maximum of 6 days until processed in the lab. A subsample of composite soil from each plot was sieved to 2 mm for chemical and biological analysis and 8 mm for soil aggregate isolation. Fresh, 2 mm-sieved soil was used to measure gravimetric soil moisture, microbial respiration, microbial biomass and net N mineralization (details below). Air-dried, 2 mm-sieved soil was used to measure total soil % C and % N by combustion (Costech ESC 4010 Elemental Analyzer, Valencia, California, USA), soil pH (1:1 soil:water slurry method), and particulate organic matter (POM) C and N via density flotation (method detailed below). Additionally, soil texture was measured on air-dried, 2 mm-sieved soil from the control plots using the hydrometer method and sodium hexametaphosphate as the dispersing agent (Ashworth et al. 2001). Soil sieved to 8 mm from the control and N addition plots was air-dried and used to measure water-stable soil aggregates (details below).

Analyses: Decomposition of unoccluded SOM

We evaluated the effects of nutrient addition on microbial decomposition of unoccluded SOM by measuring microbial respiration during a long-term laboratory incubation. A subsample of fresh, 2 mm sieved soil from each plot was placed in a 120 ml specimen cup and soil moisture was adjusted to 70% field capacity. Field capacity was calculated separately for each site by pulling 20 KPa pressure on saturated soil. Microbial respiration rate (mg C g $soil^{-1}$ day^{-1}) was determined at least 17 times during the 380-day laboratory incubation. For each respiration rate measurement, the specimen cups were placed inside 1 L Mason jars and sealed for either 24- or 48-hour intervals. The CO_2 concentration in the airtight

jars was measured at the beginning and end of each interval using an infrared gas analyzer (LICOR 7000). When not being measured, specimen cups were covered with gas-permeable, low-density polyethylene film. Throughout the incubation, soil samples were maintained at 70% field capacity and kept at 20°C in the dark. We calculated cumulative C respired (mg C g $soil^{-1}$) during the incubation by averaging the respiration rate between adjacent measurement dates and multiplying by the interval between them, then summing the amount of C respired in between each rate measurement. Additionally, because we were interested in the effects of N on unoccluded SOM substrates that decay at different rates, we evaluated our respiration rate data against both one- and two-pool decay models (see *Statistical Analyses* below).

Since microbes drive the turnover of unoccluded SOM, we also assessed the effects of N addition on microbial biomass C and N at the start of the respiration incubation using chloroform fumigation extraction (Brookes et al. 1985). Briefly, replicate fresh, 2 mm-sieved soil samples were extracted with 0.5M K_2SO_4 prior to and following chloroform fumigation under vacuum for 5 days. Following filtration, extracts were analyzed for total organic C and total N (Shimadzu TOC-V, Shimadzu Corporation, Kyoto, Japan). Soil microbial biomass C (*MC*) and N (*MN*) were calculated as: $MC = EC/k_{EC}$ and $MN = EN/k_{EN}$, where *EC* is the difference between extractable C in the fumigated and unfumigated samples, *EN* is the difference between extractable N in the fumigated and unfumigated samples, k_{EC} is the C extraction efficiency coefficient, and k_{EN} is the N extraction efficiency coefficient. We used the standard extraction efficiency coefficients of 0.45 (k_{EC}) and 0.54 (k_{EN}) from the literature (Brookes et al. 1985; Beck et al. 1997).

Analyses: Aggregate-occluded and mineral-associated SOM

We measured the effects of nutrient addition on SOM stabilization in aggregate and mineral fractions using wet sieving fractionation, which isolated water stable soil aggregates (Six et al. 2000; Bach et al. 2010). Briefly, air-dried, 8 mm-sieved soil subsamples from the control and N addition treatments only were wet sieved with a 2 mm sieve for 2 minutes each to isolate large macro-aggregates (>2000 μm). Soil that passed through the sieve was wet-sieved with a 250 μm sieve to isolate small macro-aggregates

(2000 μm – 250 μm). Finally, the remaining material was wet-sieved with a 53 μm sieve to isolate micro-aggregates (250 μm – 53 μm) and mineral-associated SOM (<53 μm). During wet sieving, floating organic matter was removed so we could test for N effects on C that was *occluded* within each aggregate fraction. The isolated fractions were dried at 105 °C for 12 hours, followed by 60 °C for 48 hours. Fractions were weighed and analyzed for C and N concentration (Costech ESC 4010 Elemental Analyzer, Valencia, California, USA) and used to determine percentage of whole soil total C and N contributed by each fraction. We did not perform sand-corrections on the aggregate fractions (Elliott et al. 1991) because our primary goal was to evaluate the distribution of C and N among aggregate fractions at several grassland sites, not a direct comparison of C and N content within specific fractions across sites. The large macro-aggregate, small macro-aggregate, and micro-aggregate fractions were used to evaluate H2 (aggregate-occluded SOM), while the smallest size fraction informed H3 (mineral-associated SOM).

In order to assess mechanisms of aggregation, we supplemented our aggregate fraction data with root biomass and mycorrhizal colonization measurements. Directly following collection, the additional intact core sampled from the control and N treatment plots was washed in wire mesh tubes (0.28 mm mesh) in a rotating elutriator (Wiles et al. 1996) until all soil was removed (~3 hours). Remaining material was suspended in water and roots were captured with fine sieves and hand-picking. Root crowns were not considered root biomass and removed. Once free of soil, roots were dried at 65°C overnight and weighed to calculate dry root biomass per unit area. Colonization of root tissue by arbuscular mycorrhizal fungi was determined by the point intercept method. Roots were removed from soil cores by washing gently with water over a 53 μm sieve. Cleaned roots were stained with Trypan Blue and stored in a 1:1:1 (vol) solution of glycerin:lactic acid:water at 4°C. Roots were spread in a petri dish marked with 13 mm square grid and examined at 40x magnification to determine presence of fungal structures (hyphae and/or vesicles) at each root-grid line intersection. One hundred intersects were counted for every sample to determine the proportion of root tissue colonized, and each sample was counted twice to ensure reproducible results. Seven root samples were not prepared for mycorrhizal analysis and, consequently, are not included in the statistical analyses.

Analyses: Additional soil measurements

We also measured the effects of nutrient addition on particulate organic matter (POM) chemistry and microbial net N mineralization rate. These two soil properties can change in response to N addition due to the effects of N addition on plant substrate chemistry. Particulate organic matter was measured following gentle agitation and separation in a dense liquid (method modified after Sollins et al. 2006). Briefly, 10 ml of 1.8 g L^{-1} NaI was added to 10 g of air-dried, 2 mm sieved soil. Samples were gently shaken for 30 minutes to disperse weakly bound soil aggregates and centrifuged (2,400 rpm for 30 minutes). We separated floating light fraction material (POM) and rinsed and analyzed the fraction for % C and % N (Costech ESC 4010 Elemental Analyzer, Valencia, California, USA). Four samples were contaminated during the procedure and excluded from the statistical analyses.

To measure net N mineralization, we performed a 28-day laboratory incubation. Fresh, 2 mm sieved soil samples were extracted immediately with 2M KCl. Duplicate samples were adjusted to 70% field capacity, incubated in the laboratory at 20°C in the dark for 28 days, and then extracted with 2M KCl. Extracts were analyzed for inorganic N (ammonium and nitrate) using colorimetric methods (Hood-Nowotny et al. 2010). We calculated net N mineralization (mg N g $soil^{-1}$ day^{-1}) by subtracting the inorganic N concentration of the original soil extract from the inorganic N concentration of the incubated soil extract and dividing by the number of incubation days.

Statistical analyses

We were interested in whether N had distinct effects on more labile versus chemically complex unoccluded SOM (H1). Consequently, we evaluated whether microbial respiration data should be modeled with two pools that cycle at different rates, or a simple one-pool model (single decomposition rate). We evaluated the fit of the daily respiration rate measurements to two decomposition models: a one-pool decay model, $C_{rate}(t) = k\,(C_t\, e^{-k\,t})$; and a two-pool decay model, $C_{rate}(t) = k_f\,(C_f\, e^{-k_f\,t}) + k_s\,[(C_t - C_f)\, e^{-k_s\,t}]$. In both models, $C_{rate}(t)$ is the daily respiration rate (mg C g $soil^{-1}$ day^{-1}) at time t, t is time (days), and C_t is total soil C (mg C g $soil^{-1}$). In the one-pool model, k is the decomposition rate (day^{-1}) of the total soil C pool

(C_t). In the two-pool model, k_f and k_s are the decomposition rates (day^{-1}) of the "fast"- and "slow"-decomposing soil C pools, respectively. The MRT (day) of the fast and slow pools are k_f^{-1} and k_s^{-1}, respectively. The slow pool is defined as the difference between the total soil C pool (C_t; mg C g $soil^{-1}$) and the fast soil C pool (C_f; mg C g $soil^{-1}$).

We used maximum-likelihood estimation (MLE) to fit both decomposition models and calculate the decay rate and pool size parameters (bbmle package in R). The Akaike Information Criterion (AIC) was used as a measure of model fit. Since the two-pool model fit the data best (see *Results: Decomposition models and parameters* below), the parameter estimates from the two-pool model were used in subsequent analyses. We tested for parameter estimate equifinality – or model results where multiple combinations of parameters produce equally good model fits (Beven 2006) – by randomly generating 50,000 parameter combinations for each sample and fitting all parameter combinations to the one- and two-pool models. We evaluated model goodness-of-fit (R^2) by comparing the predicted respiration rates from these random parameter combinations against the actual respiration rate data of each soil sample. This test showed no evidence in support of parameter estimate equifinality using MLE (see Appendix 2 – Supplement S2.1 for further details on equifinality evaluation methods and results).

We evaluated the effects of N on the unoccluded SOM pool (H1) by testing for the effects of site and nutrient addition (N x P x K) on the two-pool model parameter estimates (k_f, k_s, C_f, and C_s) and cumulative C respired using ANOVA. We evaluated N effects on the aggregate-occluded and mineral-associated SOM pools (H2 and H3) by testing the effects of site and N on the proportion of total C contained in each of the aggregate fractions isolated via wet sieving. We also tested for the effects of site and nutrient addition (N x P x K) on total soil C, N and C:N ratio; microbial biomass C, N, and C:N ratio; POM C, N, and C:N ratio; soil pH; and net N mineralization; as well as the effects of site and N on root biomass and mycorrhizal colonization using ANOVA. Response variables were natural log transformed when needed to meet normality assumptions (tested using the Shapiro-Wilke normality test). Additionally, we assessed the regression assumptions of normality and homogeneity of variance by plotting the residual values versus fitted values and quantile-quantile residual plots of each model.

For all responses, we examined alternate model structures that included covariates (e.g., soil, climate, or plant variables) instead of site identity as main effects along with nutrient treatments (data not shown). None of the available covariates provided as much or more explanatory power than site. Furthermore, the covariate models did not describe the site by nutrient response interactions when they occurred. Consequently, for all models we included site as a fixed effect to account for known (e.g., climatic, pedologic, and plant community) differences among the study sites. Block was included as a random effect (nlme package in R).

For each model, we tested for site by nutrient interactions, and included them in the final model structure when significant. When there was a significant interaction, we used the lsmeans package in R to perform post-hoc comparisons; p-values were Bonferroni corrected for multiple comparisons. Both marginal and conditional R^2 – or the variance explained by fixed effects only versus both fixed and random effects – were calculated for each model using the MuMIn package in R (Nakagawa and Schielzeth 2013). All analyses were performed in R (R version 3.0.1; R Foundation for Statistical Computing 2013).

For the samples collected from the full nutrient factorial (N x P x K plots), the N treatment dominated the observed responses (see *Results* below) and the +NP, +NK, and +NPK plots enhanced the N response. Consequently, we report significant interactions with P and K below when they occurred, but we focus on the effects of N.

RESULTS

Unoccluded SOM response to N enrichment (H1)

We evaluated our long-term microbial respiration incubation against two decomposition models. Based on AIC, the two-pool decomposition model was the best fit for 85.6% of the samples (137/160) and the one- and two-pool models were indistinguishable (difference in AIC <3) for 14.4% of the samples (23/160). The one-pool model was never the best fit. Model fit for the two-pool model ranged from 0.54 – 0.99 R^2, with a mean R^2 value of 0.85 and median R^2 of 0.88. The effects of N addition on the two-pool parameter estimates are reported here.

On average, decomposition rates of the "fast" decomposing soil C pool (k_f) increased with N addition (ambient N average = 0.1008 day^{-1}; added N average = 0.1173 day^{-1}) and the fast pool MRT decreased with N addition (ambient N average = 9.92 days; added N average = 8.53 days), although the direction and magnitude of response varied by site (site by N interaction: p = 0.001; Figure 2.1a; see Appendix 2 – Table S2.3 for decomposition parameter ANOVA tables). Post-hoc tests revealed that N addition significantly increased k_f at the most northern and sandiest soil sites (Iowa, p = 0.0001; Minnesota, p = 0.03), had no significant effect on k_f in Kansas and Nebraska, and significantly decreased k_f in Colorado (p = 0.007). In response to N addition, there was a marginally significant decrease in the size of the fast pool (C_f) on average (ambient N average = 0.30 mg C g $soil^{-1}$; added N average = 0.23 mg C g $soil^{-1}$; N effect: p = 0.07; Figure 2.1b).

In contrast to the fast pool (k_f), the decomposition rate of the "slow" decomposing soil C pool (k_s) decreased with N addition on average (ambient N average = 2.95 x 10^{-4} day^{-1}; added N average = 2.63 x 10^{-4} day^{-1}) and the slow pool MRT increased with N addition on average (ambient N average = 9.30 years; added N average = 10.42 years). Responses to N addition varied among the sites, having either negative or neutral effects (site by N interaction: p = 0.03; Figure 2.1c). Nitrogen significantly decreased k_s in Nebraska (p=0.05), Kansas (p=0.03) and Colorado (p=0.003), but had no effect at the two sandiest sites (Iowa and Minnesota). The size of the slow pool (C_s) did not change significantly (N effect, p > 0.1; Figure 2.1d).

Nitrogen addition either significantly decreased or did not change cumulative C respired (site by N interaction: p = 0.02; Figure 2.1e). Post-hoc tests showed that N significantly decreased cumulative respiration in Nebraska by 23% (p=0.002) and Kansas by 29% (p=0.0001), but had no effect at the other three sites. The trend toward decreasing cumulative respired C in response to N addition at Nebraska and Kansas was the same whether evaluated on a per g soil or per g microbial C basis (i.e., metabolic quotient, qCO_2; data not shown). Microbial biomass C concentration (per mass soil and per mass soil C) did not change in response to N addition (p > 0.1; see Table 2.2 for microbial biomass data and Appendix 2 – Table S2.5 for microbial biomass ANOVA tables).

Aggregate-occluded and mineral-associated soil fraction responses to N enrichment (H2 and H3)

At the four coarse-textured sites (Colorado, Iowa, Minnesota, Nebraska), at least half of the total C content was associated with the middle aggregate size classes: small macro-aggregates (2000 – 250 μm) and micro-aggregates (250 – 53 μm; Figure 2.2). In contrast, at the site with the greatest soil clay content (Kansas), more than 50% of total soil C was in the large macro-aggregate fraction (> 2000 μm). On average, N addition increased the proportion of C in large macro-aggregates (ambient N average: 0.16 g macro-aggregate C g soil C^{-1}; added N average: 0.18 g macro-aggregate C g soil C^{-1}), but the difference was only marginally significant (N effect: $p = 0.07$). None of the other aggregate fractions or the mineral-associated fraction had statistically significant N effects ($p > 0.1$; see Appendix 2 – Table S2.4 for aggregate fraction ANOVA tables).

To assess mechanisms that could lead to the N responses in the aggregate-occluded and mineral-associated fractions, we also evaluated root and mycorrhizal abundance (Table 2.3), as well as soil pH (Table 2.2). N addition had no significant effect on root biomass ($p > 0.1$; see Appendix 2 – Table S2.6 for root variable ANOVA tables). However, N addition caused a marginally significant increase in average percent root colonization by mycorrhizae (ambient N average: 50.96%; added N average: 59.44%; N effect: $p = 0.052$), as well as a statistically significant increase in the absolute root biomass colonized by mycorrhizae (ambient N average: 4.72 mg cm^{-3}; added N average: 4.92 mg cm^{-3}; N effect: $p = 0.05$). Finally, average soil pH decreased with N addition (ambient N average: 6.0; added N average: 5.8), especially in the presence of either P or K (N by P interaction: $p = 0.02$; N by K interaction: $p = 0.04$; see Appendix 2 – Table S2.5 for pH ANOVA table).

Additional soil variables

In addition to evaluating multiple SOM pools, we measured variables that may influence site responses to N addition (Table 2.2). Particulate organic matter C concentration increased significantly in response to N, but only in P addition plots (N by P interaction: $p = 0.008$; ambient N/added P = 3.20 mg POM C g $soil^{-1}$, added N/added P = 4.48 mg POM C g $soil^{-1}$). Particulate organic matter N concentration

increased significantly with N addition (N main effect: $p = 0.001$; ambient N average: 0.32 mg N g soil^{-1}; added N average: 0.38 mg N g soil^{-1}). The POM C:N ratio also declined significantly with N, but only under ambient P (N by P interaction: $p = 0.009$; ambient N/ambient P = 11.51, added N/ambient P = 9.73). Nitrogen addition significantly increased net N mineralization rates at three sites (site by N interaction: $p < 0.0001$): Iowa ($p = 0.004$), Minnesota ($p < 0.0001$), and Kansas ($p < 0.0001$). Finally, there was no statistically significant effect of N on total soil C concentration, but on average, N addition increased total soil N concentration by 6% (N effect: $p = 0.04$) and decreased the soil C:N ratio by 3% (N effect: $p = 0.0008$) relative to control plots (see Appendix 2 – Table S2.5 for ancillary soil variable ANOVA tables).

DISCUSSION

Overall, we confirmed that N enrichment affects SOM pools with variable MRTs differentially, but our pool-specific hypotheses were only partially supported. In the unoccluded SOM pools, our results supported our hypothesis: on average, N enrichment increased the decomposition rate of the most quickly decomposing unoccluded pool ("fast" decomposing soil C pool) and decreased the decomposition rate of the more slowly decomposing unoccluded pool ("slow" decomposing soil C pool) (average fast pool MRT decreased from 9.92 to 8.53 days, average slow pool MRT increased from 9.30 to 10.42 years with N addition). Additionally, on average cumulative C respired decreased with N addition. By contrast, in the aggregate-occluded and mineral-associated SOM pools, data did not support our hypotheses: the proportion of C occluded within the largest aggregate fraction (> 2000 μm) actually *increased* slightly with N addition and there were no effects of N addition on the mineral-associated soil fraction. Despite some agreement with our hypotheses, the effects of N on multiple SOM pools were not consistent across the five grassland sites studied. Our results suggest that, while C sequestration could increase in response to N, the effect is not universal across grasslands.

Nitrogen enrichment changed the decomposition rates of unoccluded SOM

We found partial support for our first hypothesis (H1): in line with our prediction, N enrichment increased the decomposition rate (k_f) and decreased the pool size (C_f) of the "fast" decomposing soil C in the unoccluded SOM pool at the more northern, sandy soil sites (Iowa and Minnesota). Additionally, the "slow" decomposing soil C in the unoccluded pool had a slower decomposition rate (k_s) under N enrichment at the more southern sites that did not exhibit fast pool responses (Colorado, Kansas, and Nebraska). Furthermore, N enrichment decreased cumulative C respired in Kansas and Nebraska. Overall, these divergent effects of N on more quickly versus more slowly cycling soil C are in accordance with patterns observed in leaf litter decomposition (Berg and Matzner 1997; Hobbie et al. 2012) and predicted from models of microbial activity following N addition (Ågren et al. 2001; Schimel and Weintraub 2003; Moorhead and Sinsabaugh 2006). A number of non-exclusive mechanisms have been proposed to explain increased decomposition of rapidly cycling C and decreased decomposition slowly cycling C in response to N addition. Next we evaluate these mechanisms in light of explaining our pool- and site-specific responses.

In response to N enrichment, the decomposition of the "fast" decomposing C in the unoccluded pool could increase due to increasing N concentration of plant tissue inputs to this pool. Substrates with a low C:N ratio will decompose faster than substrates with higher C:N ratio, at least in the early stages of decomposition (Cornwell et al. 2008). Studies in grasslands have shown that litter N content increases with increasing soil N availability (Wedin and Tilman 1996). Nitrogen addition can also alter plant input chemistry by increasing the abundance of species with high N content tissue (Henry et al. 2004). Although we did not measure plant input chemistry, under N enrichment we observed increased POM N concentration, decreased POM C:N ratio, and increased N mineralization rates, all of which suggest that the N content of substrate inputs increased with N addition. Interestingly, N addition significantly increased N mineralization rates at three sites, two of which also had significantly higher decay rates in the "fast" decomposing soil C pool.

Additionally, N addition could increase the decay rate of the "fast" decomposing C in the unoccluded pool through N effects on the microbial community. Previous research has shown that the

production of hydrolytic microbial enzymes, including cellulases that degrade labile organic matter, are stimulated by N addition (Carreiro et al. 2000; Sinsabaugh et al. 2002; Talbot and Treseder 2011; Hobbie et al. 2012) and lead to increased decomposition (Sinsabaugh and Moorhead 1994). Examination of microbial enzyme production, as well as the site-level factors that affect their production (e.g., the availability of non-N nutrient enzyme co-factors), would be needed to assess the relevance of this hypothesis in light of the site by N interaction we found in the response of rapidly cycling unoccluded C.

On the other hand, N addition may decrease the decomposition of the "slow" decomposing C in the unoccluded pool through N effects on microbial growth efficiency. Models (Schimel and Weintraub 2003) and empirical studies (Thiet et al. 2006) have shown N addition can cause more C to be allocated to microbial growth (assuming N-limited growth) instead of lost via respiration and extracellular enzymes, leading to increased microbial efficiency and decreased respiration. We did find that N decreased cumulative C respired per unit microbial biomass C (i.e., the metabolic quotient, qCO_2), and that this decline was significant at two of the three sites where N also decreased the decay rate of the slow pool. This result is consistent with a recent meta-analysis which found a significant negative relationship between microbial respiration per unit microbial biomass and litter N concentration (Spohn 2015). Although we only evaluated the microbial biomass C pool at the beginning of the laboratory incubation, this finding does suggest that changing microbial allocation to growth versus respiration may be occurring at some sites.

Additionally, N effects on microbial enzyme activity could explain the decrease in decay rates of the "slow" decomposing C pool with N addition. The production of oxidative enzymes – which decompose more complex C substrates such as lignin – can be inhibited by N addition (Fog 1988), Previous research in forest ecosystems has demonstrated that N addition leads to decreased oxidative enzyme activity and increased soil C sequestration (Waldrop et al. 2004). However, there is some evidence to suggest that oxidative enzymes are not significantly inhibited by N addition in grasslands (Keeler et al. 2008; Sinsabaugh 2010), where plant lignin content is relatively low compared to forests. Alternately, lignin may be degraded as a mechanism of N acquisition, or "N mining." Consequently, N enrichment could suppress

N mining and, consequently, decrease lignin (and slow pool) degradation (Craine et al. 2007). However, it is unclear to what extent N mining occurs in ecosystems (Spohn 2015).

Overall, we have scant evidence to explain the site by N interactions we observed for the divergent N responses of unoccluded SOM pools with "fast" versus "slow" decomposing C among our sites. Although the sites that exhibited positive N effects on fast pool decay are the sandiest, the climate and vegetation characteristics of these sites also differ in comparison with the other sites studied (Table 2.1). Further research on site differences, along with measurements of the candidate mechanisms described above, would inform site-specific responses.

Aggregate-occluded SOM increased slightly with N enrichment

Contrary to our expectation that N addition would decrease C occluded within soil aggregates (H2), we found a trend of increasing proportion of C in the largest aggregate fraction (> 2000 μm). The effect of N on macro-aggregate C was, however, consistent with the root responses we observed: we found that root biomass also increased with N addition on average (but not significantly). Although theory predicts that plant proportional allocation belowground should decrease as belowground resources (such as N) increase, previous studies have observed greater absolute root biomass under N addition even if the relative root:shoot ratio decreases (e.g., Fornara and Tilman 2012). It is possible soil texture interacts with N addition to evoke this response; within sites with coarse-textured soil (Minnesota, Iowa, Nebraska, and Colorado), root biomass increased slightly with N addition and at three of these sites (Minnesota, Iowa, and Nebraska), macro-aggregate C increased (although neither response was significant). In contrast, within the most fine-textured soil (Kansas), root biomass was 25% lower in N addition plots than control (Table 2.3). In light of the role of roots in aggregate formation (Oades and Waters 1991; Jastrow et al. 1998), the root biomass response to N enrichment, particularly in the coarse-textured sites, may have contributed to increased formation of macro-aggregates and occlusion of C. Future studies replicated across soil texture types are required to support and understand this potential interaction.

Alternately, N enrichment could have increased C content of the large macro-aggregate fraction via another aggregate-formation agent, such as fungal biomass (Guggenberger et al. 1999; Six et al. 2006; Strickland and Rousk 2010). Although we did not measure fungal biomass in this study, we did find that arbuscular mycorrhizal colonization of roots (both absolute mass abundance and percent colonized) increased with N addition. This was surprising, since a meta-analysis by Treseder (2004) found that colonization of both ecto- and arbuscular mycorrizal fungi on roots tends to decrease in response to N addition, although this was a weak relationship when data were limited to colonization rates only. Furthermore, a recent meta-analysis by Li and colleagues (2015), reported that the inhibitory effects of N on mycorrhizal colonization was strongest in forests, and insignificant in grasslands. Increased arbusucular mycorrhizal fungi colonization of roots may lead to increased large macro-aggregate C concentration through physical enmeshment of soil particle with organic matter and/or direct inputs of hyphae and exudates, such as glomalin (Rillig 2004), into aggregates.

The large macro-aggregate fraction is relatively dynamic (Yang and Wander 1998; Plante and McGill 2002) and C occluded in large macro-aggregates is not stabilized against microbial decomposition. However, this fraction has the potential to influence C sequestration on the centennial time scale and beyond via interactions with smaller aggregate fractions. Large macro-aggregates can facilitate aggregation (and stabilization) of smaller occluded fractions that cycle much more slowly (Oades 1984; Six et al. 2004). Consequently, even small increases to this aggregate fraction could lead to greater aggregate and mineral occlusion of SOM in the future, along with greater C sequestration.

Mineral-associated SOM was not changed with N enrichment

We expected N enrichment to increase mineral surface-associated C (H3), however, we observed no effect of N enrichment on mineral-associated C despite a 3% decrease in pH in N addition plots relative to control plots. Lack of N effects on the mineral-associated pool could have been caused by several factors. First, four of the five study sites had very coarse-textured soil with little silt and clay (Kansas average % silt and clay particles = 68.1%; all other sites ranged from 9.9% - 28.7% silt and clay); therefore,

potential for OM to associate with mineral surfaces in these soils is very small and detecting treatment differences in this already very small pool may be difficult. Second, the mineral-associated fraction (<53 μm) isolated during wet sieving, is itself a heterogeneous mixture of clay micro-structures and silt-sized particles, which may not uniformly associate with C. Furthermore, the site with the highest percentage clay (Kansas) had the largest amount of C occluded in macro-aggregates (and other smaller fractions therein). If there were N enrichment effects on the mineral-associated fraction at this site, they may have been undetectable as they were occluded within larger aggregates. The duration of N addition (5 years in Colorado, Kansas, Minnesota, and Nebraska; 3 years in Iowa) may not have been sufficient to detect N effects in this fraction, despite observable decreases in soil pH. For example, Gillespie et al. (2014), found that N enrichment altered the chemistry of SOM in the fine (< 5 μm) fraction following 17 years of N addition. Further investigation into the effects of N enrichment on mineral-associated OM (and the timescale of this effect) is warranted.

Conclusion

Our findings – specifically, the decrease in decomposition rate of "slow" pool unoccluded SOM, decreased cumulative C respired, and trend toward increased aggregation after only 3-5 years of nutrient addition – suggest that N enrichment will lead to increased sequestration of soil C in grassland soils, although unexplained site-to-site variation indicates that this effect may not be universal. Our decomposition results are consistent with findings from forest systems where soil C has increased under N enrichment due to suppressed microbial decomposition (e.g., Zak et al. 2008; Frey et al. 2014). In contrast to previous studies, however, we also examined mechanisms of stabilization that could sequester C beyond the decadal scale (e.g., via aggregate occlusion and mineral association): in addition to slowing microbial decomposition, N addition may increase soil C storage at grassland sites through increased C occlusion in large macro-aggregates. This could eventually lead to C accumulation in the mineral-associated SOM fractions (MRT centuries to millennia) due to the cascading effects of increased aggregation on smaller aggregate fractions. Finally, site-specific factors will influence how N affects the decomposition rates and

sizes of multiple SOM pools. Unsurprisingly, but importantly, we observed significant site-by-site variation in response to N addition in both the unoccluded and occluded SOM pools we measured. Soil texture appeared to play a significant role in these interactions; however, we caution against over-interpreting this effect since texture co-varied strongly with climatic and biotic variables among our study sites. In the future, additional multi-study sites that investigate the effects of N while varying a key state factor that influences SOM stabilization (e.g., soil parent material) will inform our understanding of soil C sequestration across ecosystems.

Table 2.1 – Characteristics of the five Nutrient Network experimental sites.

Site characteristic	Cedar Creek, Minnesota	Cedar Point, Nebraska	Chichaqua Bottoms, Iowa	Konza Prairie, Kansas	Shortgrass Steppe, Colorado
MAT (°C) [a]	6.3	9.3	9	12	8.4
MAP (mm) [a]	750	454	855	872	364
N deposition rate (kg N ha^{-1} yr^{-1}) [b]	7.0	3.1	18.0	9.8	3.1
Elevation (m)	270	965	275	440	1650
Plant biomass ($g\ m^{-2}$) [c]	217.6 (15.9)	361.5 (36.9)	464.1 (39.6)	265.2 (19.0)	42.5 (3.7)
Habitat	Tallgrass prairie	Shortgrass prairie	Tallgrass prairie	Tallgrass prairie	Shortgrass prairie
Soil texture [d]					
Sand %	90.1 (0.1)	71.4 (0.5)	87.5 (0.4)	31.9 (0.8)	71.3 (0.2)
Silt %	5.6 (0.2)	18.1 (0.7)	7.4 (0.4)	49.8 (1.2)	15.1 (0.2)
Clay %	4.3 (0.1)	10.5 (0.5)	5.1 (0.2)	18.3 (0.4)	13.6 (0.4)
Soil bulk density (g dry soil cm^{-3}) [e]	1.22 (0.05)	1.58 (0.04)	1.08 (0.03)	1.52 (0.06)	1.17 (0.06)
Duration of nutrient addition treatments (yrs)	5	5	3	5	5

[a] Mean annual temperature (MAT) and mean annual precipitation (MAP) are from the WorldClim database (Hijmans et al. 2005).

[b] Modeled N deposition rates are from the Oak Ridge National Laboratory Distributed Active Archive Center (Dentener 2006).

[c] Site mean (standard error in parentheses) plant aboveground biomass sampled in 2012; sampling methods in Borer et al. (2014); data from the Nutrient Network.

[d] Site mean (standard error in parentheses) soil texture sampled in 2012 (this study) and measured using the hydrometer method (Ashworth et al. 2001). One plot per block sampled at each site (Minnesota, n = 5; Nebraska, n = 3; Iowa, n = 6; Kansas, n = 3; Colorado, n = 3).

[e] Site mean (standard error in parentheses) soil bulk density sampled in 2012 (this study). One core per block sampled at each site (Minnesota, n = 5; Nebraska, n = 3; Iowa, n = 6; Kansas, n = 3; Colorado, n = 3).

Table 2.2 –Effects of nitrogen addition on soil variables.

	Overall (across sites)		Cedar Creek, Minnesota		Cedar Point, Nebraska		Chichaqua Bottoms, Iowa		Konza Prairie, Kansas		Shortgrass Steppe, Colorado	
Analysis	Ambient N [a]	Added N [a]	Ambient N [a]	Added N [a]	Ambient N [a]	Added N [a]	Ambient N [a]	Added N [a]	Ambient N [a]	Added N [a]	Ambient N [a]	Added N [a]
Total soil C (mg C g soil^{-1})	15.82 (1.32)	16.33 (1.27)	12.51 (1.03)	15.50 (2.04)	16.34 (1.26)	14.71 (1.37)	8.28 (0.34)	8.93 (0.39)	41.49 (0.98)	39.45 (1.20)	9.95 (0.55)	11.02 (0.49)
Total soil N (mg N g soil^{-1})	1.20 (0.10)	1.28 (0.09)	0.83 (0.07)	1.08 (0.13)	1.35 (0.10)	1.24 (0.11)	0.67 (0.02)	0.73 (0.03)	3.05 (0.08)	3.02 (0.10)	0.87 (0.04)	1.00 (0.04)
Total soil C:N ratio	12.99 (0.17)	12.56 (0.15)	15.08 (0.26)	14.00 (0.27)	12.10 (0.18)	11.90 (0.22)	12.25 (0.13)	12.16 (0.15)	13.62 (0.14)	13.08 (0.14)	11.42 (0.26)	11.09 (0.24)
Microbial C (µg C g soil^{-1})	264.50 (18.46)	254.41 (15.18)	255.21 (17.27)	271.45 (22.76)	305.87 (25.63)	252.57 (26.96)	148.80 (6.33)	180.45 (15.79)	581.31 (34.16)	490.44 (18.84)	152.44 (16.41)	139.76 (10.62)
Microbial N (µg N g soil^{-1})	44.50 (3.17)	52.29 (5.03)	43.87 (2.99)	54.27 (4.92)	43.49 (3.63)	42.24 (4.93)	25.56 (0.93)	50.40 (14.09)	102.60 (4.81)	91.91 (4.76)	26.39 (1.66)	22.37 (2.24)
Microbial C:N ratio	5.99 (0.11)	5.65 (1.68)	5.88 (0.14)	5.10 (0.12)	7.13 (0.31)	6.23 (0.31)	5.85 (0.20)	5.26 (0.37)	5.66 (0.18)	5.45 (0.27)	5.63 (0.28)	6.97 (0.79)
POM C (mg C g soil^{-1}) [b]	3.52 (0.32)	4.17 (3.07)	3.94 (0.91)	5.11 (0.96)	4.97 (0.65)	5.77 (0.93)	1.71 (0.24)	1.90 (0.19)	6.08 (0.58)	5.81 (0.30)	2.48 (0.53)	3.90 (0.51)
POM N (mg N g soil^{-1}) [b]	0.32 (0.02)	0.38 (0.02)	0.31 (0.07)	0.43 (0.05)	0.49 (0.05)	0.54 (0.06)	0.20 (0.02)	0.23 (0.02)	0.41 (0.04)	0.45 (0.03)	0.30 (0.03)	0.39 (0.03)
POM C:N ratio [b]	10.30 (0.58)	10.09 (0.42)	11.64 (1.15)	10.40 (0.99)	9.87 (0.49)	10.10 (0.87)	8.10 (0.78)	8.37 (0.75)	15.71 (1.75)	13.22 (0.78)	7.60 (1.32)	9.88 (1.49)
Soil pH	6.0 (0.1)	5.8 (0.1)	5.5 (0.0)	5.2 (0.0)	6.4 (0.1)	6.2 (0.1)	6.4 (0.1)	6.3 (0.1)	6.2 (0.1)	6.1 (0.1)	5.7 (0.1)	5.4 (0.1)
Net N mineralization (mg	2.65 E-4 (3.05 E-	7.34 E-4 (7.73 E-	8.08 E-5 (2.27 E-	7.10 E-4 (7.80 E-	7.10 E-4 (6.29 E-	1.11 E-3 (7.74 E-	1.47 E-4 (1.32 E-	4.42 E-4 (1.66 E-	7.13 E-5 (1.89 E-	5.61 E-4 (1.03 E-	5.42 E-4 (3.14 E-	1.16 E-3 (2.99 E-

N g soil^{-1} day^{-1})	5)	5)	5)	5)	5)	5)	5)	4)	5)	4)	5)	4)

Values are mean (and standard error in parentheses).

[a] Treatment codes: ambient N includes all plots where N was not added (control, +P, +K, +PK plots); added N includes all N addition plots (+N, +NP, +NK, +NPK plots). See Appendix 2 – Table S2.2 for sample numbers.

[b] POM: particulate organic matter.

Table 2.3 – Effects of nitrogen addition on root variables.

	Overall (across sites)		Cedar Creek, Minnesota		Cedar Point, Nebraska		Chichaqua Bottoms, Iowa		Konza Prairie, Kansas		Shortgrass Steppe, Colorado	
Analysis	Control [a]	+N [a]	Control [a]	+N [a]	Control [a]	+N [a]	Control [a]	+N [a]	Control [a]	+N [a]	Control [a]	+N [a]
Root biomass (mg cm^{-3})	9.26 (1.55)	10.11 (1.27)	7.01 (0.46)	8.85 (0.85)	15.98 (1.89)	16.51 (3.89)	3.72 (0.38)	7.10 (2.43)	21.27 (2.77)	15.67 (1.23)	5.37 (1.20)	6.27 (0.50)
Root biomass colonized by mycorrhizae (absolute; mg cm^{-3})	4.72 (0.80)	4.92 (0.61)	4.49 (0.20)	4.99 (0.36)	7.87 (1.06)	NA	1.85 (0.24)	2.77 (0.49)	8.27 (2.71)	9.01 (0.99)	3.59 (0.96)	4.30 (0.51)
Root biomass colonized by mycorrhizae (percentage; %)	50.96 (2.93)	59.44 (1.84)	65.52 (4.67)	57.50 (3.69)	49.07 (0.81)	NA	50.03 (4.10)	57.40 (2.15)	37.39 (7.98)	57.33 (3.93)	55.05 (5.93)	68.17 (3.49)

Values are mean (and standard error in parentheses).

[a] Only Control and +N plots were analyzed. See Appendix 2 – Table S2.2 for sample numbers.

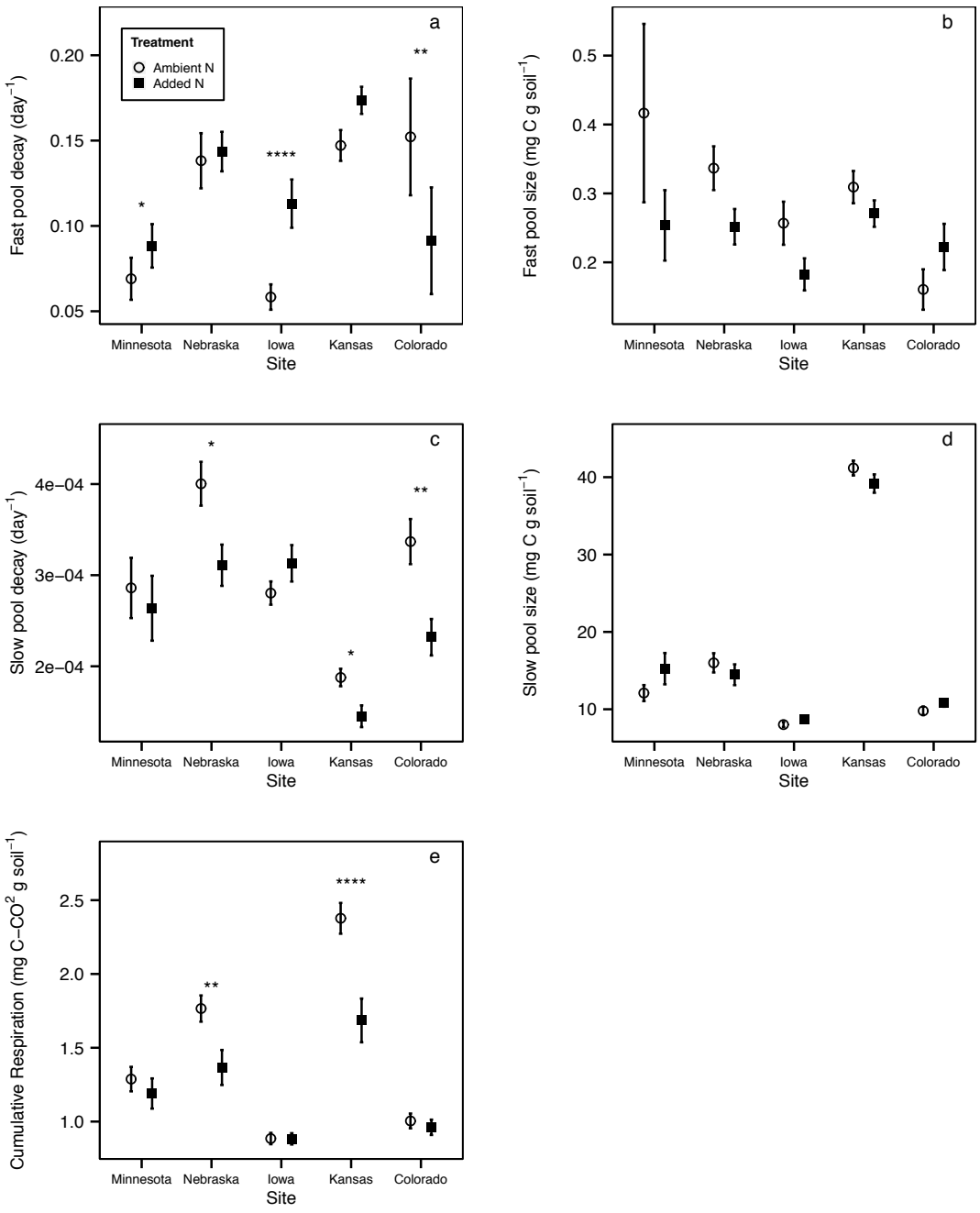

Figure 2.1 – Effect of nitrogen addition on decay rate (a) and size (b) of the "fast" decomposing C pool, decay rate (c) and size (d) of the "slow" decomposing C pool, and cumulative C respired (e) measured with a long-term microbial respiration incubation. All panels show mean plus/minus one standard error. Stars indicate significance from post-hoc pairwise comparisons: * $p \leq 0.05$, ** $p \leq 0.01$, ***

$p \leq 0.001$, **** $p \leq 0.0001$. Treatment codes are the same across all panels. Treatment codes: ambient N (open circles) include all plots where N was not added (control, +P, +K, +PK plots); added N (closed squares) include all N addition plots (+N, +NP, +NK, +NPK). See Appendix 2 – Table S2.2 for sample numbers.

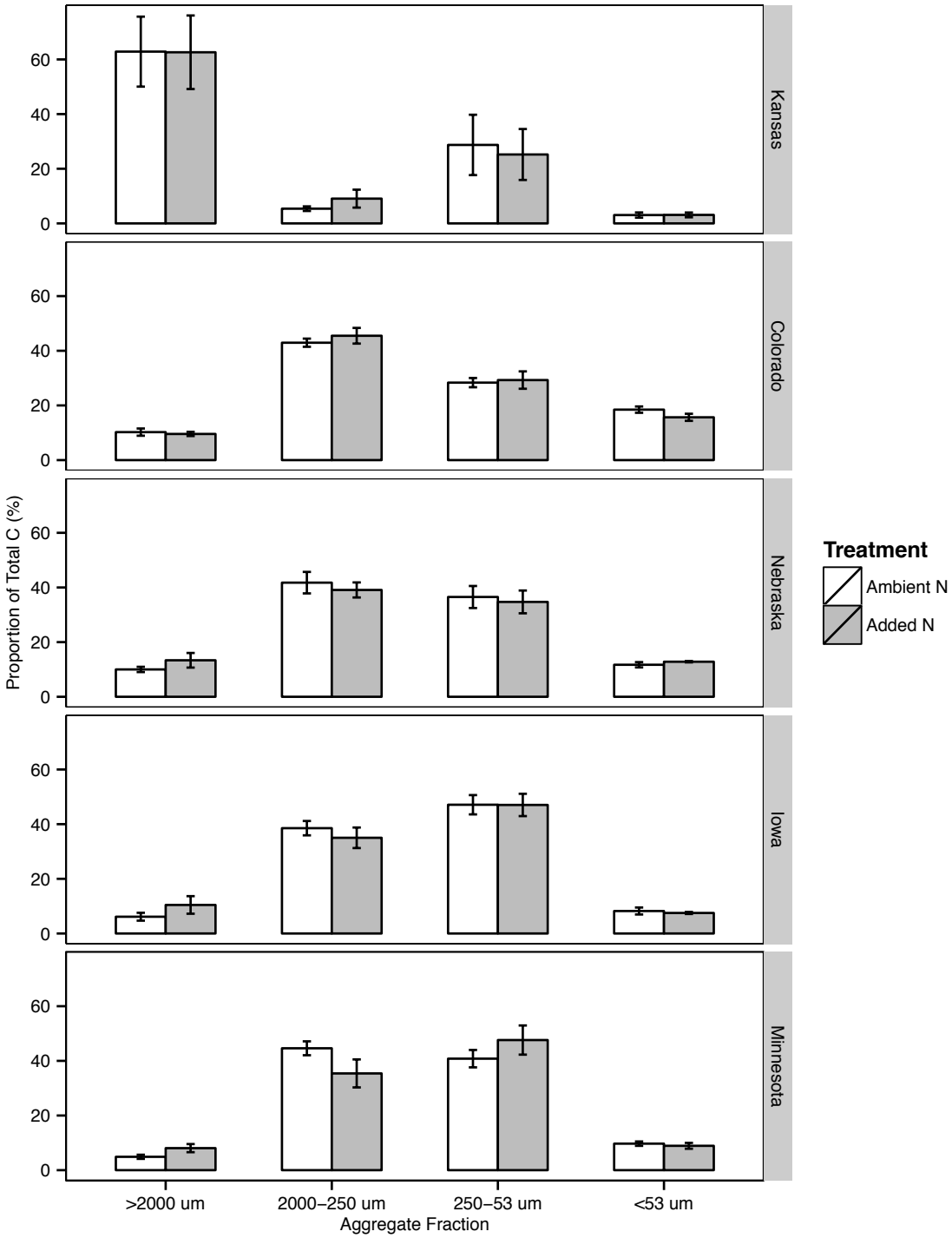

Figure 2.2 – Effects of nitrogen addition on soil carbon concentration (as % of total soil C) across soil aggregate size classes: large macro-aggregates (> 2000 µm), small macro-aggregates (2000 – 250 µm),

micro-aggregates (250 – 53 µm), and mineral-associated C (<53 µm). Figure shows mean plus/minus one standard error. Treatment codes: ambient N (white) = control plots; added N (gray) = +N plots. Sites are ordered from high to low clay content. See Appendix 2 – Table S2.2 for sample numbers. See Appendix 2 – Table S2.7 for additional metrics of soil aggregation.

CHAPTER 3

Mechanisms driving the soil organic matter decomposition response to nitrogen enrichment in grassland soils

Empirical studies show that nitrogen (N) addition often reduces microbial decomposition of soil organic matter (SOM) and microbial respiration of CO_2. Although predictions from theoretical models support these findings, the mechanisms that drive this response remain uncertain. To address this uncertainty, we sampled three grassland sites in the U.S. Central Great Plains that have each received seven years of continuous nutrient addition in the field. Nitrogen addition significantly decreased the decomposition rate of soil organic matter and the cumulative carbon (C) respired per mass soil C. We evaluated whether this effect of N addition on microbial respiration resulted from: 1) increasing microbial carbon use efficiency (CUE), 2) decreasing microbial oxidative enzyme activity, or 3) decreasing microbial biomass due to plant and/or soil mediated responses to N enrichment. However, in contrast to our hypotheses – as well as results from N addition studies in forest ecosystems – N did not increase microbial CUE or decrease microbial oxidative enzyme activity. Instead, the inhibitory effects of N on respiration were best explained by decreased microbial biomass in response to N addition. Identifying what factors drive this decreased microbial biomass response remains a question for further inquiry.

INTRODUCTION

The increased availability of biologically reactive nitrogen (N) has widespread effects on terrestrial ecosystems; N enrichment can lead to biodiversity loss, soil acidification, as well as stimulated plant growth (Vitousek et al. 1997; Suding et al. 2005; LeBauer and Treseder 2008). However, interactions between the carbon (C) cycle and nutrient cycles (such as N) are poorly understood (Ciais et al. 2013) and the extent to which increasing nutrients may feed back and influence the global C cycle remain uncertain (Wieder et al. 2015). This uncertainty is especially important to resolve for the decomposition of soil organic matter (SOM) by soil microorganisms, a process that releases C to the atmosphere as carbon dioxide (CO_2). Soil organic matter contains a significant reservoir of organic C and changes in decomposition rate in response to N enrichment will impact the net CO_2 exchange between the atmosphere and biosphere.

The effects of N enrichment on microbial decomposition have received considerable research attention to date. Studies of leaf litter (Berg and Matzner 1997; Hobbie et al. 2012), forest soil (Janssens et al. 2010), and grassland soil (Chapter 2) have found that N enrichment sometimes, but not always, decreases microbial decomposition and respiration. Although these results are in accordance with predictions from models of microbial activity following N addition (Ågren et al., 2001; Moorhead and Sinsabaugh, 2006), the biological and chemical mechanisms that underlie the response of SOM decomposition to added nutrients remain uncertain. Are the inhibitory effects of N on decomposition due to plant, microbial, or soil chemistry-mediated changes that occur in response to N addition? Identifying the mechanisms that control the decomposition response to N is key for elucidating how N enrichment will influence soil C sequestration, soil CO_2 emissions, and the global C cycle.

A number of mechanisms have been proposed to explain why microbial respiration decreases in response to N addition (Figure 3.1). Microbial respiration associated with organic matter decomposition is influenced by decomposer carbon use efficiency (CUE; C allocation to anabolism (e.g., microbial growth) or catabolism (e.g., decomposition); Figure 3.1, Mechanism 1), the enzyme activity of those decomposers (Figure 3.1, Mechanism 2), the chemistry of the substrate being oxidized (Figure 3.1, Mechanism 3), as well as the biomass of decomposing microorganisms (Figure 3.1, Mechanisms 2-4; Cornwell et al. 2008;

Burns et al. 2013). Nitrogen addition can alter microbial community composition and functioning, plant community composition and functioning, as well as soil chemistry. Each of these ecosystem components can, in turn, influence microbial respiration of soil C via their effects on microbial CUE and enzyme activity (Figure 3.1, Mechanisms 1 and 2), substrate quality (Figure 3.1, Mechanism 3), and the amount of C available to microbes (Figure 3.1, Mechanism 4).

First, N might decrease microbial respiration by increasing microbial CUE (Figure 3.1, Mechanism 1; Ågren et al. 2001; Schimel and Weintraub 2003). Once microbes acquire C, N addition can alter the allocation of that C to new biomass, enzymes, or maintenance respiration. For example, Schimel and Weintraub (2003) constructed a microbial decomposition model that accounted for microbial growth, enzyme production, and maintenance respiration. They predicted that N addition causes more C to be allocated to microbial growth (assuming N-limited growth) instead of lost via respiration and extracellular enzymes, leading to increased microbial efficiency. Furthermore, Schimel and Weintraub's model predicted that this should result in reduced respiration following N addition. A few empirical studies have also suggested that microbial CUE increases with N addition (e.g., Thiet et al. 2006), although clear patterns across terrestrial N availability gradients or from N addition studies are lacking. For example, Manzoni et al. (2012) surveyed microbial CUE results from natural gradients of soil organic N and found that microbial CUE increased with increasing N concentration; however, the relationship between CUE and N availability was opposite (negative) when Manzoni et al. examined N addition studies.

Second, N addition might reduce decomposition because N directly inhibits oxidative enzymes, which decompose more complex C substrates such as lignin (Fog 1988; Figure 3.1, Mechanism 2). Researchers have suggested that lignin degradation may be inhibited by added N if lignin degradation is a mechanism of N acquisition or "N mining" (Craine et al. 2007). Many studies have demonstrated decreased activity of oxidative enzymes under N addition. For example, in a northern temperate forest study system, N decreased phenol oxidase activity (DeForest et al. 2004a; Waldrop et al. 2004), the abundance of functional genes involved in the depolymerization of a variety of complex C molecules (such as lignin; Eisenlord et al. 2013), as well as the expression of ligninolytic genes (Edwards et al. 2011). However, it is unclear to what extent these enzyme-based mechanisms that lead to decreased respiration hold true in non-

forest systems (Sinsabaugh 2010). The inhibitory effect of N on enzyme activity is not consistent across all sites and systems (e.g., Keeler et al. 2008).

Third, N addition could reduce microbial respiration by changing the chemistry and input rate of plant-derived organic matter substrates for decomposition (Figure 3.1, Mechanism 3). It is well known the N addition alters plant community composition, as well as plant tissue chemistry (Suding et al. 2005). We expect litter N content to increase with soil N supply (Xia and Wan 2008) and lead to faster decomposition rates and greater microbial respiration, especially during the early stages of decomposition (Berg and Matzner 1997). However, there could be additional changes to substrate chemistry that would lead to an increase in more complex (or "recalcitrant") C compounds in plant tissues, and consequently, slower decomposition rates and reduced microbial respiration. Roots and leaf litter have distinct C chemistry and decomposability; for example, Freschet et al. (2012) found that fine roots decompose more slowly compared to leaf litter. Since N addition can increase fine-root production and the absolute amount of root biomass inputs (Adair et al. 2009; Yuan and Chen 2012), N addition could increase the overall inputs of complex C and lead to decreased decomposition and microbial respiration.

Finally, N addition may reduce respiration due to the effects of changing soil chemistry on microbial biomass, microbial enzyme activity, and/or microbial access to C (Figure 3.1, Mechanism 4). Nitrogen addition leads to soil acidification (decreasing pH), the loss of base cations (e.g., Mg^{2+} and Ca^{2+}), and increased solubility of hydrolyzing cations (e.g., Al^{3+} and Fe^{3+}; Tian and Niu 2015). Positive relationships between soil pH and microbial biomass are well established (Wardle 1992; Aciego Pietri and Brookes 2008) and microbial biomass may be lower in more acidic soils due to the biologically toxic effects of increased Al^{3+} (Flis et al. 1993). Alternately, low pH soils may inhibit microbial extracellular enzyme activity (Sinsabaugh et al. 2008), leading to decreased microbial access to C and reduced respiration. Finally, the increased solubility of hydrolyzing cations at low pH can increase the stabilization of C in organic matter-metal complexes that are inaccessible to microbes, and lead to decreased C availability to microbes and, subsequently, reduced decomposition (Scheel et al. 2008; Mueller et al. 2012).

Our objective was to evaluate the mechanisms by which N addition leads to decreased microbial respiration of SOM. In particular, we examined whether N addition caused decreased microbial respiration by 1) increasing microbial CUE, 2) decreasing microbial oxidative enzyme activity, or 3) decreasing

microbial biomass due to plant and/or soil mediated responses to N enrichment. To address our hypotheses, we re-sampled soils from a long-term nutrient addition study where we previously observed inhibitory effects of N on microbial decomposition and respiration (Chapter 2).

MATERIAL AND METHODS

Soil sampling and processing

In August 2014, we collected soil samples from three multi-factorial nutrient addition experiments in the U.S. Central Great Plains (Table 3.1): Cedar Point Biological Station (Ogallala, Nebraska; 41.2°, -101.63°); Konza Prairie Biological Station (Manhattan, Kansas; 39.07°, -95.58°); and Shortgrass Steppe (Nunn, Colorado; 40.82°, -104.77°). These three sites were previously sampled in 2012 when we analyzed the effects of N on SOM pool sizes and cycling (reported in Chapter 2). The experiments are participatory sites of the Nutrient Network (NutNet), a global network of nutrient addition and herbivore exclosure experiments (Borer et al. 2014).

At each site, nutrient additions of nitrogen (N), phosphorus (P), and potassium plus micronutrients (K), were replicated across three blocks in a full factorial design. At the three sites examined in this study, N, P, and K have been applied annually (10 g m^{-2} yr^{-1}) since 2008; the micronutrient mix in the K plots was applied once in 2008. Nitrogen was applied as time-release urea [$(NH_2)_2CO$], P as triple-super phosphate [$Ca(H_2PO_4)_2$], and K as potassium sulfate [K_2SO_4]. The micronutrient mix included Fe (15%), S (14%), Mg (1.5%), Mn (2.5%), Cu (1%), Zn (1%), B (0.2%), and Mo (0.05%), applied as iron sulfate, calcium magnesium carbonate (dolomite), manganese sulfate, copper sulfate penthydrate, zinc sulfate anhydrous, sodium borate, and sodium molybdate. We refer to the treatments as “N”, “P”, and “K” throughout, but acknowledge that they included the addition of other nutrients (e.g., Ca in addition to P in the case of the P treatment, and K plus micronutrients in the K treatment).

At each plot, six 0-10 cm cores (2 cm diameter) were sampled and kept on ice or in the refrigerator until processed in the lab. Within four days, soils samples from each plot were composited and sieved to 2 mm. Fresh, 2 mm sieved soil was subsampled for further analysis (see below). Air-dried, 2 mm sieved soil was used to measure total soil % C and % N by combustion (COSTECH ESC 4010 Elemental Analyzer, Valencia, CA, USA) and soil pH (1:1 soil:water slurry). Two plots in Nebraska contained inorganic C

(determined by acid pre-treatment) and were excluded from all statistical analyses. For the plots included in the analyses, total soil C is equivalent to total soil organic C. Three plots in Kansas did not contain sufficient sample to measure soil pH and were not included in the pH analysis. Fine root samples from a subsample of fresh soil were collected via flotation, along with roots captured on the 2 mm sieve; roots were cleaned of soil debris, dried, and analyzed for % C and % N by combustion (COSTECH ESC 4010 Elemental Analyzer, Valencia, CA, USA).

Microbial biomass C and N and dissolved organic C

Microbial biomass C and N were analyzed using a chloroform fumigation extraction procedure (Brookes et al. 1985). Briefly, fresh, 2 mm sieved soil was extracted with .05 M K_2SO_4 and filtered. A replicate soil sample was fumigated with chloroform in a vacuum, extracted with .05 M K_2SO_4 and filtered. Filtered extracts were analyzed for total organic C and total N (Shimadzu TOC-V, Shimadzu Corporation, Kyoto, Japan). Soil microbial biomass C (*MC*) and soil microbial biomass N (MN) were calculated as: $MC = EC/k_{EC}$ and $MN = EN/k_{EN}$, where *EC* is the difference between extractable C in the fumigated and unfumigated samples, *EN* is the difference between extractable N in the fumigated and unfumigated samples, k_{EC} is the C extraction efficiency coefficient, and k_{EN} is the N extraction efficiency coefficient. We used extraction efficiency coefficients of 0.45 (k_{EC}) and 0.54 (k_{EN}) from the literature (Brookes et al. 1985; Beck et al. 1997). Additionally, we used the extractable C measurement from the unfumigated sample as a measure of dissolved organic carbon (DOC) concentration in each sample.

Microbial respiration and decomposition parameters

We measured microbial respiration and decomposition rates during a long-term laboratory incubation. A 50 g subsample of fresh, 2 mm sieved soil from each plot was adjusted to 70% field capacity and stored at 20 °C in the dark. Field capacity was calculated separately for each site by pulling 20 KPa pressure on saturated soil. Microbial respiration rate (mg C-CO_2 g^{-1} soil day^{-1}) was calculated by measuring the accumulation of CO_2 in airtight mason jars during 24-48 hour intervals on days 1, 3, 6, 9, 12, 18, 24, 31, 38, 45, 60, 74, 96, 124, 152, 180, 208, and 236 of the incubation. An infrared gas analyzer (LICOR 7000)

was used to measure CO_2 concentration. One sample was contaminated during the long-term respiration incubation and excluded from the analysis.

We calculated cumulative C respired (mg C-CO_2 g^{-1} soil) on day 236 by averaging the respiration rate between adjacent measurement dates, multiplying the average by the interval between measurements, and then summing. Additionally, we estimated decay rate parameters by fitting our respiration rate data (Appendix 3 – Figure S3.1) to a two-pool decomposition model: $C_{rate}(t) = k_f (C_f e^{-k_f t}) + k_s [(C_t - C_f) e^{-k_s t}]$. $C_{rate}(t)$ is the daily respiration rate (mg C g^{-1} soil day^{-1}) at time t, t is time (days), k_f and C_f are the decomposition rate (day^{-1}) and size (mg C g^{-1} soil) of the "fast"-cycling soil C pool, k_s is the decomposition rate (day^{-1}) of the "slow"-cycling soil C pool, and C_t is total soil C (mg C g^{-1} soil). The size of the slow-cycling pool (C_s; mg C g^{-1} soil) is the difference between the total soil C pool and the fast-cycling soil C pool. We evaluated our data against both a one- and two-pool model and used the Akaike Information Criterion (AIC) as a measure of model fit. The two-pool model was always the best model fit (see *Results*), and consequently we fit our data to the two-pool model.

We used maximum-likelihood estimation (MLE) to fit the models from the respiration rate data and calculate the decay rate and pool size parameters of each sample (bbmle package in R). We evaluated model goodness-of-fit (R^2) by comparing respiration rates predicted from the parameter estimates against the actual respiration rate data. Finally, we also tested for and found no evidence of MLE parameter equifinality – or cases where multiple parameter combinations result in the equally good model fits (Beven 2006; see Appendix 3 – Supplement S3.1 and Figure S3.2 for equifinality evaluation methods and results).

Microbial carbon use efficiency – isotope addition experiment

To examine the effects of N addition and substrate chemistry on microbial CUE, we used a laboratory labeled substrate addition assay (Thiet et al. 2006). Six replicate subsamples from each plot were prepared (10 g fresh, 2 mm sieved soil samples) and brought to 70% field capacity with the addition of a C substrate solution (60 µg C g^{-1} dry soil). The following substrate solutions were added to two subsamples each: 1) a ^{13}C labeled glucose solution (D-Glucose-$^{13}C_6$ (49.5 atom % ^{13}C); Sigma Aldrich), 2) a ^{13}C labeled vanillin solution (Vanillin-(phenyl-$^{13}C_6$) (49.5 atom % ^{13}C); Sigma Aldrich), and 3) an unlabeled glucose solution. Soils were placed in airtight mason jars and respired CO_2 was sampled at 0 and 24 hours.

At both sampling times, 16 ml gas samples were collected by syringe and stored in 12 ml evacuated exetainers (Labco Limited, Lampeter, Wales, United Kingdom) for 17 weeks prior to analysis. CO_2, and its atom % ^{13}C, were measured by gas chromatograph-isotope ratio-mass spectrometer (University of California, Davis, Stable Isotope Lab). Additionally, ^{13}C incorporation into microbial biomass was measured using the chloroform fumigation method described above: one replicate subsample from each substrate solution treatment was extracted immediately and one was fumigated and then extracted. A subsample of each salt extract was analyzed for TOC (Shimadzu TOC-V, Shimadzu Corporation Kyoto, Japan). The remaining salt extract was dried for > 48 hours in a 60 °C forced-air drying oven and analyzed for atom % ^{13}C by elemental analyzer-isotope ratio-mass spectrometer (University of California, Davis, Stable Isotope Lab). Microbial biomass C was low for samples from Colorado and below the isotope detection limits; consequently the isotope measurements from Colorado were discarded from the analyses and we analyzed data from Kansas and Nebraska only.

Microbial CUE was calculated as: microbial biomass ^{13}C/(microbial biomass ^{13}C + respired ^{13}C). We determined microbial biomass ^{13}C and respired ^{13}C using the methods detailed in DeForest et al. (2004b). The amount of ^{13}C in microbial biomass was determined by multiplying the moles of C in microbial biomass by the atom percent excess (APE) ^{13}C microbial biomass, where APE ^{13}C microbial biomass equals the difference between the microbial biomass atom % ^{13}C in the isotope addition treatment (^{13}C glucose or ^{13}C vanillin) and the microbial biomass natural abundance atom % ^{13}C of the control treatment (unlabeled glucose addition). The amount of ^{13}C respired was calculated the same way: moles C respired x APE ^{13}C respired, where APE ^{13}C respired equals the difference between the respired atom % ^{13}C in the isotope addition treatment (^{13}C glucose or ^{13}C vanillin) and the respired natural abundance atom % ^{13}C of the control treatment (unlabeled glucose addition).

Microbial extracellular enzyme potential activity

To characterize the microbial enzyme response to N enrichment, we measured the potential activity of microbial extracellular enzymes using standard laboratory methods (German et al. 2011). The methods, which are widely utilized, use a fluorescent or color-linked substrate to measure the enzymatically depolymerized product either fluorometrically (for hydrolytic enzymes) or

spectrophotometrically (for oxidative enzymes) in controlled laboratory conditions (German et al. 2011). The activities of two oxidative enzymes (phenol oxidase [PO; EC 1.10.3.2] and peroxidase [PX; EC 1.11.1.7]) were measured colorimetrically on a spectrophotometer using the substrate L-3,4-dihydroxyphenylalanine (L-DOPA). Furthermore, the activities of six hydrolytic enzymes were assessed using 4-Methylumbelliferone (MUB)-linked substrates and measured on a fluorometer. The hydrolytic enzymes assayed degrade a variety of C compounds including cellulose (cellobiohydrolase [CBH; EC 3.2.1.91] and β-glucosidase [BG; EC 3.2.1.21]), hemicellulose (β-xylosidase [BX; EC 3.2.1.37]), starch (α-glucosidase [AG; EC 3.2.1.20]), and chitin (N-acetyl-β-d-glucosaminidase [NAG; EC 3.1.6.1]). We also assayed one hydrolytic enzyme that catalyzes the conversion of organic P to phosphate (acid phosphatase [AP; EC 3.1.3.2]).

Samples for analysis were prepared by adding 1 g equivalent dry mass soil (2 mm sieved soil stored at -20 °C for 16 weeks prior to analysis) to 125 ml of 25 mM maleate buffer adjusted to pH 6 and blending thoroughly for 1 min. For the hydrolytic enzymes, 200 µl of this soil homogenate was added, along with 50 µl of a MUB-linked fluorimetric substrate for each target enzyme, into 96-well microplates with 8 replicate wells per sample per substrate. Substrate concentrations were selected during initial tests so that substrate amount did not limit activity (1000 mM for CBH, BG, AG, and NAG; 2000 mM for BX and AP). Blank wells contained 200 µl homogenate and 50 µl buffer. Standard wells contained 50 µl MUB standard ranging from 0.05 – 5 µm, along with either 200 µl homogenate (quenched curve) or 200 µl of buffer (unquenched curve). Plates were incubated at 20 °C for 2 hrs, assays were terminated with 10 µl of 1 M NaOH in each well, and read at 365 nm excitation and 450 nm emission.

For the oxidative enzymes, we incubated samples with substrates in 2 ml centrifuge tubes for 20-24 hrs, centrifuged the tubes at 3,600 rpm for 5 mins, decanted the supernatant in 96-well plates (4 replicate wells per samples) and then read the plates at 460 nm absorbance according to Madritch et al. (2007). For the phenol oxidase assay, 1.4 ml homogenate was added to .35 ml 5 mM L-DOPA. The peroxidase assay contained 1.4 ml homogenate, .35 ml 5 mM L-DOPA, and .07 ml .3% H_2O_2. Blanks contained 1.4 ml homogenate and .35 ml buffer.

Activity of each extracellular enzyme (nmol mg^{-1} soil C hr^{-1} and nmol μg^{-1} microbial biomass C hr^{-1}) was calculated according to the equations documented in German et al. (2011). For the oxidative assays, we used the L-DOPA extinction coefficient determined by Bach et al. (2013): 7.9.

Additional variables

The amount of root material collected in 2014 was small and insufficient for additional analyses of root chemistry. Consequently, we supplemented our root dataset with measurements of root samples collected from control and N addition plots at the same sites in 2012 (after five years of nutrient addition; root sampling methods detailed in Chapter 2). We measured root C chemistry using an ANKOM Fiber Analyzer (Ankom Technology, Macedon, New York, USA), which analyzed the percent composition of the following C fractions from dried, ground root material: soluble cell contents (SCC); cellulose (CELL); hemicellulose plus bound proteins (HBP); and lignin plus other recalcitrant compounds (LR).

Additionally, we analyzed data on soil micronutrient concentration from soil samples taken from all plots in 2011 (after four years of nutrient addition). Sampling methods are described in Borer et al. (2014). Soil micronutrient concentration was analyzed by Mehlich-3 extraction (A&L Analytical Laboratory, Memphis, TN), which extracts "available" micronutrients from soils (Zheng and Zhang 2012). Although there are positive linear relationships between Mehlich-3 micronutrients and other measures of available or exchangeable nutrients (e.g., anion exchange resin P), the strength of the relationship depends on underlying soil characteristics (Burt et al. 2002). Consequently, throughout we refer to soil micronutrient concentration analyzed by Mehlich-3 as "extractable" and consider this measurement to be *suggestive* of how nutrient treatments could affect soil exchangeable and available nutrient concentrations.

Statistical analyses

We evaluated the effects of nutrient addition (N x P x K) on soil C and N, microbial biomass C and N, DOC, microbial respiration (cumulative C respired, k_f, k_s, C_f, and C_s), microbial extracellular enzyme activity, microbial CUE, root chemistry, and soil chemistry using ANOVA (nlme package in R). In all models we included site as a fixed effect to account for known (e.g., climatic, pedologic, and plant community) differences among the three study sites. Block was included as a random effect. We also tested

for site by nutrient interactions; when significant, we included the interaction in the model and performed post-hoc comparisons (lsmeans package in R). P-values were Bonferroni corrected for multiple comparisons. Finally, we calculated the variance explained by fixed effects only (marginal R^2) and the variance explained by both fixed and random effects (conditional R^2; MuMIn package in R; Nakagawa and Schielzeth 2013). All analyses were performed in R (R version 3.0.1; R Foundation for Statistical Computing 2013).

We focused on the effects of N on microbial decomposition and respiration, and the mechanisms controlling the decomposition response to N enrichment. To maximize the statistical power for detecting N effects, we sampled and analyzed the full factorial of nutrient treatments. We acknowledge significant interactions with and/or main effects of P and K, however, we focus on the effects of N on the processes studied here.

RESULTS

Soil and microbial biomass C and N concentration

Nutrient addition had no effect on total soil C and N concentrations, or the soil C:N ratio (see Appendix 3 – Tables S3.1-2 for soil and microbial C and N ANOVA and data tables). By contrast, nutrient addition significantly impacted microbial biomass C and N in soils. In general, added N decreased microbial C (μg C mg^{-1} soil C), although the effects of N varied with K and site (N x K interaction: p = 0.03; site x N interaction: p = 0.01; Figure 3.2). Nitrogen addition significantly decreased microbial C (μg C mg^{-1} soil C) under ambient K by 31% on average (post-hoc comparison: $p < 0.0001$). Nitrogen addition also decreased microbial C under added K by 7% on average, although the difference was not significant (post-hoc comparison: p = 0.07). Furthermore, N addition significantly decreased microbial C (μg C mg^{-1} soil C) in Kansas and Colorado (post-hoc comparison: p = 0.001 and $p < 0.0001$, respectively), but not Nebraska (post-hoc comparison: $p > 0.1$). The effects of nutrient addition on microbial biomass N (μg N mg^{-1} soil N) were similar (N x K interaction: p = 0.02; site x N interaction: p = 0.03): N decreased microbial N by 25% on average in the ambient K plots (ambient N average = 43.9 μg microbial N mg^{-1} soil N; added average = 33.0 μg microbial N mg^{-1} soil N; post-hoc comparison: $p < 0.0001$) and had no effect on microbial N in the added K plots (post-hoc comparison: $p > 0.1$). Additionally, N addition significantly

decreased microbial N (μg N mg^{-1} soil N) in Colorado (post-hoc comparison: $p < 0.0001$), but not Kansas or Nebraska (post-hoc comparison: $p = 0.09$ and $p > 0.1$, respectively). Finally, N addition decreased the microbial biomass C:N ratio by 7.6% on average (ambient N average = 7.3; added average = 6.8; N main effect: $p = 0.004$). The effects of N on microbial biomass were the same whether analyzed per mass soil or per mass soil C or N.

N addition increased the concentration of DOC by 21% on average, from 2.13 μg DOC mg^{-1} soil C to 2.59 μg DOC mg^{-1} soil C (N main effect: $p = 0.0002$; Appendix 3 – Figure S3.3). The effects of N on DOC concentration were the same whether analyzed per mass soil or per mass soil C.

Microbial respiration and decomposition parameters

Nitrogen addition decreased the cumulative C respired per mass soil C by 21% on average (N main effect: $p = 0.0001$; Figure 3.3; see Appendix 3 – Tables S3.3-4 for respiration and decomposition parameter ANOVA and data tables). The effects of N on cumulative respiration were the same whether analyzed per mass soil C or per mass soil. By contrast, the effects of N on microbial mass-specific respiration (mg C-CO_2 respired μg^{-1} microbial C, also known as qCO_2; Anderson and Domsch 1993) were site-specific (site x N interaction: $p = 0.05$): N decreased mass-specific microbial respiration in Kansas (post-hoc comparison: $p = 0.02$) and had no effect on mass specific respiration in Nebraska and Colorado (post-hoc comparison: $p > 0.1$; Figure 3.4).

There was a significant positive effect of K on microbial mass-specific respiration: K addition increased cumulative mass-specific respiration (mg C-CO_2 respired μg^{-1} microbial C) by 16.6% on average (K main effect: $p = 0.0008$; Appendix 3 – Figure S3.4). By contrast, the K treatment had no effect on cumulative respiration per mass soil C or per mass soil (K main effect: $p > 0.1$).

In order to evaluate how nutrient addition affected the decomposition rates of distinct soil organic matter pools (e.g., pools that decompose quickly versus pools that decompose more slowly), we fit our respiration rate data to both one- and two-pool decay models. The two-pool model was the best fit for 96% of the samples (difference in AIC between models was > 11). For 4% of the samples, the models were indistinguishable (difference in AIC < 1). Fit of the two-pool model ranged from 0.75 – 0.99 R^2, with a mean and median $R^2 = 0.94$.

Nitrogen addition had variable effects on the decay rate of the fast pool (site x N interaction: p = 0.0003): N decreased k_f in Kansas (post-hoc comparison: $p < 0.001$) and had no effect on k_f in Colorado and Nebraska (Figure 3.5a). Similarly, N increased the size of the fast pool in Kansas only (site x N interaction: $p = 0.005$; post-hoc comparison (Kansas): $p < 0.001$; Figure 3.5b). By contrast, the effects of N of the slow pool were consistent across sites. Nitrogen decreased the decay rate of the slow pool (N main effect: $p <$ 0.0001; Figure 3.5c) and had no effect on the size of the slow pool (N main effect: $p > 0.1$; Figure 3.5d). Other nutrient treatments had no effect on the decay rates of the fast and slow pools.

Microbial carbon use efficiency

Microbial CUE of ^{13}C glucose was greater than microbial CUE of ^{13}C vanillin at both sites analyzed (Nebraska and Kansas; Figure 3.6). The effects of N on CUE varied with substrate type and site (see Appendix 3 – Tables S3.5-6 for microbial CUE ANOVA and data tables). The CUE of ^{13}C glucose decreased with N addition in Nebraska, but not Kansas (site x N interaction: $p = 0.03$; post-hoc comparisons: $p = 0.0003$ (Nebraska) and $p > 0.1$ (Kansas)). Additionally, there was a significant negative main effect of N on ^{13}C vanillin CUE (N main effect: $p = 0.007$). Phosphorus addition significantly decreased ^{13}C glucose CUE (P main effect: $p = 0.0006$) and ^{13}C vanillin CUE (P main effect: $p = 0.03$; Appendix 3 – Figure S3.5).

The number of moles of ^{13}C that were incorporated into microbial biomass during the assay generally followed the same trends in response to N addition that microbial biomass C did. Nitrogen addition decreased the moles of ^{13}C glucose (N main effect: $p = 0.0008$) and ^{13}C vanillin (N main effect: p = 0.05) incorporated into microbial biomass, but this effect was proportional to the decrease in microbial biomass C observed (no effect of N addition on concentration of moles ^{13}C microbial biomass per moles C microbial biomass; N main effect: $p > 0.1$). By contrast, P addition decreased the moles of ^{13}C glucose (P main effect: $p < 0.0001$) and ^{13}C vanillin (P main effect: $p = 0.0546$) incorporated into microbial biomass, and there was also an effect of P addition on the concentration of moles ^{13}C glucose microbial biomass per moles C microbial biomass (P main effect: $p = 0.04$).

The number of moles ^{13}C that were respired during the assay also followed the general trends in response to N that cumulative C respired did: added N decreased ^{13}C glucose respired when no K was

added and had no effect under added K (N x K interaction: $p = 0.01$; post-hoc comparisons: $p = 0.001$ (ambient K), $p > 0.1$ (added K)). There was no effect of N addition on moles ^{13}C vanillin respired. Phosphorus addition did not affect ^{13}C respiration of either glucose or vanillin.

Microbial extracellular enzyme potential activity

Overall, N addition affected the activity of the oxidative enzymes (although not in the expected way), but not the hydrolytic enzymes (see Appendix 3 – Tables S3.7-8 for microbial extracellular enzyme ANOVA and data tables). Nitrogen addition significantly increased the activity of PX per mass soil C by 20% on average (N main effect: $p = 0.02$) and PX activity per mass microbial C by 58% on average (N main effect: $p < 0.0001$; Figure 3.7; Appendix 3 – Figure S3.6). The K treatment also significantly positively increased PX activity per mass soil C by 24% on average (K main effect: $p = 0.02$) and per mass microbial C by 37% on average (K main effect: $p = 0.0002$). There was a significant interactive effect of N, P, and K on PO activity per mass soil C (N x P x K interaction: $p = 0.04$) and per mass microbial C (N x P x K interaction: $p = 0.01$; Figure 3.8; Appendix 3 – Figure S3.7). Nutrient addition tended to increase the activity of PO, especially in the +NPK treatment: on average, there was a 34% increase in PO activity per mass soil C in the +NPK plots compared to the control plots; additionally, PO activity per mass microbial C was ~1.2x larger in the +NPK plots compared to the control plots, on average.

Phosphorus addition, but not N or K addition, significantly impacted the activity of the hydrolytic enzymes. In general, P addition increased the activity of CBH, BG, AG, NAG, and BX, although the magnitude (and significance) of the effect depended on whether activity was measured per mass soil C or per mass microbial C (Appendix 3 – Figure S3.8-9). Phosphorus addition significantly increased CBH activity per mass soil C (P main effect: $p = 0.01$) and per mass microbial C (P main effect: $p = 0.0005$); moderately significantly increased BG activity per mass soil C (P main effect: $p = 0.07$) and significantly increased BG activity per mass microbial C (P main effect: $p = 0.005$); significantly increased AG activity per mass soil C in Kansas (site x P interaction = 0.03; post-hoc comparison (Kansas): $p = 0.0005$) and AG activity per mass microbial C (P main effect: $p = 0.01$); moderately significantly increased NAG activity per mass microbial C (P main effect: $p = 0.07$); and significantly increased BX activity per mass microbial

C (P main effect: $p = 0.05$). There were no nutrient effects on AP activity (per mass soil C and per mass microbial C).

Plant substrate chemistry

Nutrient addition significantly increased the concentration of N in plant roots at all three sites (site x N interaction: $p = 0.0001$; see Appendix 3 – Tables S3.9-12 for root chemistry ANOVA and data tables). The N effect was greatest in Kansas (post-hoc comparison: $p < 0.0001$), followed by Colorado (post-hoc comparison: $p = 0.0001$) and Nebraska (post-hoc comparison: $p = 0.01$). Root lignin concentration also increased in response to N addition in Kansas (site x N interaction: $p = 0.04$; post-hoc comparison (Kansas): $p = 0.0003$). There were no N effects on the other C fractions. Nitrogen addition significantly decreased the root lignin:N ratio at all three sites (site x N interaction: $p = 0.01$; post-hoc comparisons: $p < 0.0001$ (Nebraska); $p < 0.0001$ (Kansas); $p = 0.006$ (Colorado); Appendix 3 – Figure S3.10).

Soil chemistry

Nutrient treatments significantly altered soil pH and soil extractable micronutrient concentration (see Appendix 3 – Tables S3.13-14 for soil chemistry ANOVA and data tables). Nitrogen addition significantly decreased soil pH (N x site interaction: $p = 0.005$; Appendix 3 – Figure S3.11) from an average of 6.7 in ambient N plots to 6.3 in added N plots in Nebraska (post-hoc comparison: $p < .0001$), from 6.1 to 6.0 in Kansas (post-hoc comparison: $p = 0.0004$), and from 5.8 to 5.6 in Colorado (post-hoc comparison: $p < .0001$). Furthermore, N addition significantly decreased soil extractable Ca concentration (N main effect: $p = 0.0005$), but not soil extractable Mg concentration. Nitrogen addition increased soil extractable Mg concentration in Kansas and had no effect on soil extractable Mg concentration in Nebraska and Colorado (site x N interaction: $p = 0.02$; post-hoc comparisons: $p = 0.02$ (Kansas) and $p > 0.1$ (Nebraska and Colorado)). Phosphorus addition significantly increased the concentration of soil extractable P (site x P interaction: $p = 0.0006$; post-hoc comparisons: $p < 0.0001$ at each site). There was a significant interactive effect of P and K addition on soil extractable Fe concentration (P x K interaction: $p = 0.01$): P addition and K addition decreased soil extractable Fe concentration when applied alone, but did not change soil extractable Fe concentration when applied together. Finally, K addition significantly increased the

concentration of soil extractable K (site x K interaction: $p = 0.02$; post-hoc comparisons: $p < 0.0001$ at each site), as well as soil extractable B (K main effect: $p = 0.002$), Cu (K main effect: $p = 0.0001$), and Mn (K main effect: $p = 0.0003$).

DISCUSSION

Nitrogen addition decreased the decomposition rate of the slowly cycling soil C pool and decreased microbial respiration at three grassland sites in the U.S. Central Great Plains region. These results are in line with our previous research (Chapter 2), as well as results from forest systems (Janssens et al. 2010). However, our hypotheses about the mechanisms that explain this decomposition response were only partially supported. In contrast to our predictions, N addition did not increase microbial CUE or decrease oxidative enzyme activity. These results are in contrast with theoretical models that predict decreased respiration due to increasing microbial CUE in response to N addition (Ågren et al. 2001; Schimel and Weintraub 2003), a meta-analysis that reports a positive relationship between microbial CUE and soil N:C ratio (Manzoni et al. 2012), as well as studies of forests soils that report decreased SOM decomposition in response to N due to reductions in oxidative enzyme activity (Waldrop et al. 2004). Instead, we found consistent negative effects of N addition on microbial biomass, which accounted for the decreased cumulative respiration at two of the three sites studied (Colorado and Nebraska had no change in mass-specific respiration). At the third site (Kansas), we hypothesize that in addition to decreasing microbial biomass, N addition decreased C availability to microbes leading to decreased mass-specific respiration. Overall, our results suggest that in grassland soils decreased SOM decomposition with N addition may be due to the negative effects of N on microbial biomass via plant or soil mediated responses to N addition (Treseder 2008).

Nitrogen addition and substrate complexity decreased microbial CUE

In contrast to our hypothesis, N addition did not significantly increase microbial CUE. Theoretical models predict that CUE will increase as the availability of nutrients increases (Ågren et al. 2001; Schimel and Weintraub 2003; Manzoni et al. 2010). However, there is limited empirical evidence that this occurs for terrestrial decomposers. In a meta-analysis of microbial CUE measurements across a gradient of

terrestrial N availability, Manzoni et al. (2012) found that CUE of soil microbial communities increased as the C:N ratio of the soil or decomposing substrate decreased (i.e., as N availability increased). However, when Manzoni et al. (2012) surveyed N addition studies (as opposed to natural N gradients), they found that the relationship between CUE and soil C:N switched: CUE tended to decrease as N availability increased. Why would CUE decrease in response to N addition? This could occur if other nutrients required for biomass construction are limiting. For example, if N is supplied in excess of the C and P (or another nutrient) required for biomass, excess C will be respired and the CUE will decrease. Alternately, CUE could decrease in response to N addition if the composition of the microbial community shifts towards dominance by organisms with lower CUEs. For example, bacteria have lower C to nutrient biomass requirements, slower biomass turnover rates, and lower CUE compared to fungi (Six et al. 2006). Consequently, a decrease in the fungi:bacteria ratio could decrease microbial CUE. We did observe a significant decrease in the microbial biomass C:N ratio, which tends to decline as the ratio of fungi:bacteria declines (Waring et al. 2013). Therefore, an increase in bacteria relative to fungi under N enrichment may explain the observed decrease in CUE in response to N addition.

We also found that microbial CUE of ^{13}C glucose was higher than the microbial CUE of ^{13}C vanillin, as expected. This is unsurprising since increasing substrate complexity demands more metabolic steps for substrate degradation (Ågren and Bosatta 1987), which will lead to decreased microbial growth efficiency (Brant et al. 2006). Given that N addition changes the chemistry and input rate of plant tissues (Wedin and Tilman 1996; Yuan and Chen 2012), overall microbial CUE (and consequently C respired) could change due to microbial responses to changing substrate chemistry, as opposed to the direct effects of N on microbial growth efficiency or microbial community composition. For example, changes in microbial CUE could explain decreased respiration in response to N addition if N addition increased the input of relatively simple C compounds, leading to increased microbial CUE and decreased C respired.

Nitrogen addition did not decrease microbial extracellular oxidative enzyme activity

In contrast to our prediction, N did not significantly decrease oxidative enzyme activity, nor did it affect hydrolytic enzyme activity. Instead, N addition significantly increased the activity of one of the oxidative enzymes we studied: peroxidase (PX). While the inhibitory effects of N on oxidative enzymes in

forest systems are well established (e.g., DeForest et al. 2004a; Waldrop et al. 2004; Edwards et al. 2011; Eisenlord et al. 2013), this N effect may be system-specific (Keeler et al. 2008; Sinsabaugh 2010). The negative effects of N on oxidative enzymes may be minimal or non-existent in grassland systems where glomeromycota and ascomycetes (and opposed to basidomycetes) dominate and lignin content of the plant community is less relative to forests (Sinsabaugh 2010). Interestingly, DOC increased in response to N, which in forest systems has been interpreted as a result of decreased ligninolytic activity and incomplete lignin degradation by actinobacteria (Zak et al. 2008).

Why might N addition have increased oxidative enzyme activity, as opposed to decreased activity as predicted? First, extracellular enzymes are also comprised of N; consequently, N availability could limit the production of these enzymes (Allison and Vitousek 2005). Increasing activity following N addition is common among hydrolytic enzymes in litter decomposition studies (e.g., Carreiro et al. 2000; Sinsabaugh et al. 2002; Waldrop et al. 2004; Talbot and Treseder 2011; Hobbie et al. 2012), although we did not observe such an increase here. Alternately, assays of oxidative enzyme activity may not be accurate measures of microbial enzyme activity, since they do not distinguish microbial activity from abiotic processes that can contribute significantly to whole soil oxidation activity (e.g., Hall and Silver 2013; Sinsabaugh 2010; Bach et al. 2013). Nitrogen addition could increase oxidative activity of the soil indirectly through changing soil chemistry (e.g., pH) that changes the availability of reactive minerals. Or N addition could increase oxidative activity by increasing the stabilization (or protection) of extracellular enzymes against degradation on mineral surfaces or in aggregates (Allison 2006).

Nitrogen effects on microbial biomass explained the microbial respiration response

Since N did not increase microbial CUE or decrease oxidative enzyme activity, it seems most likely that the negative effects of N addition on microbial biomass explain the inhibitory effects of N on microbial decomposition and decreased microbial respiration. At all three sites, N addition significantly decreased microbial biomass. Furthermore, in Colorado and Nebraska, N did not significantly change mass-specific respiration (cumulative C respired per mass microbial biomass C) and microbial respiration decreased in proportion to the decreases in microbial biomass. Consequently, the negative effects of N on microbial biomass alone explain the decreased decomposition rate and the cumulative C respired in

Colorado and Nebraska, and the negative effects of N on microbial biomass (as well as additional factors detailed below) explain the decreased decomposition rate and cumulative C respired in Kansas.

As detailed earlier (Figure 3.1), plant and soil mediated changes in response to N addition could also negatively affect microbial biomass (Treseder 2008). First, N addition could decrease microbial biomass via changing the chemistry and abundance of plant inputs (Figure 3.1, Mechanism 3). Nitrogen affects plant species composition (Suding et al. 2005), plant tissue chemistry (Xia and Wan 2008), and the relative allocation to (and consequently inputs from) aboveground versus belowground tissues (Yuan and Chen 2012); all of these factors influence the rate at which substrates decompose and are incorporated into microbial biomass. If N addition leads to a decrease in substrate "quality" (e.g., an increase in chemically complex substrates), microbial biomass could decrease. Alternately, N addition can cause soil acidification, which may lead to Al toxicity of microbes or decreased C availability to those microbes (Flis et al. 1993; Mueller et al. 2012), and ultimately, decreased microbial biomass (Figure 3.1, Mechanism 4). These mechanisms are not mutually exclusive and we explore the evidence in support of each in turn.

First, N effects on microbial biomass could occur via changes in the chemistry of decomposition substrates. Specifically, N addition could decrease microbial biomass if changes in decomposition substrate chemistry in response to N lead to decreased C availability to microbes for biomass production. We found that N addition increased root N concentration, consistent with other studies the reported increased aboveground plant N concentration in response to N addition (e.g., Wedin and Tilman 1996). Increased N concentrations are known to stimulate microbial decomposition in the short term (Melillo et al. 1982), but can slow decomposition in its later stages (Berg and Matzner 1997). Since slower decomposition rates can decrease C availability to microbes for biomass production, an increase in N concentration could lead to decreased microbial biomass. However, we also found that N addition decreased the root lignin:N ratio, which tends to increase the decay rate of litter (Talbot and Treseder 2011). Consequently, we expect the observed shifts in root substrate chemistry to lead to greater, not lesser, C availability for microbes.

Additionally, the inhibitory effects of N on microbial biomass via plant substrates seem unlikely since we expect N addition to increase total C inputs belowground. This should ultimately *increase* the total amount of C available to microbes for biomass. Although we did not measure root inputs at these sites, results from a meta-analysis and grassland field study, respectively, have shown that N tends to increase

fine root production (Yuan and Chen 2012) and total belowground C allocation (Adair et al. 2009). In the latter study, the effects of N on root production were driven by the increase in total belowground biomass in response to N. Previously we found that N tended to increase the total root biomass stock in Colorado and Nebraska (Chapter 2). At those sites belowground inputs likely have increased as well, potentially leading to an increase in C inputs for microbial biomass and, consequently, microbial biomass. Consequently, it seems unlikely that effects of N on substrate chemistry or quantity explain the decrease in microbial biomass observed following N addition.

Finally, the effects of N on soil chemistry could have decreased microbial biomass. We found that N addition decreased soil pH. However, N only partly changed concentrations of base and hydrolyzing cations in ways we would expect in response to pH changes: after 3 years of nutrient addition soil extractable Ca, but not Mg, decreased in response to N addition and N did not affect soil extractable Fe. Calcium availability could limit microbial growth under more acidic pH, leading to decreased microbial biomass (Treseder 2008). Alternately, soil acidification could cause Al toxicity of microbes as the concentration of Al increases. Unfortunately, soil extractable Al concentration was not measured in this study. However, data from other studies investigating the relationship between soil pH and extractable Al concentration provides insight into whether or not Al-mediated effects on microbial toxicity or C accessibility could have influenced microbial biomass at these sites. For example, in a study of unmanipulated soils along a natural continuous pH gradient that ranged from pH 3.7 to 8.3, Aciego Pietri and Brookes (2008) found that there was a strong negative relationship between soil pH and extractable Al concentration for soils below pH 5.4 (soil extractable Al decreased significantly as the soils became more alkaline); however, above pH 5.4, soil extractable Al was near zero and did not change. Soil pH, along with soil mineralogy, is a primary control on Al availability (Bertsch and Bloom 1996). Consequently, it seems unlikely that pH changes in response to N addition could have increased extractable Al, caused microbial toxicity and/or changed C accessibility, and led to decreased microbial biomass since our lowest average pH under N addition was above the threshold identified by Aciego Pietri and Brookes (2008; added N average pH at Colorado was 5.6). Overall, the mechanism driving the decline in microbial biomass in response to N remains uncertain.

Interestingly, the negative effects of N on microbial biomass do not fully account for decreased cumulative respiration in Kansas: at that site, N enrichment significantly decreased mass specific respiration in addition to significantly decreasing microbial biomass. This suggests that an additional mechanism, besides decreased microbial biomass, is necessary to explain decreased respiration in Kansas. The soils at Konza Prairie (the Kansas site) are extremely fine-textured and highly aggregated in comparison with the soils at either Shortgrass Steppe (Colorado) or Cedar Point (Nebraska; Chapter 2). Consequently, N effects on mechanisms that influence C accessibility in aggregates or on mineral surfaces may be relevant here (Dungait et al. 2012). Previously we found that N addition had a moderate positive effect on aggregation at these grassland sites, particularly for large macro-aggregates (Chapter 2). Nitrogen addition can increase aggregation by increasing the abundance of aggregate "binding" agents (e.g., roots or fungal biomass; Wilson et al. 2009; King 2011; Gupta and Germida 2015). Although large macro-aggregates were destroyed prior to the respiration incubation due to pre-incubation sieving, increased macro-aggregation can facilitate aggregation and (stabilization) of the smaller aggregate fractions that *would* be present in the lab incubated soil (Oades 1984; Six et al. 2004). If N addition increased aggregation (and protection) of these smaller aggregate fractions too, aggregate occlusion of C may have decreased the C available to microbes, leading to decreased respiration of the Kansas soil samples in addition to the decreased respiration due to decreased microbial biomass.

Phosphorus and potassium addition affected soil microbes, but did not influence soil organic matter decay

We did not observe P or K effects on the decay rates of the fast and slow cycling soil organic matter pools in this or a previous study (Chapter 2). This was surprising since we *did* observe significant effects of these nutrients on other measures of microbial activity (CUE and extracellular enzymes; P effect) and microbial biomass (K effect). The lack of an effect on decay rate suggests that the P and K effects on soil microbes were limited and did not influence respiration. Whether the effects strengthen over time and lead to changes in the decomposition rates of soil C remains to be seen.

Many of the same mechanisms that explain N effects on microbial activity and biomass may explain how P and K addition influenced these variables. Specifically, the K treatment could decrease

microbial biomass through alterations to soil chemistry (e.g., micronutrient availability) that influence C accessibility or are directly toxic to microbes (Flis et al. 1993; Mueller et al. 2012). Phosphorus addition, on the other hand, could increase microbial extracellular enzyme activity if P availability limits enzyme production (Allison and Vitousek 2005; Bradford et al. 2008b). Likewise, microbial CUE could decrease if microbes allocated more C to enzyme production under P addition (Manzoni et al. 2012), a C pool we did not track in our ^{13}C tracer experiment. If chronic P and K addition leads to changes in SOM decay rates in the future, further understanding of these microbial processes will be warranted.

Conclusions

Overall, we found that seven years of N addition decreased the decomposition rate and cumulative C respired at three grassland sites in the U.S. Central Great Plains. In contrast with studies of forest soils, as well as predictions from theoretical models, N addition did not decrease microbial decomposition and respiration due to decreased oxidative enzyme activity or increased microbial CUE. Instead, in these grassland soils, the negative effect of N on microbial biomass explains the decreased decomposition and respiration of SOM at two of the three study sites. At the third site, additional factors that influence microbial access to C (such as aggregation) likely contributed to the decreased respiration in addition to decreased microbial biomass. Ultimately, by altering the rate at which CO_2 is released to the atmosphere during microbial decomposition, N addition will contribute to increased sequestration of soil C in these grassland soils.

Table 3.1 – Characteristics of the three Nutrient Network experimental sites sampled.

Site characteristic	Cedar Point, Nebraska	Konza Prairie, Kansas	Shortgrass Steppe, Colorado
MAT (°C) [a]	9.3	12	8.4
MAP (mm) [a]	454	872	364
N deposition rate (kg N ha^{-1} yr^{-1}) [b]	3.1	9.8	3.1
Elevation (m)	965	440	1650
Plant biomass ($g\ m^{-2}$) [c]	137.51 (15.61)	352.73 (28.72)	102.02 (10.89)
Habitat	Shortgrass prairie	Tallgrass prairie	Shortgrass prairie
Soil C (mg C g^{-1} soil) [d]	14.01 (2.26)	37.19 (5.13)	9.27 (1.24)
Soil N (mg N g^{-1} soil) [d]	1.11 (0.20)	2.83 (0.31)	0.82 (0.13)
Soil C:N ratio [d]	12.71 (0.36)	13.12 (0.36)	11.37 (0.52)
Soil texture [e]			
Sand %	71.4 (0.5)	31.9 (0.8)	71.3 (0.2)
Silt %	18.1 (0.7)	49.8 (1.2)	15.1 (0.2)
Clay %	10.5 (0.5)	18.3 (0.4)	13.6 (0.4)
Soil bulk density (g dry soil cm^{-3}) [f]	1.58 (0.04)	1.52 (0.06)	1.17 (0.06)
Nutrient addition treatment duration (yrs)	7	7	7

[a] Mean annual temperature (MAT) and mean annual precipitation (MAP) are from the WorldClim database (Hijmans et al. 2005).

[b] Modeled N deposition rates are from the Oak Ridge National Laboratory Distributed Active Archive Center (Dentener 2006).

[c] Site mean (standard error in parentheses) plant aboveground biomass sampled in control plots (2007-2012); sampling methods in Borer et al. (2014); data from the Nutrient Network; n = 3.

[d] Site mean (standard error in parentheses) soil carbon and nitrogen sampled in control plots (this study); n = 3.

[e] Site mean (standard error in parentheses) soil texture sampled in 2012 (Chapter 2) and measured using the hydrometer method (Ashworth et al. 2001). One plot per block sampled at each site (n = 3).

[f] Site mean (standard error in parentheses) soil bulk density sampled in 2012 (Chapter 2). One core per block sampled at each site (n = 3).

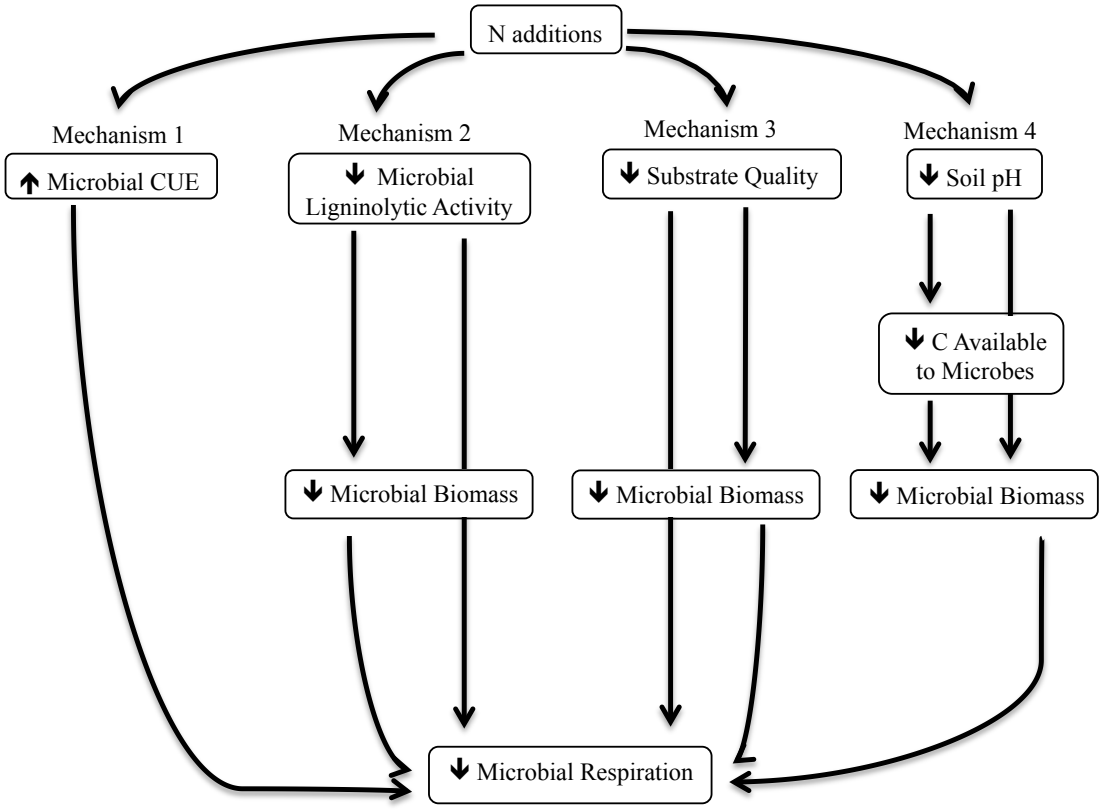

Figure 3.1 – Hypothesized mechanisms that explain the negative effects of nitrogen addition on microbial respiration. N = nitrogen; C = carbon.

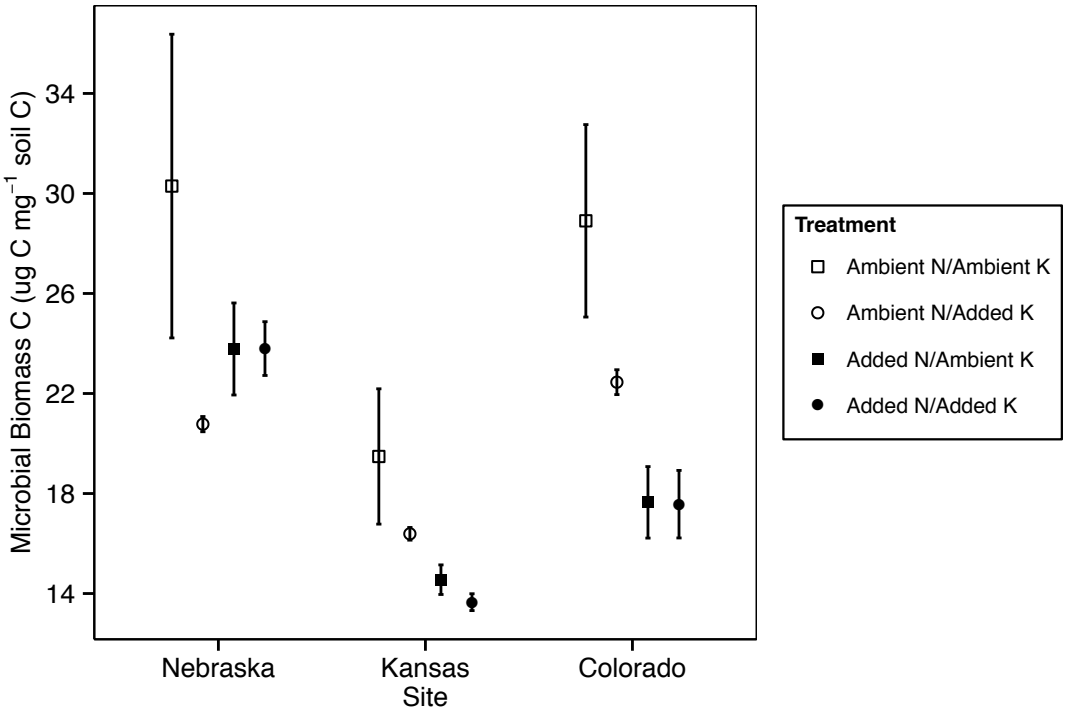

Figure 3.2 – Treatment effects on microbial biomass carbon per mass soil carbon. Figure shows mean plus/minus one standard error. Treatment codes: open symbols = ambient N; shaded symbols = added N; squares = ambient K; circles = added K.

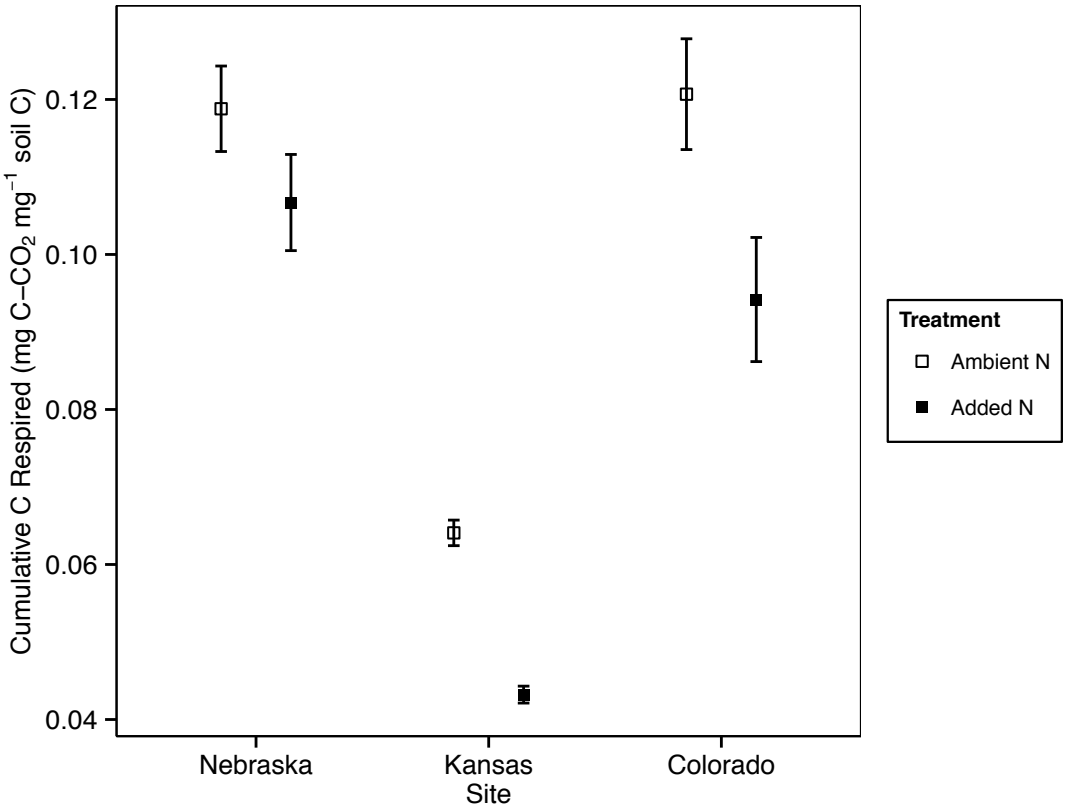

Figure 3.3 – Nitrogen treatment effects on cumulative carbon respired per mass soil carbon. Figure shows mean plus/minus one standard error. Treatment codes: open symbols = ambient N; shaded symbols = added N.

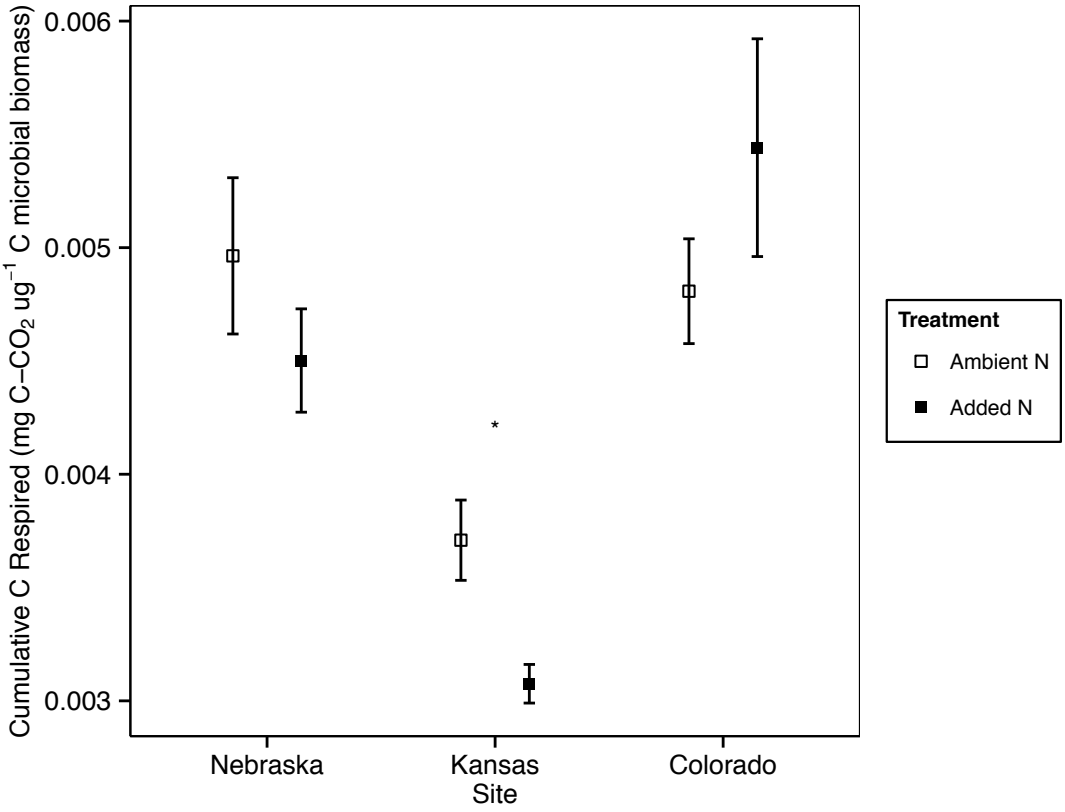

Figure 3.4 – Nitrogen treatment effects on mass-specific cumulative carbon respired per mass microbial carbon. Figure shows mean plus/minus one standard error. Stars indicate significance from post-hoc pairwise comparisons: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Treatment codes: open symbols = ambient N; shaded symbols = added N.

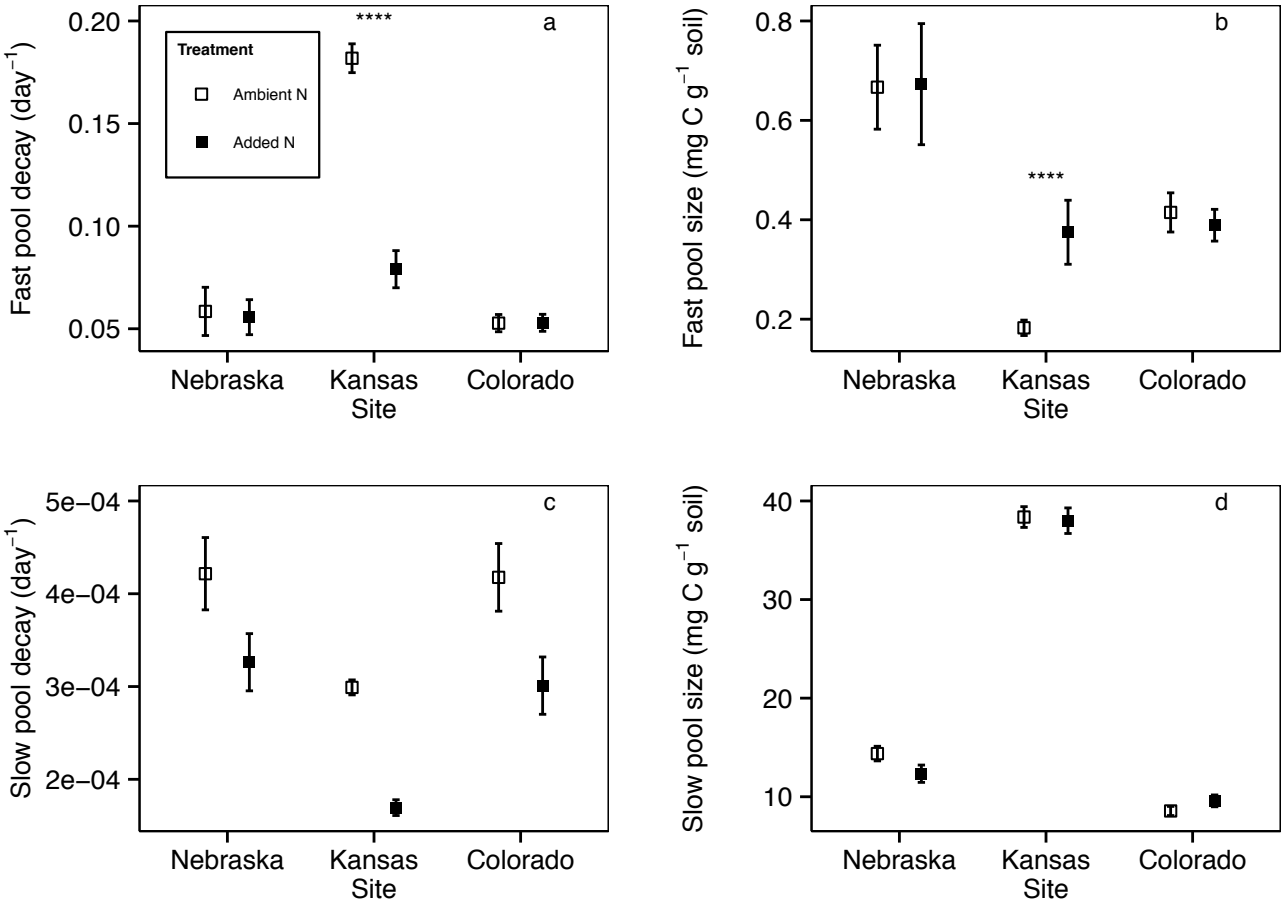
Fast pool decay (day^{-1})
0.20
0.15
0.10
0.05
****
a
Treatment
Ambient N
Added N
Nebraska
Kansas
Colorado
Site
Fast pool size (mg C g^{-1} soil)
0.8
0.6
0.4
0.2
****
b
Nebraska
Kansas
Colorado
Site
Slow pool decay (day^{-1})
5e−04
4e−04
3e−04
2e−04
c
Nebraska
Kansas
Colorado
Site
Slow pool size (mg C g^{-1} soil)
40
30
20
10
d
Nebraska
Kansas
Colorado
Site

Figure 3.5 – Nitrogen treatment effects on the decay rate (a) and size (b) of the fast decomposing C pool, decay rate (c) and size (d) of the slow decomposing C pool measured with a long-term microbial respiration incubation. All panels shows mean plus/minus one standard error. Stars indicate significance from post-hoc pairwise comparisons: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Treatment codes are the same across all panels. Treatment codes: open symbols = ambient N; shaded symbols = added N.

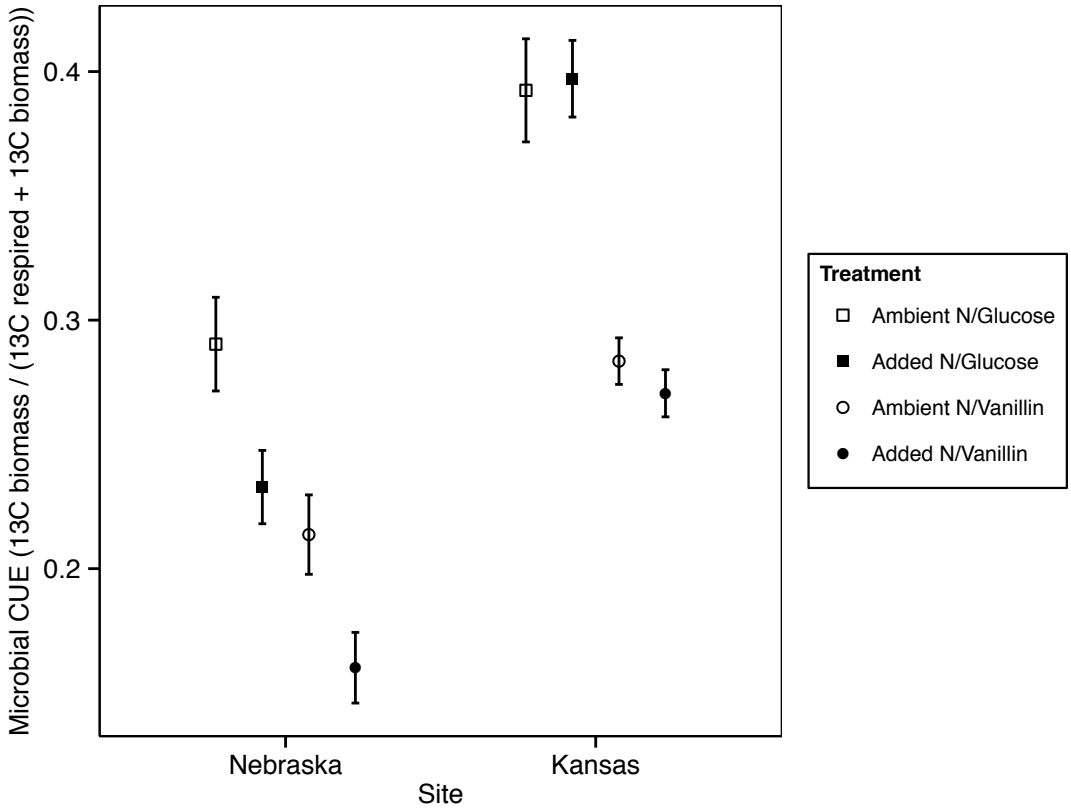

Figure 3.6 – Nitrogen treatment effects on microbial carbon use efficiency of ^{13}C glucose and ^{13}C vanillin from soil collected in Nebraska and Kansas. Figure shows mean plus/minus one standard error. CUE = carbon use efficiency. Treatment codes: open symbols = ambient N; shaded symbols = added N; squares = ^{13}C glucose; circles = ^{13}C vanillin.

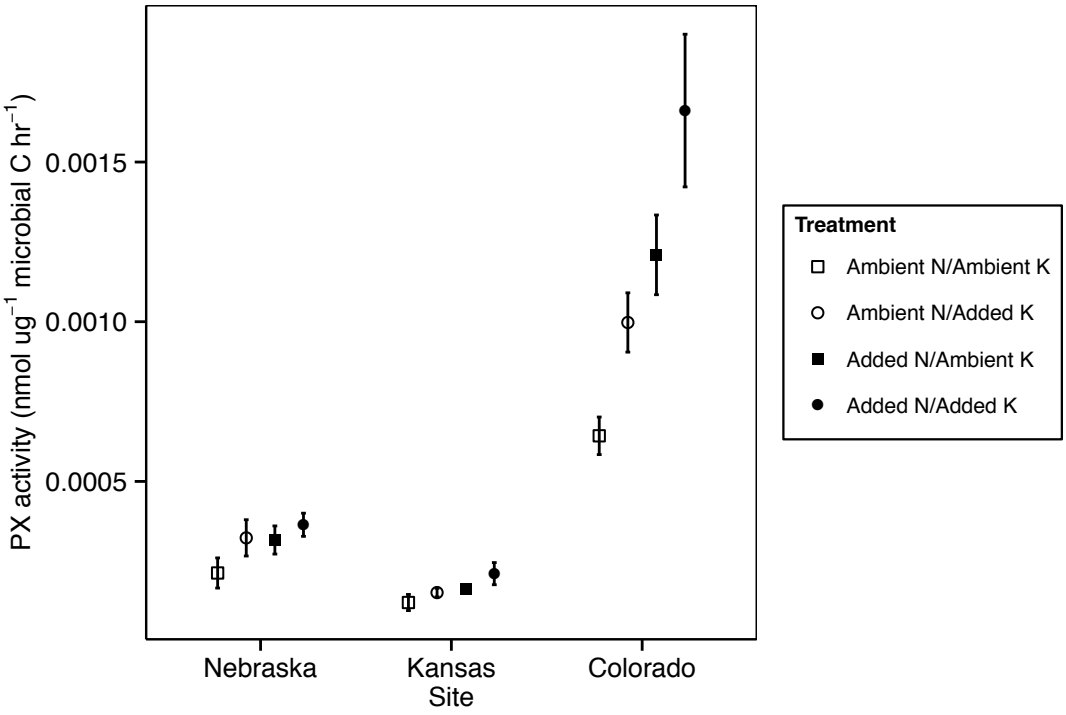

Figure 3.7 – Treatment effects on peroxidase activity per mass microbial carbon. Figure shows mean plus/minus one standard error. PX = peroxidase. Treatment codes: open symbols = ambient N; shaded symbols = added N; squares = ambient K; circles = added K.

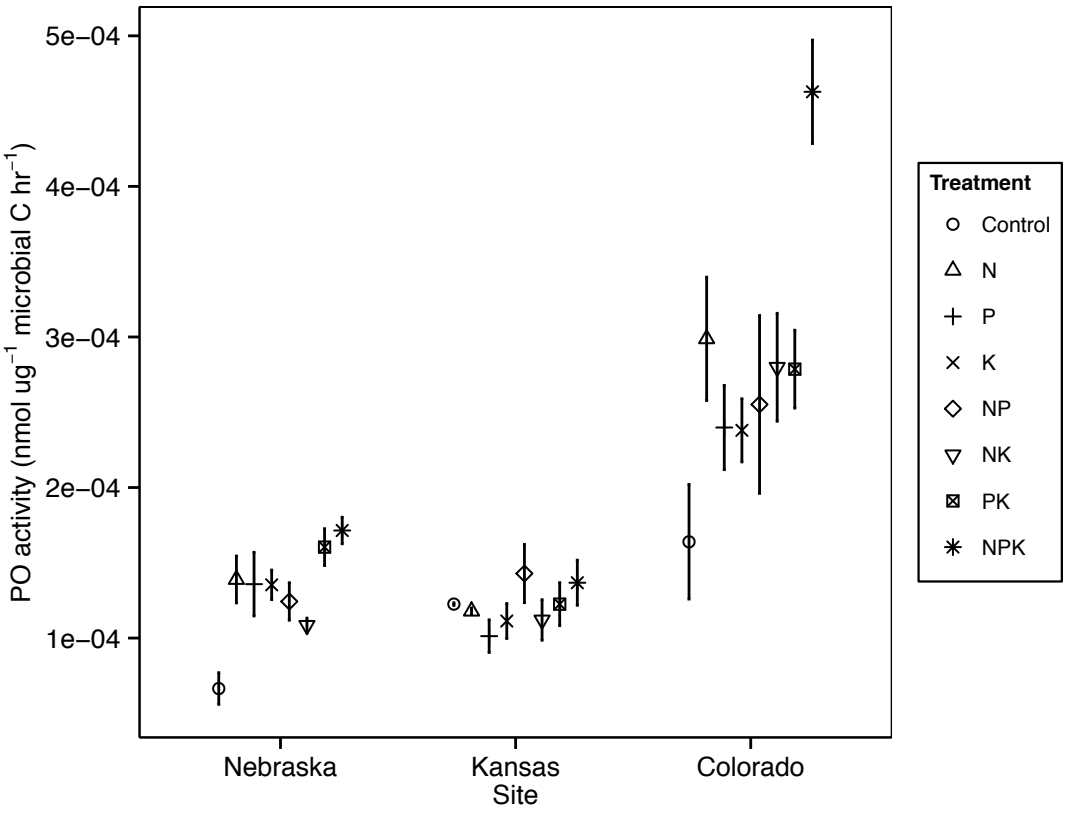

Figure 3.8 – Treatment effects on phenol oxidase activity per mass microbial carbon. Figure shows mean plus/minus one standard error. PO = phenol oxidase. Treatment codes: Control = control plots; N = nitrogen (N) addition plots; P = phosphorus (P) addition plots; K = potassium (K) addition plots; NP = nitrogen and phosphorus addition plots; NK = nitrogen and potassium addition plots; PK = phosphorus and potassium addition plots; NPK = nitrogen, phosphorus, and potassium addition plots.

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APPENDIX 1 – SUPPLEMENTAL MATERIAL FOR CHAPTER 1

Table S1.1 – Nutrient Network experimental sites included in this study.

Site characteristics	Bunchgrass	Bogong	Burrawan	Cedar Creek	Cedar Point	Cowichan	Elliot Chaparral
Site code	bnch.us	bogong.au	burrawan.au	cdcr.us	cdpt.us	cowi.ca	elliot.us
Country	US	AU	AU	US	US	CA	US
Latitude (degree)	44.28	-36.878	-27.738	45.40	41.2	48.46	32.88
Longitude (degree)	-121.97	147.25	151.14	-93.20	-101.63	-123.38	-117.05
Elevation (m)	1318	1760	425	270	965	50	200
MAP (mm)	1647	1592	683	750	445	764	331
MAT (°C)	5.5	5.7	18.4	6.3	9.5	9.8	17.2
Total soil C (mg C g^{-1} soil) [a]	84.87 (5.31)	59.58 (11.95)	15.74 (2.11)	10.17 (0.79)	12.79 (0.72)	54.65 (1.22)	28.24 (10.50)
Total soil N (mg N g^{-1} soil) [b]	5.91 (0.46)	4.09 (1.04)	0.74 (0.03)	0.75 (0.07)	1.00 (0.07)	4.11 (0.04)	1.90 (0.53)
Plant aboveground biomass (g m^{-2}) [c]	142.42 (27.12)	567.7 (147.9)	179.92 (41.30)	241.25 (109.75)	244.17 (61.30)	758.4 (90.43)	312.13 (78.04)
Plant root biomass (g m^{-2}) [d]	1263.60 (167.99)	311.15 (65.52)	136.34 (33.87)	858.70 (671.43)	960.20 (215.54)	1675.18 (244.48)	128.38 (45.19)
Soil pH [e]	5.7 (0.2)	4.7 (0.0)	6.1 (0.2)	6.1 (0.1)	7.2 (0.2)	5.9 (0.2)	6.4 (0.0)

Sand content (%) [f]	70.43 (0.72)	63.70 (0.10)	82.2 (1.35)	88.75 (0.05)	67.97 (6.54)	32.73 (3.72)	54.23 (2.62)
Silt content (%) [g]	26.53 (0.62)	18.05 (1.05)	9.07 (1.13)	8.65 (1.05)	21.73 (5.06)	39.27 (1.97)	25.57 (1.10)
Clay content (%) [h]	2.93 (1.33)	18.15 (0.95)	8.63 (1.76)	2.50 (1.00)	10.17 (1.33)	27.90 (1.80)	20.10 (1.78)
Soil bulk density (g cm^{-3})	0.62 (0.07)	0.68 (0.08)	0.93 (0.02)	NA	0.85 (0.13)	0.75 (0.03)	NA
Nutrient addition duration (yrs)	4	3	4	5	4	4	3
Plots per treatment (n)	3	3	3	3	3	3	3

Site characteristics	**Fruebuel**	**Hall's Prairie**	**Konza Prairie**	**Lancaster**	**Lookout**	**Mt. Caroline**
Site code	frue.ch	hall.us	konz.us	lancaster.uk	look.us	mtca.au
Country	CH	US	US	UK	US	AU
Latitude (degree)	47.11	36.87	39.07	53.99	44.21	-31.78
Longitude (degree)	8.54	-86.70	-96.58	-2.63	-122.13	117.61
Elevation (m)	995	194	440	180	1501	285
MAP (mm)	1355	1282	877	1322	1898	330
MAT (°C)	6.5	13.6	11.9	8	4.8	17.3
Total soil C (mg C g^{-1} soil) [a]	30.55 (2.36)	12.02 (0.28)	42.67 (2.10)	192.81 (22.75)	173.13 (13.61)	11.22 (2.83)
Total soil N (mg N g^{-1} soil) [b]	3.27 (0.28)	1.23 (0.08)	3.10 (0.06)	10.54 (1.21)	12.26 (0.98)	0.71 (0.17)

Plant aboveground biomass (g m^{-2}) [c]	1126.28 (75.07)	392.27 (124.68)	489.85 (130.55)	122.70 (28.00)	42.70 (15.62)	172.13 (30.57)
Plant root biomass (g m^{-2}) [d]	222.30 (75.09)	103.68 (26.71)	211.57 (45.25)	545.56 (61.57)	988.19 (306.69)	122.47 (58.54)
Soil pH [e]	5.2 (0.2)	5.5 (0.1)	6.7 (0.3)	4.8 (0.1)	4.8 (0.1)	5.9 (0.1)
Sand content (%) [f]	38.23 (4.40)	25.07 (1.97)	31.85 (4.59)	50.17 (0.38)	70.00 (1.15)	82.00 (1.00)
Silt content (%) [g]	41.03 (1.18)	59.33 (1.97)	50.21 (6.83)	31.10 (1.05)	29.10 (1.15)	11.30 (1.53)
Clay content (%) [h]	20.63 (3.33)	15.50 (0.00)	17.94 (2.25)	18.63 (0.67)	0.80 (0.00)	6.60 (0.58)
Soil bulk density (g cm^{-3})	0.70 (0.04)	1.06 (0.05)	0.53 (0.05)	0.27 (0.09)	0.39 (0.06)	0.98 (0.03)
Nutrient addition duration (yrs)	3	4	4	3	4	3
Plots per treatment (n)	3	3	3	3	3	3

Site characteristics	**Shortgrass Steppe**	**Sierra Foothills**	**Spindletop**	**Trelease**	**Ukulinga**	**Duke Forest**
Site code	sgs.us	sier.us	spin.us	trel.us	ukul.za	unc.us
Country	US	US	US	US	ZA	US
Latitude (degree)	40.82	39.24	38.14	40.08	-29.67	36.01
Longitude (degree)	-104.77	-121.28	-84.50	-88.83	30.40	-79.02
Elevation (m)	1650	197	271	200	843	141

MAP (mm)	365	935	1140	982	880	1163
MAT (°C)	8.4	15.6	12.5	11	18.1	14.6
Total soil C (mg C g^{-1} soil) [a]	12.55 (1.75)	21.09 (2.69)	23.34 (0.73)	29.96 (0.90)	46.27 (1.52)	20.17 (1.57)
Total soil N (mg N g^{-1} soil) [b]	1.00 (0.11)	1.90 (0.16)	2.61 (0.04)	2.63 (0.05)	3.16 (0.06)	1.47 (0.12)
Plant aboveground biomass (g m^{-2}) [c]	87.47 (10.61)	324.12 (64.61)	238.43 (66.87)	1609.00 (134.50)	696.53 (144.17)	317.08 (89.08)
Plant root biomass (g m^{-2}) [d]	404.66 (127.62)	59.68 (18.58)	859.25 (456.54)	113.98 (35.57)	177.85 (119.45)	355.74 (187.23)
Soil pH [e]	6.6 (0.1)	5.9 (0.2)	6.1 (0.1)	5.5 (0.0)	5.4 (0.1)	5.5 (0.1)
Sand content (%) [f]	74.67 (1.76)	38.67 (7.06)	29.33 (3.71)	22.20 (4.28)	18.28 (0.66)	56.00 (2.60)
Silt content (%) [g]	14.43 (1.33)	42.43 (4.81)	49.77 (4.06)	62.13 (4.24)	36.98 (1.52)	22.63 (0.57)
Clay content (%) [h]	10.80 (2.00)	18.80 (2.31)	20.80 (1.15)	15.60 (0.00)	44.63 (0.88)	21.27 (2.03)
Soil bulk density (g cm^{-3})	1.07 (0.09)	0.77 (0.03)	1.08 (0.03)	0.59 (0.04)	1.24 (0.09)	0.72 (0.04)
Nutrient addition duration (yrs)	4	4	4	3	3	4
Plots per treatment (n)	3	3	3	3	3	3

[a] Average control plot total soil C concentration (standard error in parentheses).

[b] Average control plot total soil N concentration (standard error in parentheses).

[c] Average control plot plant aboveground biomass (standard error in parentheses).

[d] Average control plot plant root biomass (standard error in parentheses).

[e] Average control plot soil pH (standard error in parentheses).

[f] Average control plot sand content (standard error in parentheses).

[g] Average control plot silt content (standard error in parentheses).

[h] Average control plot clay content (standard error in parentheses).

Table S1.2 – Average observed proportion change in total soil C concentration (g C g^{-1} soil) in response to nutrient treatments relative to the control treatment. [a]

Treatment	Proportion change [a]
Control	0
N	0.088
P	0.023
K	0.091
NP	0.018
NK	0.090
PK	0.018
NPK	0.127

[a] Proportion change in soil C = ((treatment plot soil C – control plot soil C)/control plot soil C)

Table S1.3 – Average observed proportion change in plant aboveground biomass (g m^{-2}) in response to nutrient treatments relative to the control treatment. [a]

Treatment	Proportion change [a]
Control	0
N	0.455
P	0.560
K	0.284
NP	0.673
NK	0.594
PK	0.652
NPK	0.887

[a] Proportion change in aboveground biomass = ((treatment plot aboveground biomass – control plot aboveground biomass)/control plot aboveground biomass)

Table S1.4 – Effects of nutrient addition on plot-level covariates: ANOVA tables.

Effect	Plant aboveground biomass [c]	Plant root biomass [d]	Soil pH	Soil P [e]	Soil K [e]	Soil Ca [e]	Soil Mg [e]
N	**		****			**	*
P	****			****	†	****	
K			*		****		
N:P							
N:K							
P:K							†
N:P:K		*				†	
Marginal R^{2} [a]	0.02	0.01	0.02	0.37	0.12	0.01	0.00
Conditional R^{2} [b]	0.79	0.67	0.79	0.8	0.73	0.89	0.92

Effect	Soil S [e]	Soil Na [e]	Soil Zn [e]	Soil Mn [e]	Soil Fe [e]	Soil Cu [e]	Soil B [e]
N	*			†			
P			*		**		
K	****		***	*	*	****	****
N:P							
N:K							
P:K	*			†			

N:P:K			*				
Marginal $R^{2\,a}$	0.07	0.00	0.01	0.01	0.01	0.15	0.02
Conditional $R^{2\,b}$	0.64	0.86	0.71	0.78	0.79	0.65	0.79

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

[a] Marginal R^2 represents the variance that is explained by fixed effects only.

[b] Conditional R^2 represents the variance that is explained by both fixed and random effects.

[c] Plant aboveground biomass ($g\ m^{-2}$)

[d] Plant root biomass ($g\ m^{-2}$)

[e] Soil exchangeable nutrient concentration (ppm)

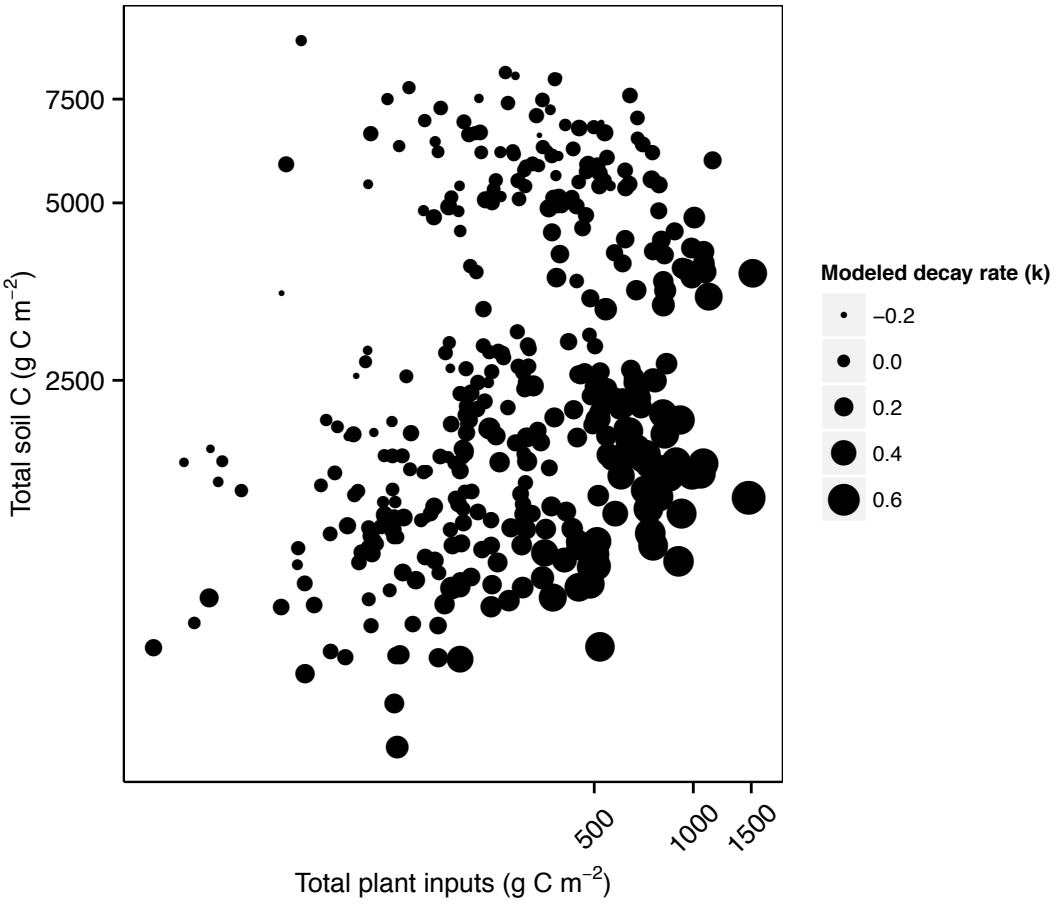

Figure S1.1 – Relationship between modeled decay rate (*k*, yr^{-1}), observed total soil C (g C m^{-2}), and total plant inputs from above- and belowground biomass (g C m^{-2}). Decay rates were computed from a numerical soil C model of observed plant inputs and soil C stocks in each plot.

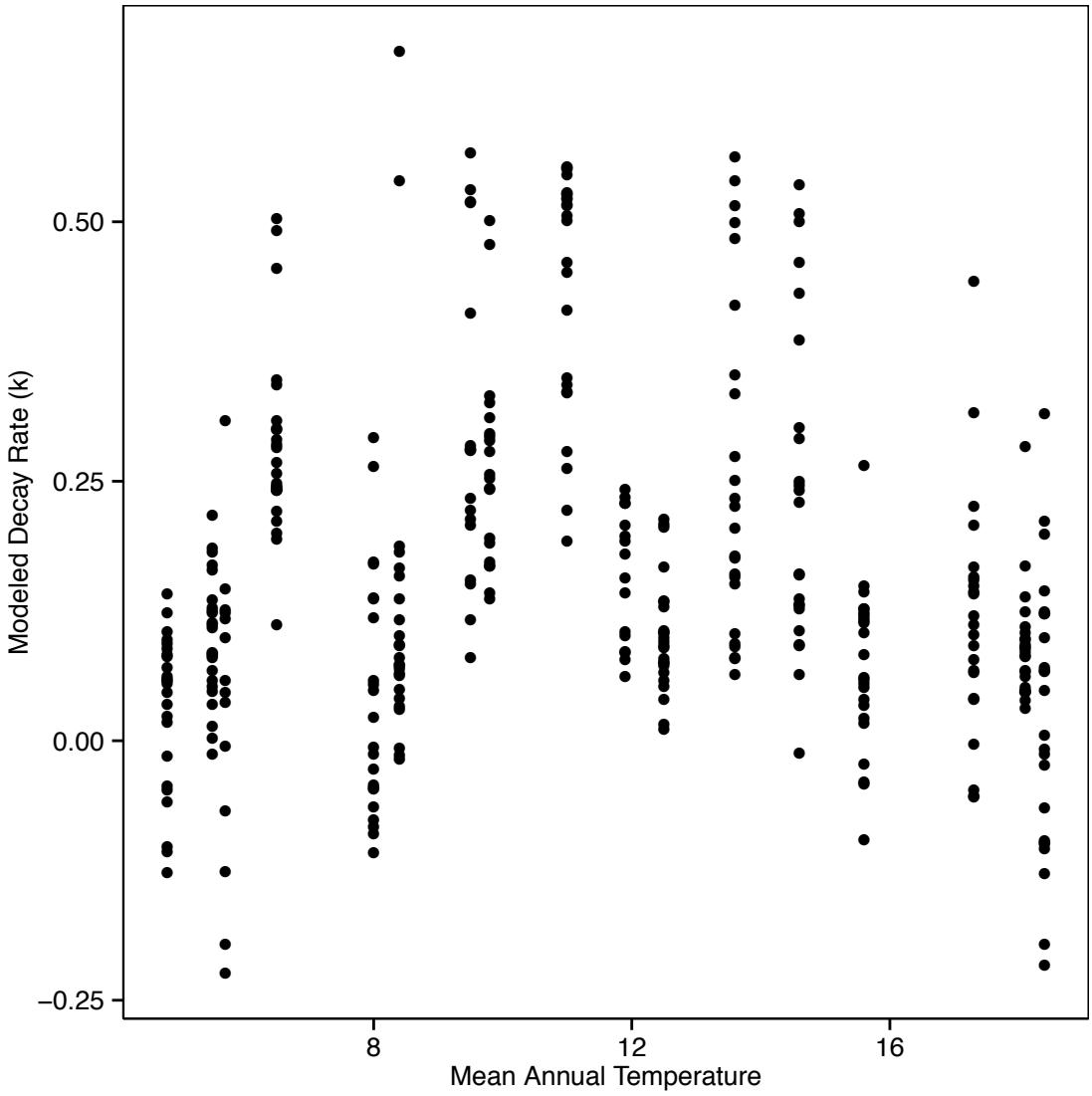

Figure S1.2 – Relationship between modeled decay rate (*k*, yr^{-1}) and mean annual temperature (°C). Decay rates were computed from a numerical soil C model of observed plant inputs and soil C stocks in each plot.

Table S1.5 – Modeled decay rates (*k*): data table.

	Numerical model results: k (yr^{-1})	
Treatment	**Mean**	**Standard Error**
Control	0.1464	0.0181
N	0.1298	0.0244
P	0.1807	0.0199
K	0.1219	0.0245
NP	0.1876	0.0256
NK	0.1398	0.0255
PK	0.1788	0.0226
NPK	0.1710	0.0266

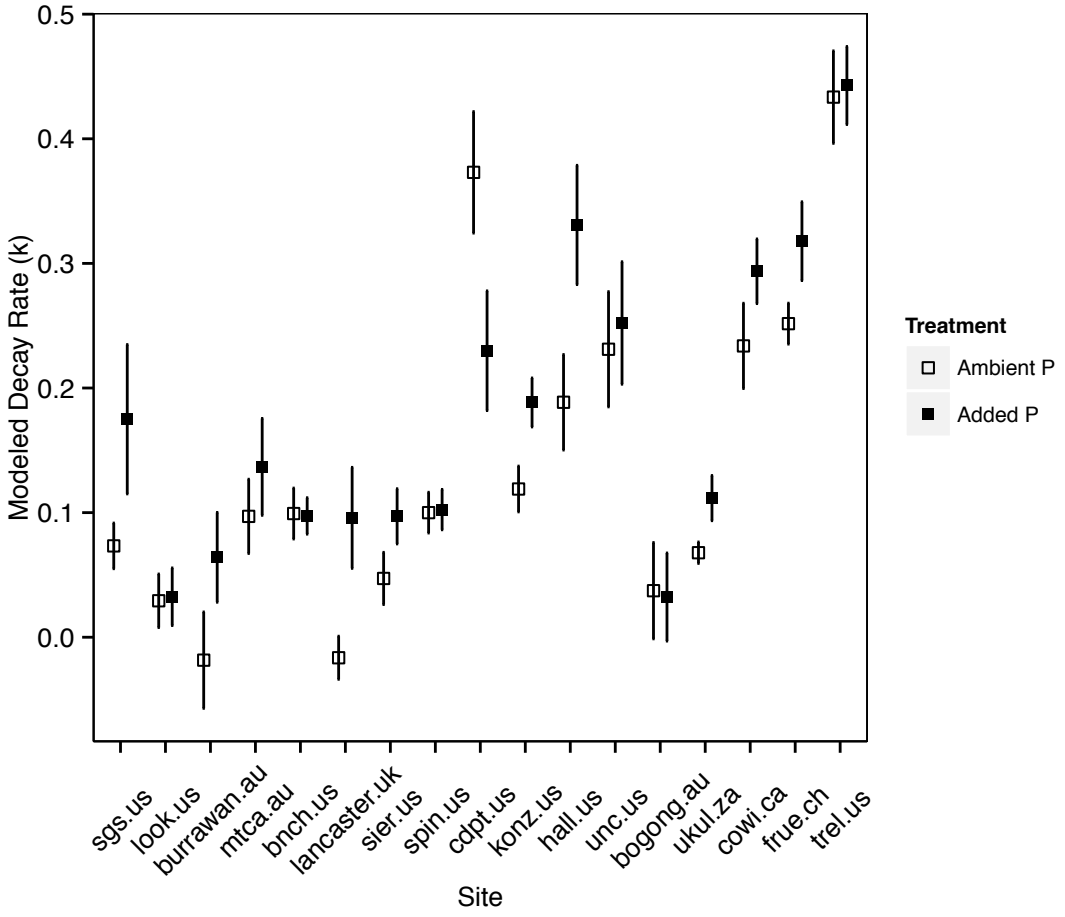

Figure S1.3 – Effects of phosphorus addition on modeled decay rate. Decay rates (k, yr^{-1}) were computed from a numerical soil C model of observed plant inputs and soil C stocks in each plot. Figure shows mean (plus/minus standard error) modeled decay rates (k). Sites are ordered from low to high average plant biomass. Treatment codes: open squares = ambient P plots (Control, N, K, and NK treatments); closed squares = added P plots (P, NP, PK, and NPK treatments).

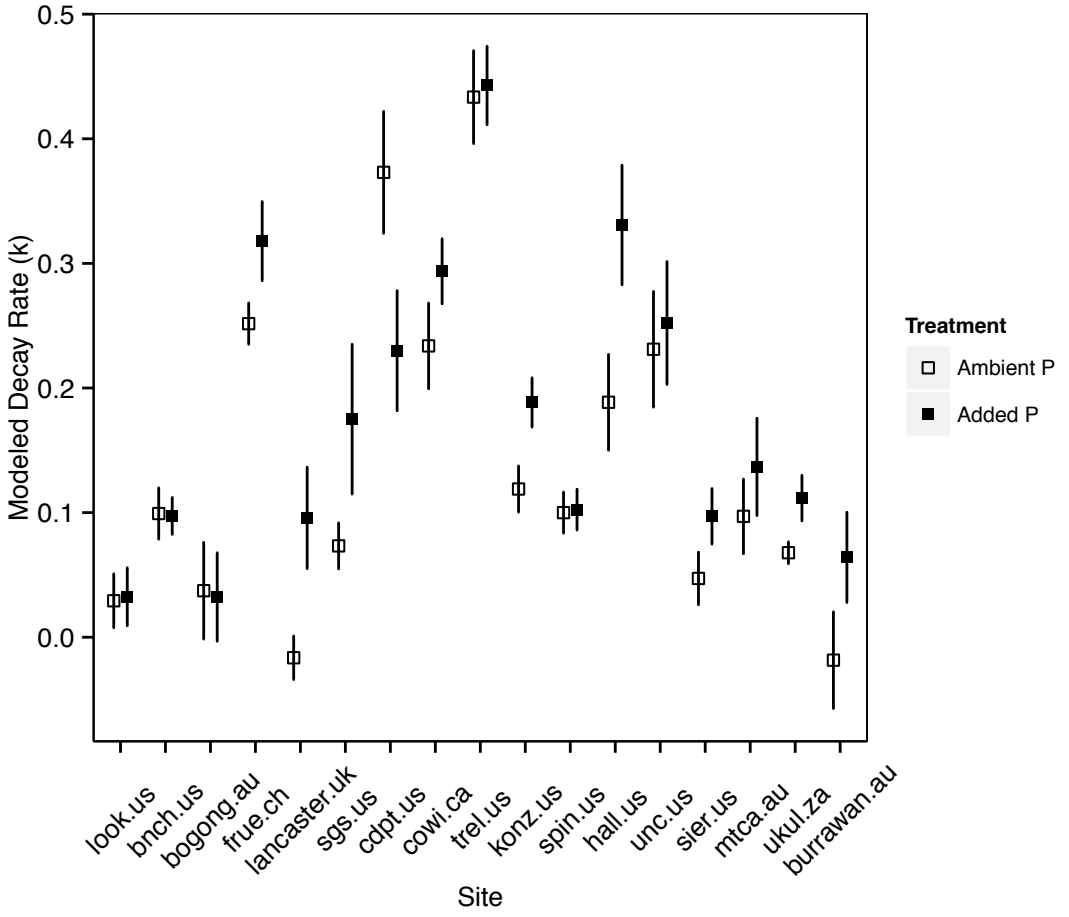

Figure S1.4 – Effects of phosphorus addition on modeled decay rate. Decay rates (k, yr^{-1}) were computed from a numerical soil C model of observed plant inputs and soil C stocks in each plot. Figure shows mean (plus/minus standard error) modeled decay rates (k). Sites are ordered from low to high mean annual temperature. Treatment codes: open squares = ambient P plots (Control, N, K, and NK treatments); closed squares = added P plots (P, NP, PK, and NPK treatments).

Supplement S1.1 – Numerical soil C model sensitivity analyses

The numerical soil C model described in Chapter 1 included several simplifying assumptions due to uncertainty underlying the initial parameters. This was especially true for our estimates of plant biomass inputs. While these simplifying assumptions allowed for hypothesis exploration – namely estimating decay rates given observed plant biomass and total soil C stocks– we also wanted to explore *k* result sensitivity. We explored three variations to the numerical soil C model described in Chapter 1; each variation is described here in turn.

Model variation 1 – In the original model, we used plot-level estimates of root biomass. Since these data are likely associated with a large amount of sampling error (only one core per plot was sampled to measure root biomass stock), we were concerned that extreme values observed at the plot-level would significantly impact modeled decay rate. In model variation 1, we examined model sensitivity to plot-level root biomass estimates by using the average root biomass value at each site instead of the plot-level root biomass value for *B*.

Model variation 2 -- Our original model also assumed that the magnitude of plant biomass inputs (and, consequently, the aboveground biomass response to treatments) was constant across the three years modeled and that all of the aboveground biomass is immediately incorporated into soil. We constructed an alternate model (model variation 2), to produce a "lower bound estimate" of inputs. We used this model to evaluate the effects of nutrient addition on modeled decay rate given that we expect plant biomass response to treatments to increase over time and we expect a time lag between yearly plant biomass production and incorporation into soil.

To produce a "lower bound estimate" of inputs, we used plot aboveground biomass measurements collected after one year of nutrient addition, instead of after three years of nutrient addition, for *A*. The effects of nutrient addition on aboveground biomass after one year of nutrient addition were very small; in particular N and P addition did *not* significantly increase aboveground biomass when applied singly and in combination (Figure S1.8). This is a significant contrast to aboveground biomass responses to nutrient addition observed after three years of nutrient addition (Figure 1.6). Consequently, this model evaluated the effects of minimal nutrient effects on aboveground biomass for modeled decay rate.

Model variation 3 – In the original model, the turnover rate of the root biomass inputs (*B*) was kept constant across all treatments (.52 yr^{-1}) since we did not have information on treatment-specific root turnover rates. In model variation 3, we changed *B* so that root biomass inputs increased in response to nutrient addition. The proportion increase was based on the average increase in fine root production in response to +N, +P, and +NP addition reported in a meta-analysis (Yuan and Chen 2012). Specifically, in the +N treatment, the root biomass turnover rate increased from .52 to .66 yr^{-1} (27 % increase in fine root production in response to N addition from Yuan and Chen (2012)); in the +P treatment, the root biomass turnover rate increased from .52 to .63 yr^{-1} (21 % increase in fine root production in response to P addition from Yuan and Chen (2012)); and in the +NP treatment, the root biomass turnover rate increased from .52 to .73 yr^{-1} (40 % increase in fine root production in response to N and addition from Yuan and Chen (2012)). Since Yuan and Chen (2012) did not have data on +K responses, nor +NK, +PK, and +NPK responses, we made those treatments identical to the +P and +NP treatments, respectively.

Results

Model variations 1-3 resulted in slight differences in estimated *k* values compared to the original numerical soil C model (Figures S1.5-7; Table S1.6). Phosphorus had a stimulating effect on decomposition for all models. The effect was significant in model variations 1 (P main effect: $p = 0.001$) and 2 (P main effect: $p < 0.0001$; see Table S1.7 for ANOVA tables). In model variation 3, there was a moderately significant interactive effect of nutrient addition (N x P x K interaction: $p = 0.07$): the modeled decay rates increased relative to the control treatment for the +P, +NP, and +PK treatments and decreased relative to the control for the +N treatment, on average. Overall, these model variations demonstrate that the stimulating effect of P addition on decomposition (as reported in Chapter 1) is robust across these model differences.

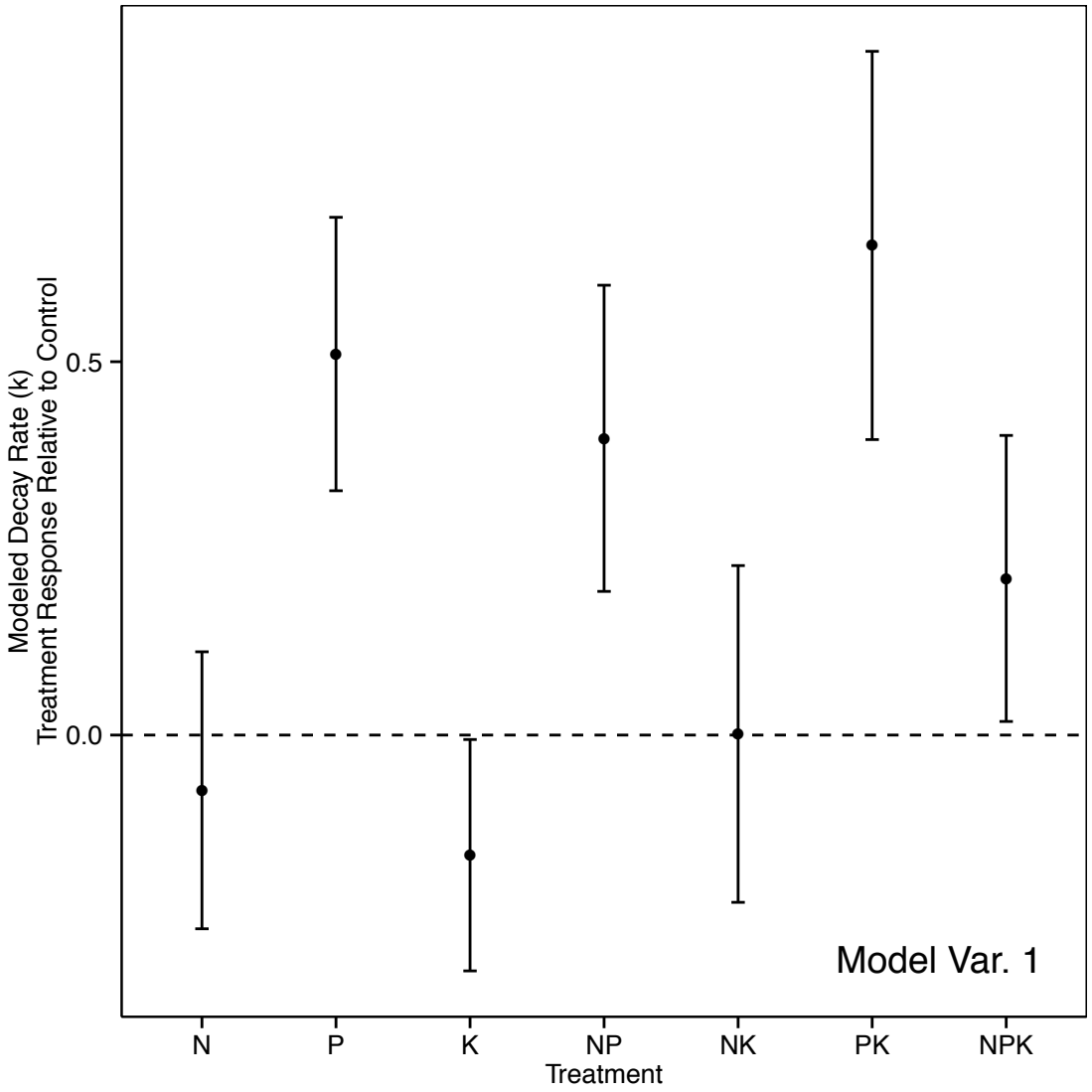

Figure S1.5 – Effects of nutrient addition on proportion change in modeled decay rate (model variation 1). Decay rates (k, yr^{-1}) were computed from a numerical soil C model of observed plant inputs and soil C stocks in each plot. Figure shows mean (plus/minus standard error) modeled decay rates (k) in each treatment relative to the control plot modeled decay rate of each block. Treatment codes: N = nitrogen addition plots; P = phosphorus addition plots; K = potassium plus micronutrient addition plots; NP = nitrogen and phosphorus addition plots; NK = nitrogen and potassium plus micronutrient addition plots; PK = phosphorus and potassium plus micronutrient addition plots; NPK = nitrogen, phosphorus, and potassium plus micronutrient addition plots.

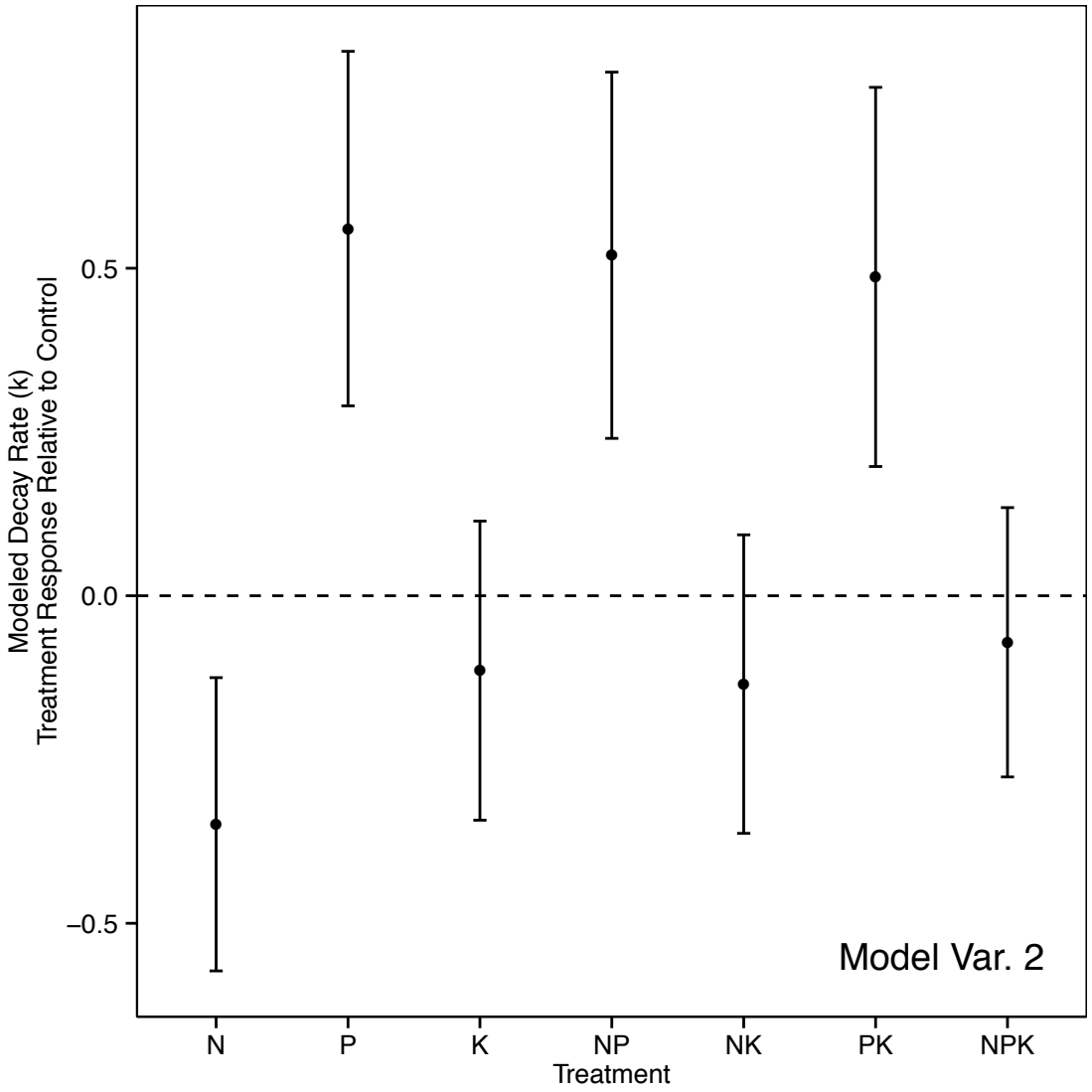

Figure S1.6 – Effects of nutrient addition on proportion change in modeled decay rate (model variation 2). Decay rates (k, yr^{-1}) were computed from a numerical soil C model of observed plant inputs and soil C stocks in each plot. Figure shows mean (plus/minus standard error) modeled decay rates (k) in each treatment relative to the control plot modeled decay rate of each block. Treatment codes: N = nitrogen addition plots; P = phosphorus addition plots; K = potassium plus micronutrient addition plots; NP = nitrogen and phosphorus addition plots; NK = nitrogen and potassium plus micronutrient addition plots; PK = phosphorus and potassium plus micronutrient addition plots; NPK = nitrogen, phosphorus, and potassium plus micronutrient addition plots.

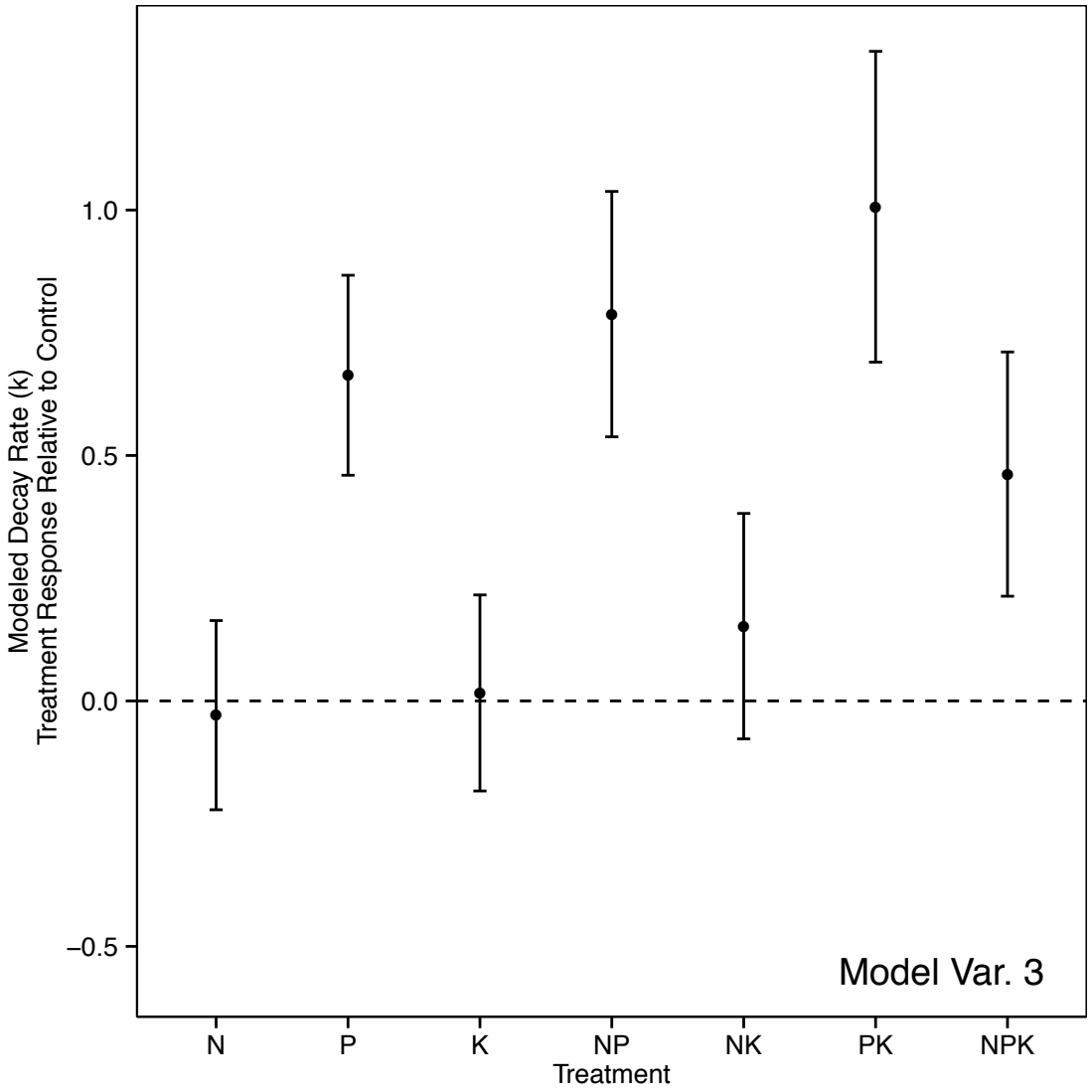

Figure S1.7 – Effects of nutrient addition on proportion change in modeled decay rate (model variation 3). Decay rates (k, yr^{-1}) were computed from a numerical soil C model of observed plant inputs and soil C stocks in each plot. Figure shows mean (plus/minus standard error) modeled decay rates (k) in each treatment relative to the control plot modeled decay rate of each block. Treatment codes: N = nitrogen addition plots; P = phosphorus addition plots; K = potassium plus micronutrient addition plots; NP = nitrogen and phosphorus addition plots; NK = nitrogen and potassium plus micronutrient addition plots; PK = phosphorus and potassium plus micronutrient addition plots; NPK = nitrogen, phosphorus, and potassium plus micronutrient addition plots.

Table S1.6 – Modeled decay rates (*k*): data tables. [a]

Treatment	Model variation 1	Model variation 2	Model variation 3
Control	0.1430 (0.0168)	0.1501 (0.0210)	0.1446 (0.0178)
N	0.1368 (0.0231)	0.1103 (0.0222)	0.1406 (0.0249)
P	0.1743 (0.0184)	0.1462 (0.0195)	0.1886 (0.0201)
K	0.1214 (0.0224)	0.1272 (0.0267)	0.1295 (0.0247)
NP	0.1758 (0.0239)	0.1513 (0.0244)	0.2028 (0.0257)
NK	0.1450 (0.0254)	0.1373 (0.0255)	0.1558 (0.0252)
PK	0.1745 (0.0223)	0.1572 (0.0243)	0.1910 (0.0229)
NPK	0.1636 (0.0241)	0.1340 (0.0263)	0.1862 (0.0274)

[a] Average modeled decay rates in each treatment (standard error in parentheses).

Table S1.7 – Modeled decay rates (*k*): ANOVA tables.

Effect	Model variation 1	Model variation 2	Model variation 3
N			
P	***		****
K			
N:P			
N:K			
P:K			
N:P:K		†	
Marginal R^2 [a]	0.02	0.01	0.03
Conditional R^2 [b]	0.56	0.55	0.53

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

[a] Marginal R^2 represents the variance that is explained by fixed effects only.

[b] Conditional R^2 represents the variance that is explained by both fixed and random effects.

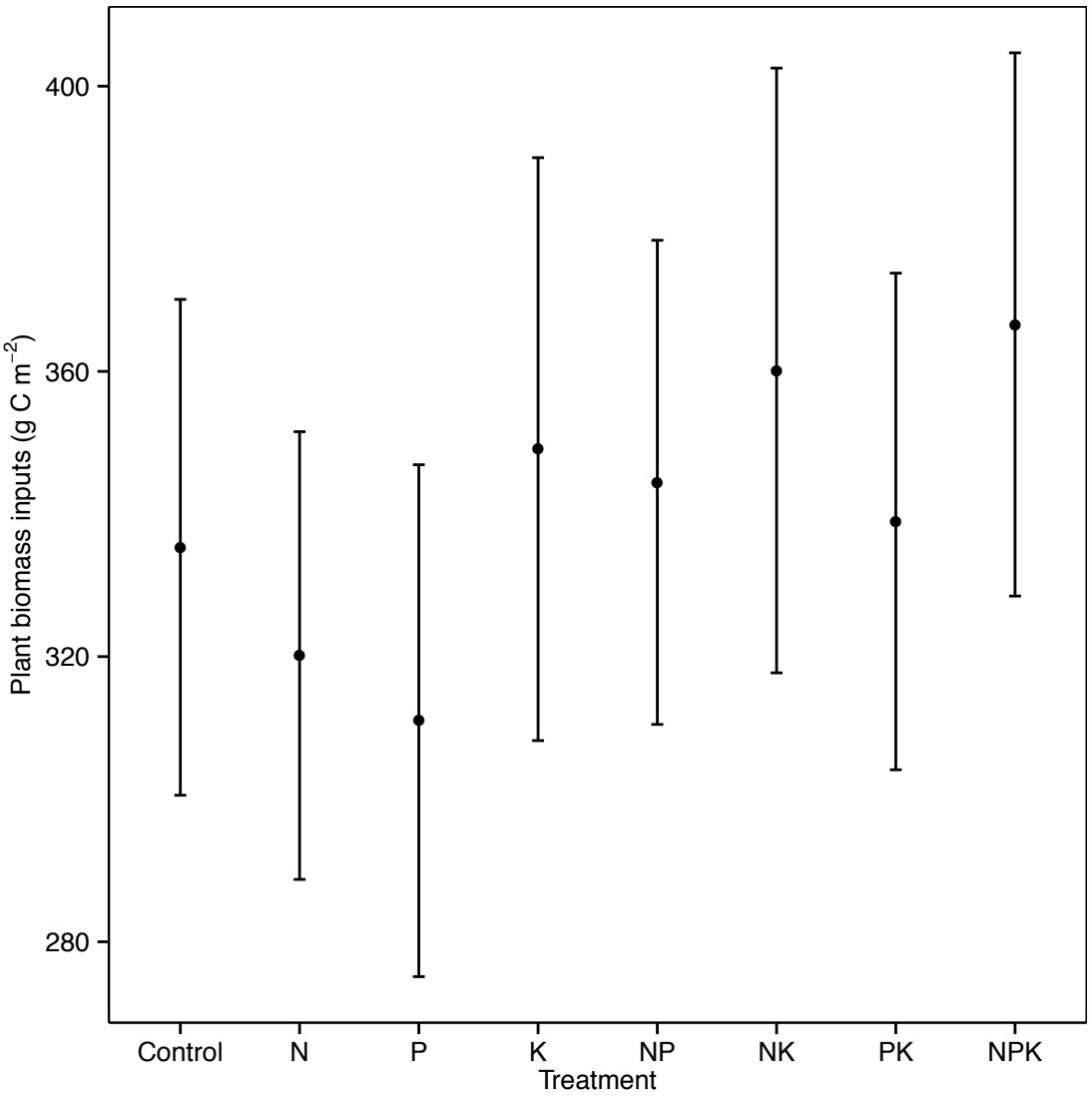

Figure S1.8 – Nutrient treatment effect on plant biomass inputs (g C m^{-2}) after one year of nutrient addition. Figure shows mean +/- one standard error. Treatment codes: Control = control plots; N = nitrogen addition plots; P = phosphorus addition plots; K = potassium plus micronutrient addition plots; NP = nitrogen and phosphorus addition plots; NK = nitrogen and potassium plus micronutrient addition plots; PK = phosphorus and potassium plus micronutrient addition plots; NPK = nitrogen, phosphorus, and potassium plus micronutrient addition plots.

APPENDIX 2 – SUPPLEMENTAL MATERIAL FOR CHAPTER 2

Table S2.1 – Experimental plots sampled for each analysis performed in this study.

Analysis	All plots [a]	Control and +N plots	Control plots only
Microbial respiration	X		
Total soil C and N	X		
Microbial biomass C and N	X		
POM C and N [b]	X		
Soil pH	X		
Net N mineralization	X		
Water-stable soil aggregates		X	
Root biomass		X	
Mycorrhizal colonization of root biomass		X	
Soil texture			X

[a] Full factorial nutrient experiment: control, +N, +P, +K, +NP, +NK, +PK, and +NPK plots.

[b] POM: particulate organic matter.

Table S2.2 – Number of experimental plots included in the statistical analysis of each variable measured. [a]

	Cedar Creek, Minnesota		Cedar Point, Nebraska		Chichaqua Bottoms, Iowa		Konza Prairie, Kansas		Shortgrass Steppe, Colorado	
Analysis	Ambient N [b]	Added N [b]	Ambient N [b]	Added N [b]	Ambient N [b]	Added N [b]	Ambient N [b]	Added N [b]	Ambient N [b]	Added N [b]
Microbial respiration	20	20	12	12	24	24	12	12	12	12
Total soil C and N	20	20	12	12	24	24	12	12	12	12
Microbial biomass C and N	20	20	12	12	24	24	12	12	12	12
POM C and N [c]	17	20	12	12	24	24	12	12	11	12
Soil pH	20	20	12	12	24	24	12	12	12	12
Net N mineralization	20	20	12	12	24	24	12	12	12	12
Water-stable soil aggregates	5	5	3	3	6	6	3	3	3	3
Root biomass	5	5	3	3	6	6	3	3	3	3
Mycorrhizal colonization of root biomass	3	5	3	0	6	5	3	3	2	3

[a] Plots were excluded from statistical analyses because the sample was missing or there was sample contamination during lab analyses.

[b] Treatment codes: For the analyses where the full nutrient factorial was sampled, ambient N includes all plots where N was not added (control, +P, +K, +PK plots); added N includes all N addition plots (+N, +NP, +NK, +NPK). For the analyses where only control and +N plots were sampled, ambient N = control plots and added N = +N plots.

[c] POM: particulate organic matter.

Supplement S2.1 – Evaluation of equifinality from maximum-likelihood estimation (MLE)

Parameter estimates fit using maximum-likelihood estimation (MLE) can result in equifinality: multiple combinations of parameters that produce equally good model fits (Beven 2006). We evaluated whether equifinality in parameter estimates was possible in the parameter space of our decomposition parameter estimates by randomly generating 50,000 parameter combinations for each sample. The parameters were randomly selected from a defined parameter space that spanned from 0.33x to 3x of the parameter values fit with MLE (using the bbmle package in R). We fit both the one-pool and two-pool models (see *Chapter 2 Methods* for model details) with these randomly generated parameters for each sample. We then compared the predicted C respiration rate (mg C g $soil^{-1}$ day^{-1}) to the actual C respiration rate of each sample to generate an R^2 value for each randomly generated parameter combination.

In all cases, the best R^2 of the randomly generated parameter combinations were no better than, but similar to, those selected using MLE. Furthermore, the best-fit models from the randomly generated parameter set converged on one area of parameter space, indicating that there are not multiple combinations of parameters that result in equally good model fits. See Figure S2.1 for illustrative examples of three samples for which we randomly generated 1,000,000 parameter combinations and evaluated model fit (R^2) against our MLE parameter values.

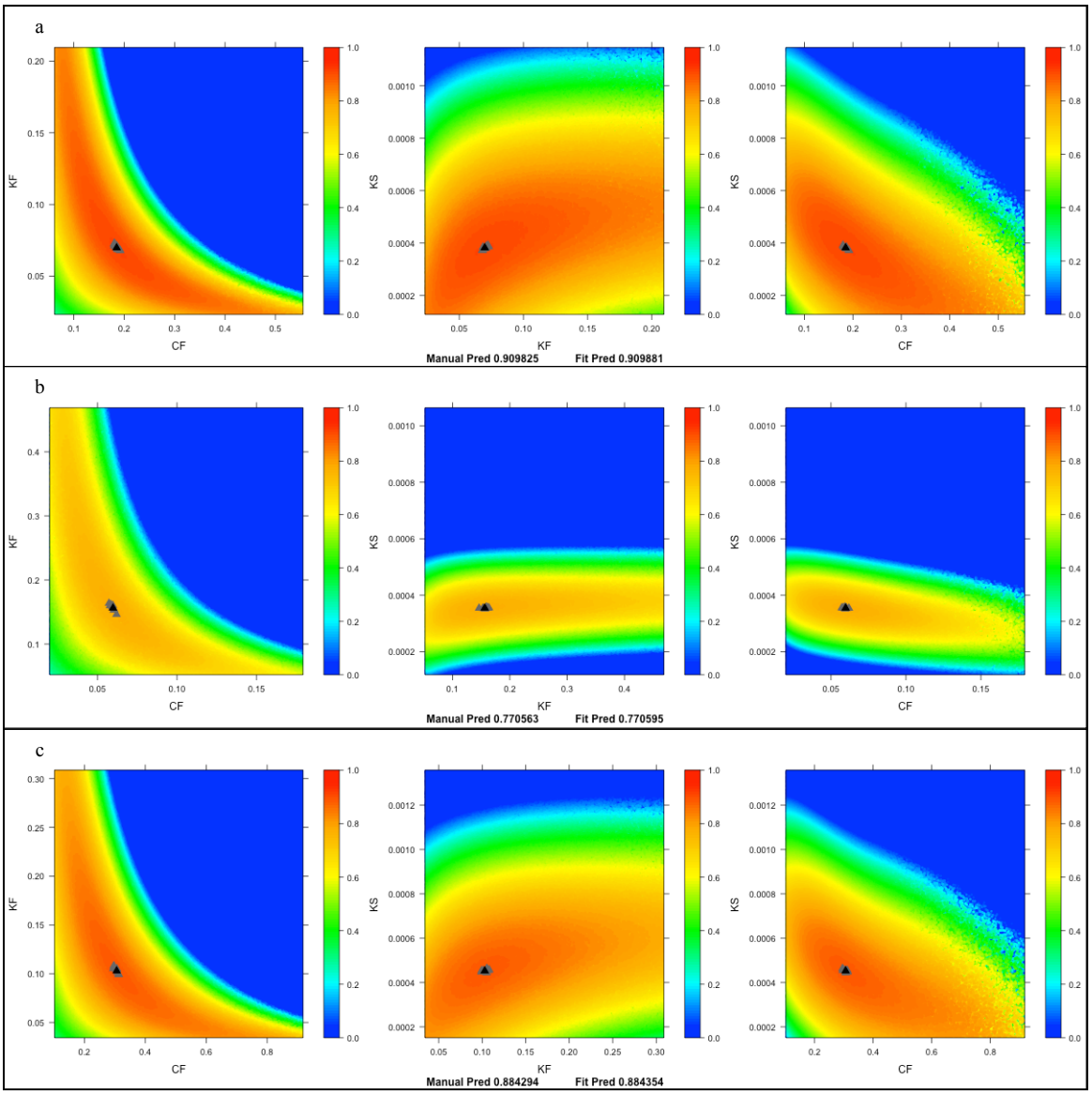

Figure S2.1 – Model fits (R^2) of randomly generated parameter combinations versus model fit (R^2) of MLE parameter values from three soil samples. In each panel, the graphs show R^2 (color coded) of 1,000,000 randomly generated combinations of the two-pool model parameters (from left to right): fast pool decay (k_f) versus fast pool size (C_f); slow pool decay (k_s) versus fast pool decay (k_f); and slow pool decay (k_s) versus fast pool size (C_f). Grey triangles are the top ten best parameter combinations (based on R^2) from the randomly generated parameters. The black triangle shows the parameter values selected with MLE. The average R^2 from the top ten best parameter combinations ("Manual Pred") was always less than the R^2 from the MLE parameters ("Fit Pred"). Panel a: Chichaqua Bottoms (Iowa) control plot, sample

number 24. Panel b: Cedar Creek (Minnesota) +K plot, sample number 57. Panel c: Cedar Point (Nebraska) +PK plot, sample number 14.

Table S2.3 – ANOVA table: decomposition parameters and cumulative respiration from the microbial respiration incubation.

Effect	k_f	k_s	C_f	C_s	Cumulative C respired
Site	***	****	*	****	****
N	*	*	†		***
P					
K					
N x P					
N x K					
P x K				*	
N x P x K					
Site x N	**	*	NA	NA	*
Marginal R^2 [a]	0.3614	0.3865	0.1723	0.7887	0.6226
Conditional R^2 [b]	0.3614	0.3958	0.2006	0.8023	0.6593

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, NA = non-significant interaction term removed from model.

[a] Marginal R^2 represents the variance that is explained by fixed effects only.

[b] Conditional R^2 represents the variance that is explained by both fixed and random effects.

Table S2.4 – ANOVA table: aggregate-occluded and mineral-associated soil fractions.

Effect	Large macro-aggregate fraction	Small macro-aggregate fraction	Micro-aggregate fraction	Mineral-associated fraction
Site	****	†	****	****
N	†			
Site x N	NA	NA	NA	NA
Marginal R^2 [a]	0.7862	0.4900	0.8784	0.8315
Conditional R^2 [b]	0.7862	0.7663	0.8842	0.9130

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, NA = non-significant interaction term removed from model.

[a] Marginal R^2 represents the variance that is explained by fixed effects only.

[b] Conditional R^2 represents the variance that is explained by both fixed and random effects.

Table S2.5 – ANOVA table: additional soil variables.

Effect	Total soil C	Total soil N	Soil C:N ratio
Site	****	****	****
N		*	***
P			
K			
N x P			
N x K			
P x K	*	*	
N x P x K			
Site x N	NA	NA	NA
Marginal R^2 [a]	0.7902	0.7966	0.6829
Conditional R^2 [b]	0.8042	0.8143	0.7081

Effect	Microbial C	Microbial N	Microbial C:N ratio
Site	****	****	*
N			
P	†		
K			
N x P			
N x K			
P x K			
N x P x K			
Site x N	NA	*	*
Marginal R^2 [a]	0.7003	0.6057	0.2023
Conditional R^2 [b]	0.7115	0.6057	0.2023

Effect	POM C [c]	POM N [c]	POM C:N ratio [c]
Site	**	***	**
N	*	**	
P			
K			
N x P	**		**
N x K			
P x K			

N x P x K			
Site x N	NA	NA	NA
Marginal $R^{2\,a}$	0.3586	0.3954	0.2879
Conditional $R^{2\,b}$	0.4607	0.5113	0.3921

Effect	Soil pH	Net N mineralization
Site	****	****
N	****	****
P		
K		
N x P	*	
N x K	*	
P x K		
N x P x K		
Site x N	NA	****
Marginal $R^{2\,a}$	0.6439	0.6495
Conditional $R^{2\,b}$	0.7318	0.6952

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, NA = non-significant interaction term removed from model.

[a] Marginal R^2 represents the variance that is explained by fixed effects only.

[b] Conditional R^2 represents the variance that is explained by both fixed and random effects.

[c] POM: particulate organic matter.

Table S2.6 – ANOVA table: root variables.

Effect	Root biomass	Mycorrhizal colonized root biomass (absolute)	Mycorrhizal colonized root biomass (%)
Site	****	****	†
N		*	†
Site x N	NA	NA	NA
Marginal R^2 [a]	0.7338	0.8057	0.3759
Conditional R^2 [a]	0.7338	0.8133	0.3759

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, NA = non-significant interaction term removed from model.

[a] Marginal R^2 represents the variance that is explained by fixed effects only.

[b] Conditional R^2 represents the variance that is explained by both fixed and random effects.

Table S2.7 – Data table: soil aggregates

Variable	Overall (across sites)		Cedar Creek, Minnesota		Cedar Point, Nebraska		Chichaqua Bottoms, Iowa		Konza Prairie, Kansas		Shortgrass Steppe, Colorado	
	Control [a]	+N [a]	Control [a]	+N [a]	Control [a]	+N [a]	Control [a]	+N [a]	Control [a]	+N [a]	Control [a]	+N [a]
Mean weighted diameter (mm) [b]	1.24 (0.23)	1.33 (0.23)	0.74 (0.02)	0.87 (0.05)	0.84 (0.08)	0.87 (0.13)	0.88 (0.04)	1.07 (0.13)	3.48 (0.55)	3.49 (0.57)	0.93 (0.01)	0.95 (0.08)
Large macro-aggregate fraction (g aggregate 100 g^{-1} soil)	14.57 (5.06)	16.62 (5.01)	2.75 (0.56)	4.14 (0.81)	9.74 (1.31)	11.26 (2.97)	4.68 (0.73)	9.20 (2.64)	63.59 (13.36)	64.40 (13.48)	9.86 (0.69)	9.84 (1.09)
Small macro-aggregate fraction (g aggregate 100 g^{-1} soil)	39.44 (3.20)	38.87 (3.44)	47.07 (3.61)	52.91 (3.98)	22.75 (1.98)	18.83 (0.92)	52.10 (1.62)	48.22 (0.54)	26.17 (10.63)	22.28 (8.74)	31.37 (2.31)	33.39 (3.72)
Micro-aggregate fraction (g aggregate 100 g^{-1} soil)	41.19 (3.89)	39.84 (3.81)	47.61 (3.10)	39.78 (4.19)	59.47 (2.30)	61.51 (2.67)	40.35 (1.85)	39.96 (3.00)	4.88 (0.75)	8.23 (3.13)	50.18 (1.66)	49.61 (4.26)
Mineral-associated fraction (g aggregate 100 g^{-}	4.80 (0.64)	4.67 (0.59)	2.57 (0.24)	3.17 (0.68)	8.04 (0.66)	8.39 (1.02)	2.86 (0.40)	2.62 (0.36)	5.35 (1.98)	5.08 (1.64)	8.60 (0.24)	7.15 (0.36)

^{1} soil)												
Large macro-aggregate fraction C concentration (mg aggregate C g^{-1} aggregate soil)	16.77 (2.29)	19.54 (3.44)	18.62 (1.99)	31.53 (9.78)	13.54 (1.10)	14.61 (2.64)	10.45 (0.92)	10.11 (1.24)	37.51 (4.21)	35.12 (2.28)	8.80 (0.36)	7.78 (0.53)
Small macro-aggregate fraction C concentration (mg aggregate C g^{-1} aggregate soil)	15.20 (2.88)	16.70 (3.01)	8.77 (1.08)	14.45 (5.48)	21.06 (2.38)	21.86 (4.50)	7.98 (1.65)	8.44 (1.00)	41.93 (3.42)	41.52 (2.35)	7.79 (0.53)	6.97 (0.34)
Micro-aggregate fraction C concentration (mg aggregate C g^{-1} aggregate soil)	13.46 (2.80)	13.72 (2.73)	9.41 (0.95)	12.46 (2.78)	9.07 (0.44)	7.5 (1.11)	7.94 (0.66)	7.72 (1.07)	41.72 (4.45)	40.34 (2.73)	7.37 (0.51)	7.4 (0.91)
Mineral-associated fraction C concentration (mg aggregate C g^{-1} aggregate soil)	25.51 (2.00)	26.08 (2.05)	38.03 (3.91)	38.78 (3.03)	18.96 (0.84)	17.99 (1.04)	23.49 (1.46)	25.64 (2.05)	22.16 (1.48)	22.64 (1.11)	18.56 (2.05)	17.30 (0.86)
Large macro-aggregate fraction C	4.02 (1.84)	4.28 (1.77)	0.50 (0.12)	1.25 (0.37)	1.31 (0.16)	1.63 (0.45)	0.52 (0.12)	1.01 (0.38)	22.74 (2.94)	22.01 (3.57)	0.87 (0.09)	0.76 (0.08)

concentration (mg aggregate C g^{-1} soil)												
Small macro-aggregate fraction C concentration (mg aggregate C g^{-1} soil)	5.08 (1.00)	5.62 (1.09)	4.05 (0.38)	7.90 (3.18)	4.81 (0.79)	4.14 (0.92)	4.10 (0.74)	4.08 (0.51)	11.68 (5.69)	9.63 (4.24)	2.45 (0.27)	2.34 (0.33)
Micro-aggregate fraction C concentration (mg aggregate C g^{-1} soil)	3.77 (0.31)	3.81 (0.33)	4.51 (0.66)	4.72 (0.84)	5.41 (0.42)	4.56 (0.52)	3.20 (0.30)	2.95 (0.25)	2.10 (0.55)	3.46 (1.51)	3.69 (0.20)	3.61 (0.29)
Mineral-associated fraction C concentration (mg aggregate C g^{-1} soil)	1.09 (0.11)	1.09 (0.12)	0.96 (0.10)	1.22 (0.28)	1.51 (0.06)	1.53 (0.28)	0.67 (0.11)	0.66 (0.08)	1.23 (0.51)	1.17 (0.42)	1.59 (0.17)	1.23 (0.07)

Values are mean (and standard error in parentheses).

[a] Only Control and +N plots were analyzed. See Appendix 2 – Table S2.2 for sample numbers.

[b] Mean weighted diameter (mm) = 5 x (P_{weight} >2000 um) + 1.125 x (P_{weight} >250 um) + .157 x (P_{weight} >53um) + .027 x (P_{weight} <53um); where P_{weight} is the proportion of total dry soil mass in each aggregate fraction.

APPENDIX 3 – SUPPLEMENTAL MATERIAL FOR CHAPTER 3

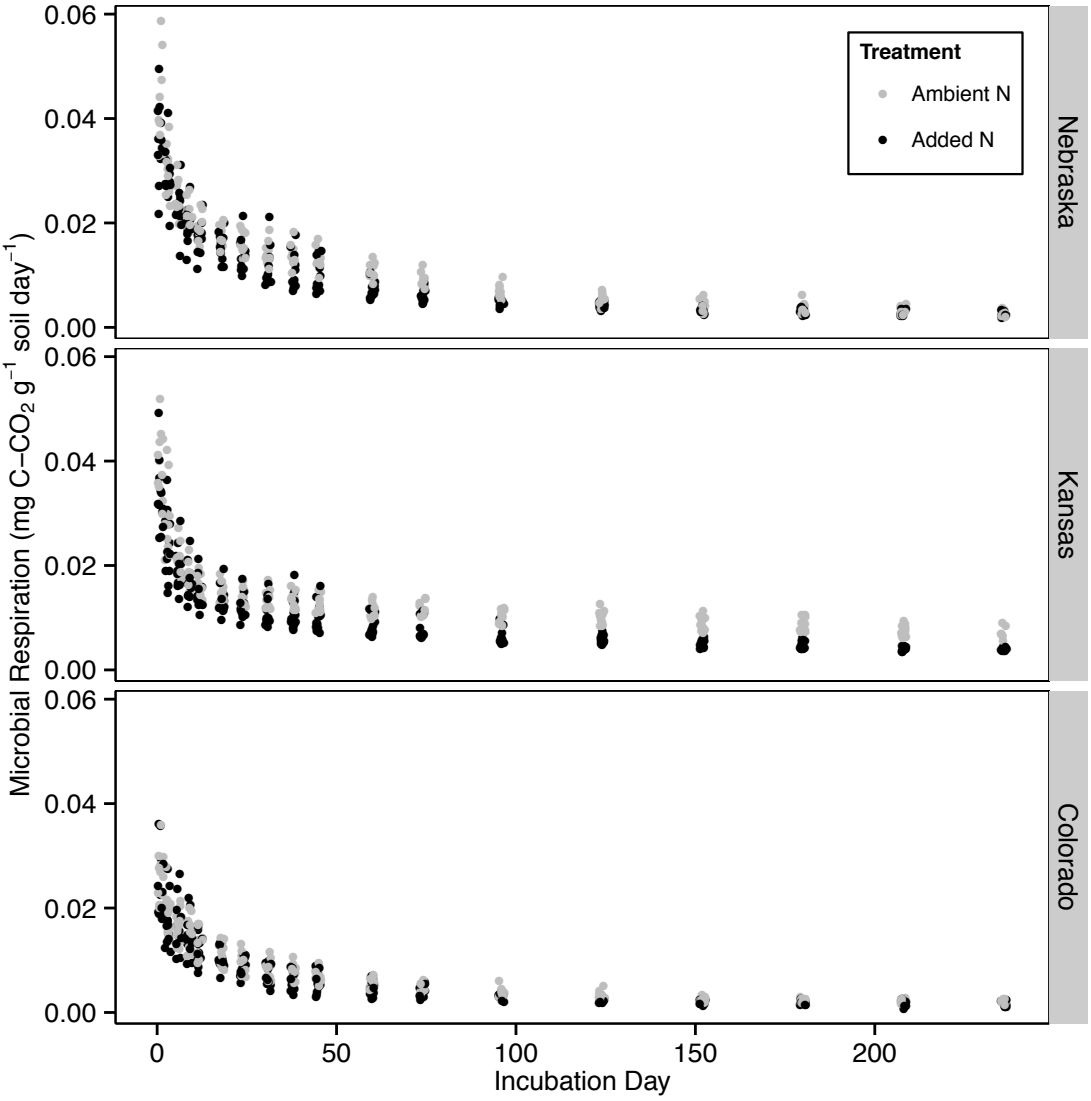

Figure S3.1 – Effect on nitrogen treatment on microbial respiration rate from a long-term laboratory incubation. Treatment codes: grey = ambient nitrogen plots; black = added nitrogen plots.

Supplement S3.1 – Evaluation of equifinality from maximum-likelihood estimation (MLE)

Parameter estimation using maximum-likelihood estimation (MLE) can result in multiple parameter combinations that produce equally good model fits, also known as equifinality (Beven 2006). We tested for equifinality by randomly generating 50,000 parameter combinations that spanned from 0.33x to 3x of the parameter values fit with MLE (using the bbmle package in R). We then used these randomly generated parameters to predict C respiration rates (mg C g $soil^{-1}$ day^{-1}) from both the one-pool and two-pool models (see *Methods* for model details). We evaluated model fit (R^2) by comparing the predicted C respiration rate from the randomly generated parameter estimates to the actual C respiration rate of each sample. The randomly generated parameters that fit that data best always converged on one area of the parameter space and were always similar to, but no better than, than the parameters selected using MLE (Appendix 3 – Figure S3.2). This suggests that there are not multiple parameter combinations that result in equally good model fits and that MLE selected the best model.

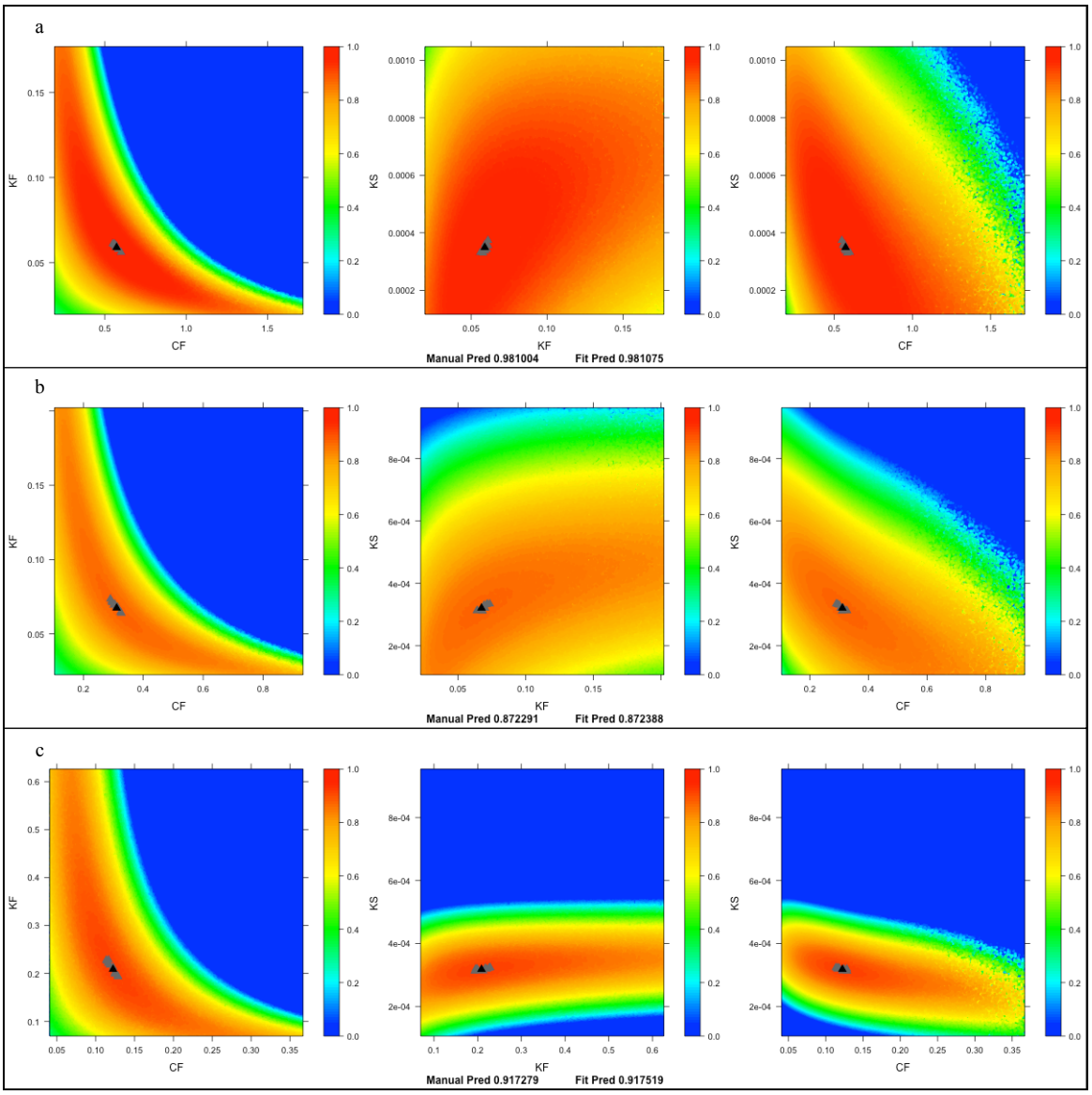

Figure S3.2 – Model fits (R^2) of randomly generated parameter combinations versus model fit (R^2) of MLE parameter values from three soil samples. In each panel, the graphs show R^2 (color coded) of 1,000,000 randomly generated combinations of the two-pool model parameters (from left to right): fast pool decay (k_f) versus fast pool size (C_f); slow pool decay (k_s) versus fast pool decay (k_f); and slow pool decay (k_s) versus fast pool size (C_f). Grey triangles are the top ten best parameter combinations (based on R^2) from the randomly generated parameters. The black triangle shows the parameter values selected with MLE. The average R^2 from the top ten best parameter combinations ("Manual Pred") was always less than the R^2 from the MLE parameters ("Fit Pred"). Panel a: Cedar Point (Nebraska) +NPK plot, sample number

10. Panel b: Shortgrass Steppe (Colorado) +NK plot, sample number 25. Panel c: Konza Prairis (Kansas) control plot, sample number 18.

Table S3.1 – Soil and microbial C and N: ANOVA tables.

Effect	Soil C (mg C g^{-1} soil)	Soil N (mg N g^{-1} soil)	Soil C:N ratio	Dissolved organic C (µg C mg^{-1} soil C)	Microbial biomass C (µg C mg^{-1} soil C)	Microbial biomass N (µg N mg^{-1} soil N)	Microbial biomass C:N ratio
Site	****	****	***	****	***	***	***
N				***	****	**	**
P							
K					*	*	
N x P							†
N x K					*	*	
P x K							
N x P x K							
Site x N	NA	NA	NA	NA	*	*	NA
Marginal R^2 [a]	0.94	0.93	0.60	0.94	0.65	0.63	0.55
Conditional R^2 [b]	0.94	0.93	0.60	0.95	0.65	0.63	0.55

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, NA = non-significant interaction term removed from model.

[a] Marginal R^2 represents the variance that is explained by fixed effects only.

[b] Conditional R^2 represents the variance that is explained by both fixed and random effects.

Table S3.2 – Soil and microbial C and N: data table. Values are mean (standard error in parentheses).

Site	Treatment	n	Soil C (mg C g^{-1} soil)	Soil N (mg N g^{-1} soil)	Soil C:N ratio	Dissolved organic C (µg C mg^{-1} soil C)
Nebraska	Control	3	14.01 (2.26)	1.11 (0.20)	12.71 (0.36)	2.05 (0.24)
	N	3	13.58 (1.40)	1.12 (0.09)	12.11 (0.32)	2.89 (0.27)
	P	3	14.84 (1.37)	1.20 (0.14)	12.46 (0.47)	2.64 (0.19)
	K	3	15.30 (1.00)	1.12 (0.18)	14.06 (1.31)	2.63 (0.47)
	NP	2	12.95 (2.45)	0.10 (0.22)	13.06 (0.40)	2.87 (0.43)
	NK	3	14.01 (0.51)	1.11 (0.02)	12.65 (0.39)	3.27 (0.73)
	PK	2	16.51 (0.28)	1.33 (0.03)	12.41 (0.07)	2.23 (0.52)
	NPK	3	13.13 (1.31)	1.06 (0.11)	12.39 (0.04)	3.67 (0.58)
Kansas	Control	3	37.34 (5.14)	2.80 (0.32)	13.31 (0.28)	0.46 (0.01)
	N	3	37.62 (3.28)	2.93 (0.23)	12.83 (0.14)	0.545 (0.04)
	P	3	37.56 (1.40)	2.82 (0.14)	13.36 (0.25)	0.50 (0.08)
	K	3	38.52 (2.11)	2.84 (0.12)	13.55 (0.20)	0.50 (0.06)
	NP	3	36.01 (1.32)	2.90 (0.11)	12.40 (0.03)	0.61 (0.05)
	NK	3	40.16 (3.08)	3.06 (0.33)	13.25 (0.67)	0.56 (0.01)
	PK	3	40.49 (1.61)	3.05 (0.10)	13.25 (0.07)	0.60 (0.08)
	NPK	3	39.70 (3.02)	3.07 (0.16)	12.91 (0.37)	0.58 (0.04)
Colorado	Control	3	9.27 (1.24)	0.82 (0.13)	11.37 (0.52)	3.15 (0.62)
	N	3	9.08 (1.15)	0.86 (0.09)	10.49 (0.47)	4.27 (0.39)
	P	3	8.36 (0.74)	0.75 (0.07)	11.23 (0.85)	3.11 (0.73)

K	3	8.66 (0.62)	0.83 (0.03)	10.42 (0.50)	2.97 (0.48)
NP	3	10.83 (1.14)	0.97 (0.05)	11.12 (0.83)	3.91 (1.12)
NK	3	11.47 (0.99)	1.00 (0.09)	11.45 (0.09)	3.47 (0.14)
PK	3	9.60 (1.44)	0.89 (0.12)	10.73 (0.36)	3.14 (0.43)
NPK	3	8.50 (1.10)	0.75 (0.05)	11.20 (0.63)	4.48 (0.88)

Site	Treatment	n	Microbial biomass C (µg C g^{-1} soil)	Microbial biomass N (µg N g^{-1} soil)	Microbial biomass C:N ratio	Microbial biomass C (µg C mg^{-1} soil C)	Microbial biomass N (µg N mg^{-1} soil N)
Nebraska	Control	3	491.07 (116.07)	62.95 (6.86)	7.67 (1.30)	37.04 (11.48)	58.62 (7.42)
	N	3	306.09 (39.78)	44.09 (8.24)	7.10 (0.43)	22.42 (0.67)	38.75 (4.06)
	P	3	346.40 (35.54)	49.26 (5.36)	7.04 (0.06)	23.55 (2.66)	42.03 (6.40)
	K	3	323.13 (24.45)	48.66 (6.24)	6.74 (0.42)	21.10 (0.28)	43.81 (1.32)
	NP	2	321.87 (2.34)	48.30 (1.98)	6.67 (0.22)	25.82 (5.06)	51.29 (13.17)
	NK	3	355.03 (26.70)	50.50 (3.42)	7.03 (0.12)	25.35 (1.68)	45.53 (2.23)
	PK	2	334.94 (3.53)	48.67 (1.88)	6.89 (0.34)	20.29 (0.56)	36.55 (0.58)
	NPK	3	291.08 (24.89)	46.97 (1.91)	6.18 (0.37)	22.25 (0.75)	44.87 (2.90)
Kansas	Control	3	621.61 (30.41)	95.18 (5.19)	6.65 (0.05)	16.73 (0.54)	34.03 (0.87)
	N	3	538.39 (35.03)	92.77 (6.05)	5.80 (0.01)	14.37 (0.55)	31.77 (1.22)
	P	3	787.85 (143.34)	109.43 (15.72)	7.13 (0.30)	21.23 (4.51)	39.16 (6.46)
	K	3	631.70 (19.61)	92.72 (3.25)	6.82 (0.18)	16.45 (0.52)	32.68 (0.35)
	NP	3	527.52 (24.04)	91.01 (4.92)	5.80 (0.09)	14.73 (1.19)	31.55 (2.85)

	NK	3	562.44 (61.65)	92.27 (11.32)	6.11 (0.07)	13.96 (0.64)	30.10 (0.40)
	PK	3	660.61 (21.75)	99.93 (5.47)	6.63 (0.15)	16.33 (0.22)	32.68 (0.68)
	NPK	3	531.62 (49.44)	93.32 (9.82)	5.71 (0.09)	13.35 (0.25)	30.28 (1.87)
Colorado	Control	3	275.34 (36.30)	37.08 (5.57)	7.47 (0.14)	31.75 (7.89)	49.82 (15.32)
	N	3	163.72 (16.16)	20.56 (1.38)	7.93 (0.36)	18.21 (0.98)	24.08 (1.28)
	P	3	215.04 (1.86)	27.09 (0.32)	7.94 (0.16)	26.07 (1.94)	36.91 (4.23)
	K	3	198.66 (10.69)	24.21 (1.72)	8.23 (0.13)	23.01 (0.77)	29.14 (1.69)
	NP	3	184.24 (35.37)	26.29 (3.59)	6.96 (0.61)	17.08 (2.99)	26.83 (2.44)
	NK	3	231.53 (28.14)	28.39 (5.09)	8.35 (0.58)	20.08 (1.18)	27.89 (2.94)
	PK	3	209.02 (27.49)	24.26 (0.75)	8.57 (0.86)	21.90 (0.58)	27.95 (2.90)
	NPK	3	125.64 (6.28)	16.21 (0.72)	7.75 (0.09)	15.07 (1.21)	21.59 (0.61)

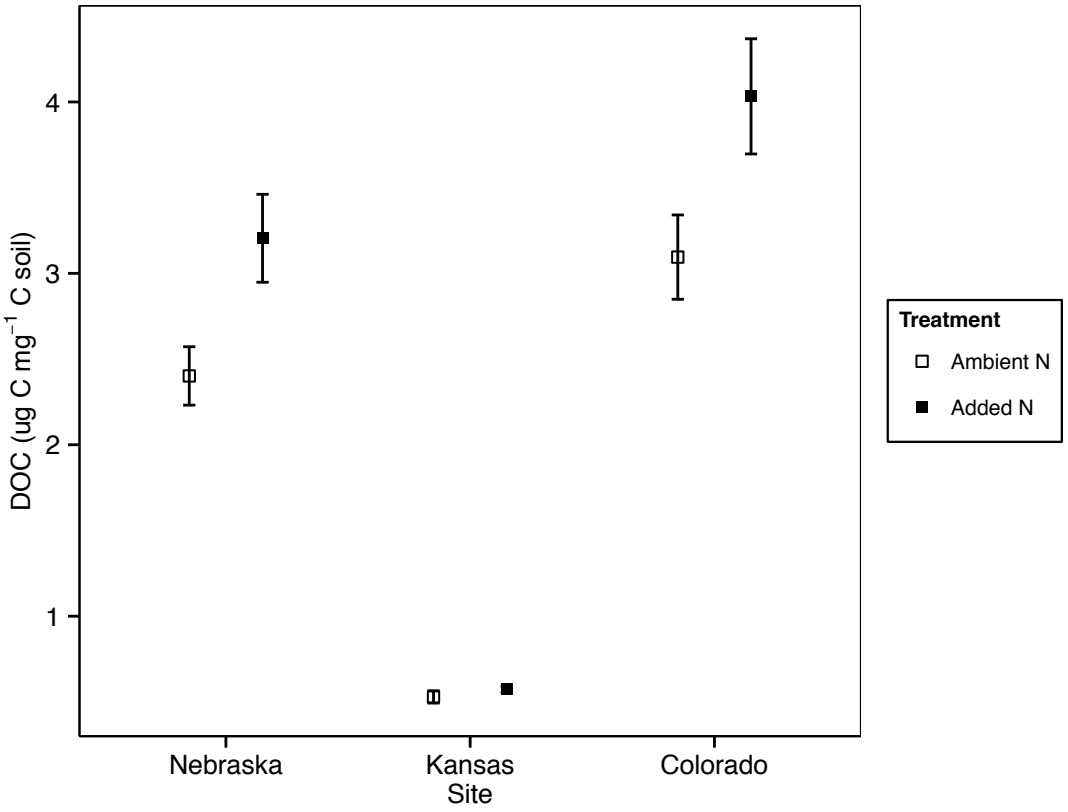

Figure S3.3 – Nitrogen treatment effects on dissolved organic C (µg DOC mg^{-1} soil C). Figure shows mean plus/minus one standard error. Treatment codes: open symbols = ambient nitrogen; shaded symbols = added nitrogen.

Table S3.3 – Microbial respiration and decomposition parameters: ANOVA tables.

Effect	Cumulative C respired (mg C-CO_2 mg^{-1} soil C)	Cumulative C respired mg C-CO_2 μg^{-1} microbial C)	Fast pool decay rate (yr^{-1})	Fast pool size (mg C g^{-1} soil)	Slow pool decay rate (yr^{-1})	Slow pool size (mg C g^{-1} soil)
Site	****	**	**	***	*	****
N	****		**	†	****	
P		†				
K		***				
N x P						
N x K						
P x K						
N x P x K		†				
Site x N	NA	†	***	**	NA	NA
Marginal R^2 [a]	0.73	0.61	0.58	0.58	0.51	0.92
Conditional R^2 [b]	0.73	0.63	0.63	0.63	0.56	0.92

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, NA = non-significant interaction term removed from model.

[a] Marginal R^2 represents the variance that is explained by fixed effects only.

[b] Conditional R^2 represents the variance that is explained by both fixed and random effects.

Table S3.4 – Microbial respiration and decomposition parameters: data table. Values are mean (standard error in parentheses).

Site	Treatment	n	Cumulative C respired (µg C-CO_2 mg^{-1} soil C)	Cumulative C respired (µg C-CO_2 $µg^{-1}$ microbial C)	Fast pool decay rate (yr^{-1})	Fast pool size (mg C g^{-1} soil)	Slow pool decay rate (yr^{-1})	Slow pool size (mg C g^{-1} soil)
Nebraska	Control	3	123.03 (11.09)	3.84 (0.91)	7.11 E-2 (4.07 E-2)	0.67 (0.19)	4.12 E-4 (5.84 E-5)	13.34 (2.45)
	N	3	96.92 (6.06)	4.34 (0.37)	5.19 E-2 (0.81 E-2)	0.45 (0.01)	3.77 E-4 (3.79 E-5)	11.96 (2.50)
	P	3	120.92 (15.80)	5.12 (0.13)	6.36 E-2 (1.89 E-2)	0.63 (0.16)	4.75 E-4 (1.29 E-4)	14.21 (1.21)
	K	3	117.30 (11.85)	5.58 (0.64)	4.78 E-2 (1.39 E-2)	0.64 (0.10)	4.17 E-4 (5.05 E-5)	14.66 (1.10)
	NP	2	114.45 (33.76)	4.34 (0.46)	4.87 E-2 (3.36 E-2)	1.03 (0.67)	1.91 E-4 (1.36 (E-4)	11.21 (3.83)
	NK	3	105.23 (7.69)	4.19 (0.45)	6.56 E-2 (2.63 E-2)	0.70 (0.21)	3.31 E-4 (4.26 E-5)	13.31 (0.33)
	PK	2	111.51 (6.61)	5.49 (0.17)	4.77 E-2 (2.02 E-2)	0.75 (0.40)	3.63 E-4 (8.66 E-5)	15.76 (0.68)
	NPK	3	112.79 (11.94)	5.08 (0.56)	5.40 E-2 (1.14 E-2)	0.63 (0.10)	3.60 E-4 (5.65 E-6)	12.50 (1.40)
Kansas	Control	2	60.23 (7.03)	3.56 (0.22)	18.77 E-2 (2.11 E-2)	0.19 (0.07)	2.78 E-4 (4.00 E-5)	37.00 (5.06)

	N	3	43.07 (2.08)	2.99 (0.03)	11.22 E-2 (1.58 E-2)	0.23 (0.06)	1.89 E-4 (1.44 E-5)	37.40 (3.22)
	P	3	66.68 (1.01)	3.41 (0.64)	16.82 E-2 (1.39 E-2)	0.20 (0.05)	3.13 E-4 (8.72 E-6)	37.36 (1.37)
	K	3	63.99 (4.76)	3.88 (0.21)	19.75 E-2 (1.68 E-2)	0.16 (0.02)	3.01 E-4 (1.84 E-5)	38.36 (2.09)
	NP	3	41.81 (2.06)	2.85 (0.09)	6.61 E-2 (1.50 E-2)	0.37 (0.08)	1.58 E-4 (4.74 E-6)	35.64 (1.38)
	NK	3	42.12 (3.05)	3.02 (0.14)	8.14 E-2 (0.91 E-2)	0.33 (0.08)	1.74 E-4 (1.15 E-5)	39.83 (3.00)
	PK	3	64.13 (1.20)	3.93 (0.13)	17.59 E-2 (0.62 E-2)	0.19 (0.01)	2.97 E-4 (2.36 E-6)	40.30 (1.60)
	NPK	3	45.85 (1.80)	3.44 (0.19)	5.64 E-2 (1.88 E-2)	0.57 (0.21)	1.57 E-4 (2.97 E-5)	39.13 (3.23)
Colorado	Control	3	135.88 (21.85)	4.49 (0.43)	5.58 E-2 (0.54 E-2)	0.41 (0.01)	5.27 E-4 (1.12 E-4)	8.85 (1.25)
	N	3	82.96 (14.60)	4.56 (0.74)	5.42 E-2 (1.26 E-2)	0.31 (0.06)	2.55 E-4 (2.99 E-5)	8.76 (1.18)
	P	3	107.81 (12.63)	4.12 (0.25)	5.14 E-2 (0.40 E-2)	0.37 (0.07)	3.49 E-4 (3.35 E-5)	7.99 (0.77)
	K	3	127.71 (1.45)	5.56 (0.22)	4.81 E-2 (0.14 E-2)	0.40 (0.03)	4.38 E-4 (5.78 E-6)	8.27 (0.59)
	NP	3	87.18 (13.75)	5.17 (0.62)	5.68 E-2 (0.39 E-2)	0.39 (0.05)	2.71 E-4 (5.65 E-5)	10.44 (1.14)

NK	3	93.91 (8.69)	4.73 (0.64)	4.92 E-2 (1.08 E-2)	0.45 (0.10)	3.03 E-4 (3.13 E-5)	11.02 (1.08)
PK	3	111.33 (14.75)	5.06 (0.53)	5.57 E-2 (1.79 E-2)	0.48 (0.16)	3.56 E-4 (7.16 E-5)	9.12 (1.34)
NPK	3	112.70 (25.97)	7.29 (1.16)	5.13 E-2 (0.85 E-2)	0.40 (0.05)	3.74 E-4 (1.08 E-4)	8.11 (1.12)

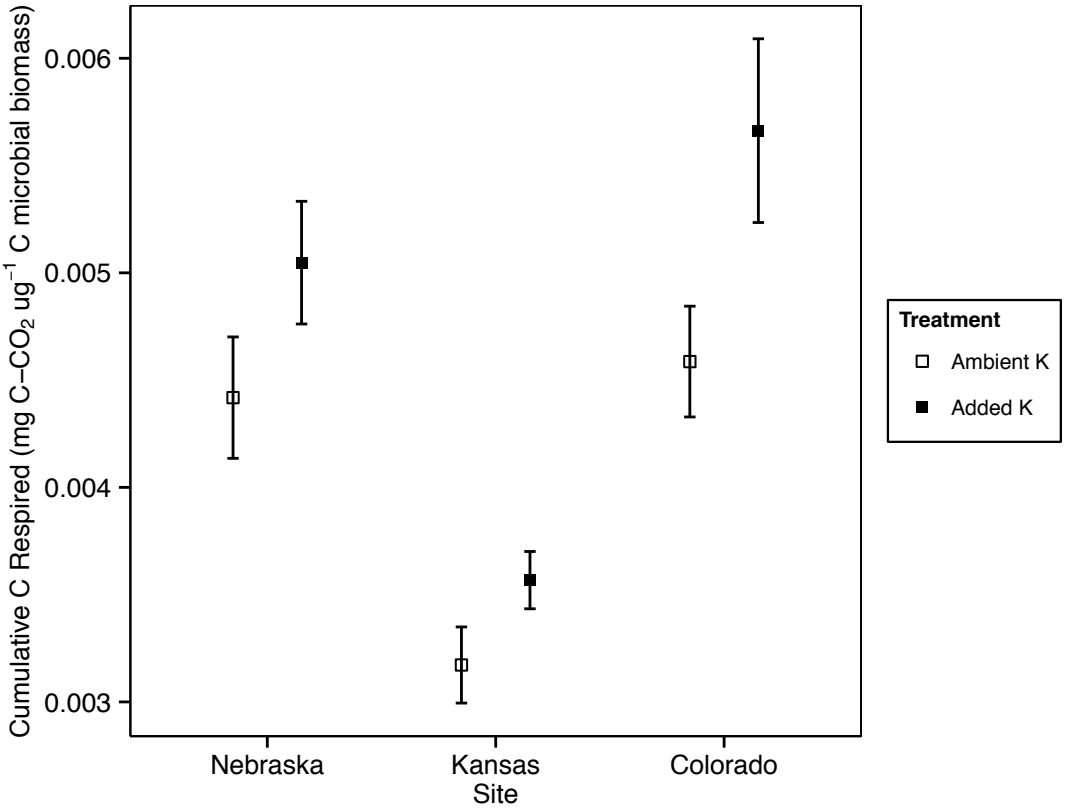

Figure S3.4 – Potassium treatment effects on mass-specific cumulative C respired (mg C-CO_2 μg^{-1} microbial biomass C). Figure shows mean plus/minus one standard error. Treatment codes: open symbols = ambient potassium; shaded symbols = added potassium.

Table S3.5 – Microbial carbon use efficiency (CUE): ANOVA tables.

Effect	^{13}C glucose CUE [c]	^{13}C vanillin CUE [c]	^{13}C glucose respired (mol C)	^{13}C vanillin respired (mol C)	^{13}C glucose microbial biomass (mol C)	^{13}C vanillin microbial biomass (mol C)
Site	**	**		*	*	**
N	*	**		†	***	†
P	***	*			****	†
K						
N x P			†			
N x K	†		**			
P x K		†				†
N x P x K						
Site x N	*	NA	NA	NA	NA	NA
Marginal R^2 [a]	0.76	0.71	0.34	0.47	0.69	0.57
Conditional R^2 [b]	0.77	0.71	0.5	0.51	0.84	0.57

† p ≤0.10, * p ≤0.05, ** p ≤0.01, *** p ≤0.001, **** p ≤0.0001, NA = non-significant interaction term removed from model.

[a] Marginal R^2 represents the variance that is explained by fixed effects only.

[b] Conditional R^2 represents the variance that is explained by both fixed and random effects.

[c] Microbial carbon use efficicney (CUE) = microbial biomass ^{13}C/(microbial biomass ^{13}C + respired ^{13}C).

Table S3.6 – Microbial carbon use efficiency (CUE): data table. Values are mean (standard error in parentheses).

Site	Treatment	n	^{13}C glucose CUE [a]	^{13}C vanillin CUE [a]	^{13}C glucose respired (mol C)	^{13}C vanillin respired (mol C)	^{13}C glucose microbial biomass (mol C)	^{13}C vanillin microbial biomass (mol C)
Nebraska	Control	3	0.35 (0.04)	0.25 (0.01)	2.51 E-5 (1.61 E-6)	1.80 E-5 (6.04 E-7)	1.33 E-5 (1.87 E-6)	5.94 E-6 (2.69 E-7)
	N	3	0.27 (0.02)	0.22 (0.02)	2.42 E-5 (2.36 E-6)	1.78 E-5 (1.88 E-6)	9.07 E-6 (1.44 E-6)	4.83 E-6 (2.45 E-7)
	P	3	0.24 (0.03)	0.17 (0.01)	2.89 E-5 (1.55 E-6)	1.95 E-5 (1.35 E-6)	9.14 E-6 (1.63 E-6)	4.06 E-6 (2.88 E-7)
	K	3	0.31 (0.01)	0.24 (0.04)	2.46 E-5 (1.84 E-6)	1.51 E-5 (1.52 E-6)	1.11 E-5 (8.28 E-7)	4.82 E-6 (8.61 E-7)
	NP	2	0.24 (0.05)	0.16 (0.01)	2.18 E-5 (1.09 E-7)	1.94 E-5 (2.20 E-6)	7.04 E-6 (1.94 E-6)	3.62 E-6 (1.19 E-7)
	NK	3	0.22 (0.02)	0.12 (0.00)	3.33 E-5 (4.96 E-6)	1.92 E-5 (1.70 E-6)	9.09 E-6 (1.32 E-6)	2.64 E-6 (1.55 E-7)
	PK	2	0.26 (0.01)	0.18 (0.03)	2.69 E-5 (1.22 E-6)	1.67 E-5 (1.85 E-6)	9.28 E-6 (7.48 E-7)	3.81 E-6 (1.29 E-6)
	NPK	3	0.21 (0.04)	0.14 (0.02)	2.86 E-5 (2.38 E-6)	2.15 E-5 (2.53 E-6)	7.61 E-6 (1.83 E-6)	3.49 E-6 (3.83 E-7)
Kansas	Control	2	0.41 (0.04)	0.29 (0.01)	2.50 E-5 (1.24 E-6)	1.37 E-5 (3.78 E-7)	1.73 E-5 (2.31 E-6)	5.67 E-6 (6.70 E-7)
	N	3	0.45 (0.04)	0.29 (0.01)	2.14 E-5 (1.40 E-6)	1.41 E-5 (1.04 E-6)	1.76 E-5 (2.02 E-6)	5.78 E-6 (2.31 E-7)
	P	3	0.35 (0.05)	0.29 (0.02)	2.81 E-5 (3.95	1.41 E-5 (1.63	1.48 E-5 (2.81	5.63 E-6 (5.19

				E-6)	E-6)	E-6)	E-7)
K	3	0.43 (0.03)	0.29 (0.01)	2.47 E-5 (2.53 E-6)	1.44 E-5 (1.21 E-6)	1.83 E-5 (1.64 E-6)	5.81 E-6 (8.58 E-7)
NP	3	0.39 (0.02)	0.25 (0.01)	2.19 E-5 (2.50 E-6)	1.51 E-5 (4.78 E-7)	1.37 E-5 (3.74 E-7)	5.11 E-6 (9.68 E-8)
NK	3	0.41 (0.02)	0.26 (0.02)	2.36 E-5 (2.68 E-6)	1.73 E-5 (1.81 E-6)	1.63 E-5 (1.18 E-6)	5.87 E-6 (2.31 E-7)
PK	3	0.40 (0.04)	0.27 (0.02)	2.38 E-5 (4.23 E-7)	1.50 E-5 (4.77 E-7)	1.60 E-5 (2.69 E-6)	5.58 E-6 (3.70 E-7)
NPK	3	0.34 (0.00)	0.28 (0.02)	2.27 E-5 (8.77 E-7)	1.52 E-5 (1.20 E-6)	1.17 E-5 (6.18 E-7)	5.84 E-6 (1.98 E-7)

[a] Microbial carbon use efficicney (CUE) = microbial biomass ^{13}C/(microbial biomass ^{13}C + respired ^{13}C).

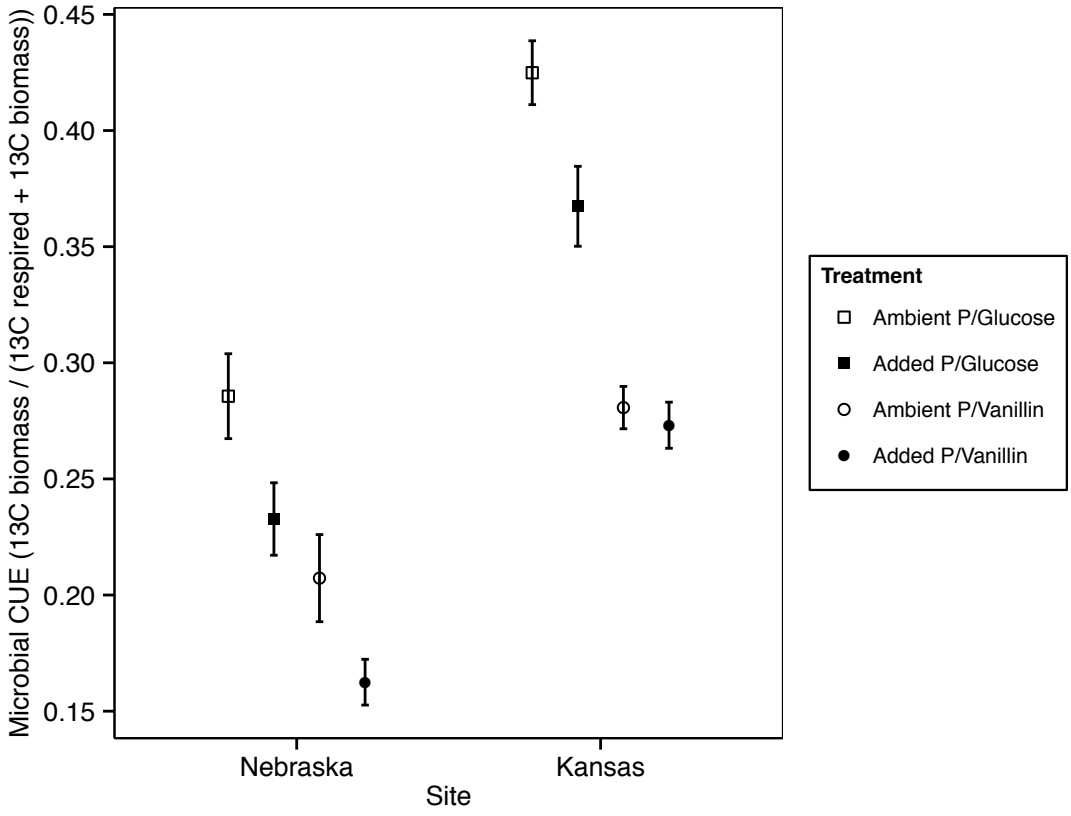

Figure S3.5 – Phosphorus treatment effects on microbial carbon use efficiency (CUE) of ^{13}C glucose and ^{13}C vanillin from soil collected in Nebraska and Kansas. Figure shows mean plus/minus one standard error. Treatment codes: open symbols = ambient phosphorus; shaded symbols = added phosphorus; squares = ^{13}C glucose; circles = ^{13}C vanillin.

Table S3.7 – Microbial extracellular enzyme activity: ANOVA tables.

Effect	PO activity (nmol mg^{-1} soil C hr^{-1}) [c]	PO activity (nmol μg^{-1} microbial C hr^{-1}) [c]	PX activity (nmol mg^{-1} soil C hr^{-1}) [c]	PX activity (nmol μg^{-1} microbial C hr^{-1}) [c]	CBH activity (nmol mg^{-1} soil C hr^{-1}) [c]	CBH activity (nmol μg^{-1} microbial C hr^{-1}) [c]	BG activity (nmol mg^{-1} soil C hr^{-1}) [c]	BG activity (nmol μg^{-1} microbial C hr^{-1}) [c]
Site	****	****	****	****	**	***	***	****
N		**	*	****		†		
P	**	**		†	*	***	†	**
K		**	*	***				
N x P								
N x K								
P x K								
N x P x K	*	*						
Site x P	NA	NA	NA	NA	NA	NA	NA	NA
Marginal R^2 [a]	0.86	0.75	0.90	0.88	0.45	0.67	0.61	0.79
Conditional R^2 [b]	0.86	0.75	0.91	0.89	0.45	0.67	0.61	0.79

Effect	AG activity (nmol mg^{-1} soil C hr^{-1}) [c]	AG activity (nmol μg^{-1} microbial C hr^{-1}) [c]	NAG activity (nmol mg^{-1} soil C hr^{-1}) [c]	NAG activity (nmol μg^{-1} microbial C hr^{-1}) [c]	BX activity (nmol mg^{-1} soil C hr^{-1}) [c]	BX activity (nmol μg^{-1} microbial C hr^{-1}) [c]	AP activity (nmol mg^{-1} soil C hr^{-1}) [c]	AP activity (nmol μg^{-1} microbial C hr^{-1}) [c]
Site	**	***	***	****	***	****	***	****
N								
P	†	*		†		*		
K								
N x P								
N x K								
P x K								
N x P x K								
Site x P	*	NA	NA	NA	NA	NA	NA	NA
Marginal R^2 [a]	0.53	0.71	0.60	0.74	0.57	0.77	0.70	0.82
Conditional R^2 [b]	0.55	0.71	0.61	0.74	0.57	0.77	0.71	0.82

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, NA = non-significant interaction term removed from model.

[a] Marginal R^2 represents the variance that is explained by fixed effects only.

[b] Conditional R^2 represents the variance that is explained by both fixed and random effects.

[c] Enzyme codes: AG = α-glucosidase; AP = acid phosphatase; BG = β-glucosidase; BX = β-xylosidase; CBH = cellobiohydrolase; NAG = N-acetyl-β-D-glucosaminidase; PO = phenol oxidase; PX = peroxidase.

Table S3.8 – Microbial extracellular enzyme activity: data table. Values are mean (standard error in parentheses).[a]

Site	Treatment	n	PO activity (nmol mg^{-1} soil C hr^{-1})	PO activity (nmol μg^{-1} microbial C hr^{-1})	PX activity (nmol mg^{-1} soil C hr^{-1})	PX activity (nmol μg^{-1} microbial C hr^{-1})
Nebraska	Control	3	2.29 E-3 (4.29 E-4)	6.65 E-5 (1.05 E-5)	4.65 E-3 (1.49 E-3)	1.38 E-4 (5.66 E-5)
	N	3	3.10 E-3 (2.72 E-4)	1.39 E-4 (1.56 E-5)	6.95 E-3 (1.37 E-3)	3.13 E-4 (6.79 E-5)
	P	3	3.10 E-3 (1.94 E-4)	1.36 E-4 (2.11 E-5)	6.56 E-3 (4.25 E-4)	2.89 E-4 (4.75 E-5)
	K	3	2.86 E-3 (2.16 E-4)	1.35 E-4 (9.82 E-6)	5.50 E-3 (1.61 E-3)	2.61 E-4 (7.66 E-5)
	NP	2	3.15 E-3 (3.08 E-4)	1.24 E-4 (1.24 E-5)	8.68 E-3 (3.55 E-3)	3.21 E-4 (7.44 E-5)
	NK	3	2.74 E-3 (1.78 E-4)	1.08 E-4 (4.84 E-6)	7.73 E-3 (6.85 E-4)	3.04 E-4 (6.92 E-6)
	PK	2	3.26 E-3 (3.38 E-4)	1.60 E-4 (1.22 E-5)	8.47 E-3 (3.85 E-4)	4.17 E-4 (7.51 E-6)
	NPK	3	3.81 E-3 (1.90 E-4)	1.71 E-4 (8.81 E-6)	9.49 E-3 (1.36 E-3)	4.24 E-4 (5.37 E-5)
Kansas	Control	2	2.07 E-3 (1.27 E-4)	1.23 E-4 (8.82 E-7)	2.57 E-3 (1.19 E-3)	1.49 E-4 (6.26 E-5)
	N	3	1.70 E-3 (9.65 E-5)	1.18 E-4 (2.15 E-6)	2.48 E-3 (2.52 E-4)	1.73 E-4 (1.88 E-5)
	P	3	2.06 E-3 (2.29 E-4)	1.01 E-4 (1.08 E-8)	2.13 E-3 (4.66 E-4)	1.02 E-4 (1.99 E-5)
	K	3	1.84 E-3 (2.54 E-4)	1.11 E-4 (1.16 E-5)	2.45 E-3 (4.60 E-4)	1.48 E-4 (2.56 E-5)
	NP	3	2.09 E-3 (2.69 E-4)	1.43 E-4 (1.93 E-5)	2.28 E-3 (2.66 E-4)	1.56 E-4 (1.80 E-5)
	NK	3	1.58 E-3 (2.44 E-4)	1.12 E-4 (1.35 E-5)	3.38 E-3 (8.01 E-4)	2.47 E-4 (6.69 E-5)
	PK	3	2.00 E-3 (2.16 E-4)	1.23 E-4 (1.43 E-5)	2.55 E-3 (2.72 E-4)	1.56 E-4 (1.53 E-5)
	NPK	3	1.83 E-3 (2.34 E-4)	1.37 E-4 (1.50 E-5)	2.34 E-3 (2.22 E-4)	1.75 E-4 (1.49 E-5)
Colorado	Control	3	4.71 E-3 (6.05 E-4)	1.64 E-4 (3.80 E-5)	1.61 E-2 (2.66 E-3)	5.37 E-4 (7.28 E-5)
	N	3	5.42 E-3 (7.49 E-4)	2.99 E-4 (4.10 E-5)	2.37 E-2 (2.90 E-3)	1.30 E-3 (1.40 E-4)

	P	3	6.15 E-3 (3.91 E-4)	2.40 E-4 (2.80 E-5)	1.96 E-2 (2.11 E-3)	7.49 E-4 (2.85 E-5)
	K	3	5.46 E-3 (3.75 E-4)	2.38 E-4 (2.10 E-5)	2.19 E-2 (7.85 E-4)	9.55 E-4 (6.29 E-5)
	NP	3	4.01 E-3 (4.49 E-4)	2.55 E-4 (5.91 E-5)	1.78 E-2 (1.53 E-3)	1.12 E-3 (2.22 E-4)
	NK	3	5.55 E-3 (4.72 E-4)	2.80 E-4 (3.58 E-5)	2.41 E-2 (3.45 E-3)	1.21 E-3 (2.06 E-4)
	PK	3	6.11 E-3 (6.22 E-4)	2.79 E-4 (2.59 E-5)	2.29 E-2 (4.50 E-3)	1.04 E-3 (1.93 E-4)
	NPK	3	7.00 E-3 (9.18 E-4)	4.63 E-4 (3.44 E-5)	3.19 E-2 (4.83 E-3)	2.11 E-3 (2.08 E-4)

Site	Treatment	n	CBH activity (nmol mg^{-1} soil C hr^{-1})	CBH activity (nmol μg^{-1} microbial C hr^{-1})	BG activity (nmol mg^{-1} soil C hr^{-1})	BG activity (nmol μg^{-1} microbial C hr^{-1})
Nebraska	Control	3	10.30 (2.38)	0.29 (0.02)	45.09 (7.87)	1.31 (0.15)
	N	3	13.36 (0.49)	0.60 (0.03)	55.33 (0.72)	2.47 (0.09)
	P	3	13.41 (1.77)	0.60 (0.13)	58.32 (9.48)	2.58 (0.56)
	K	3	10.67 (3.37)	0.51 (0.16)	46.74 (11.95)	2.22 (0.58)
	NP	2	22.11 (4.71)	0.93 (0.36)	80.01 (4.99)	3.26 (0.83)
	NK	3	13.96 (4.10)	0.54 (0.15)	58.64 (14.06)	2.29 (0.51)
	PK	2	10.70 (0.19)	0.53 (0.01)	38.68 (4.34)	1.90 (0.16)
	NPK	3	13.11 (1.29)	0.59 (0.04)	59.79 (6.51)	2.67 (0.20)
Kansas	Control	2	18.87 (4.76)	1.14 (0.34)	111.88 (26.20)	6.74 (1.92)
	N	3	18.38 (1.88)	1.27 (0.10)	105.66 (10.01)	7.35 (0.63)
	P	3	21.98 (1.19)	1.12 (0.20)	116.36 (4.95)	6.00 (1.23)
	K	3	14.14 (2.91)	0.85 (0.16)	99.15 (9.66)	6.04 (0.62)

	NP	3	21.92 (5.72)	1.57 (0.50)	129.89 (42.74)	9.31 (3.65)
	NK	3	10.75 (3.02)	0.75 (0.19)	54.92 (12.80)	3.87 (0.76)
	PK	3	19.97 (2.89)	1.22 (0.18)	121.61 (14.68)	7.45 (0.92)
	NPK	3	34.37 (3.23)	2.57 (0.24)	157.71 (29.74)	11.82 (2.24)
Colorado	Control	3	12.28 (5.13)	0.36 (0.07)	61.95 (19.44)	1.88 (0.16)
	N	3	5.86 (0.80)	0.32 (0.04)	30.25 (4.14)	1.67 (0.24)
	P	3	9.39 (2.23)	0.35 (0.06)	43.33 (5.77)	1.65 (0.11)
	K	3	8.28 (0.72)	0.36 (0.04)	35.55 (6.01)	1.56 (0.30)
	NP	3	8.64 (2.91)	0.48 (0.10)	39.25 (9.41)	2.25 (0.16)
	NK	3	7.15 (0.93)	0.36 (0.06)	38.54 (3.49)	1.92 (0.15)
	PK	3	14.73 (3.92)	0.67 (0.16)	55.41 (5.32)	2.53 (0.24)
	NPK	3	6.76 (1.80)	0.43 (0.09)	32.36 (5.23)	2.14 (0.28)

Site	Treatment	n	AG activity (nmol mg^{-1} soil C hr^{-1})	AG activity (nmol μg^{-1} microbial C hr^{-1})	NAG activity (nmol mg^{-1} soil C hr^{-1})	NAG activity (nmol μg^{-1} microbial C hr^{-1})
Nebraska	Control	3	3.35 (0.58)	0.10 (0.01)	25.20 (8.10)	0.68 (0.03)
	N	3	3.40 (0.19)	0.15 (0.01)	28.71 (1.47)	1.28 (0.07)
	P	3	3.42 (0.63)	0.15 (0.03)	30.57 (7.02)	1.34 (0.35)
	K	3	2.84 (0.79)	0.13 (0.04)	33.39 (12.18)	1.58 (0.57)
	NP	2	4.57 (1.38)	0.19 (0.09)	42.78 (11.44)	1.81 (0.80)
	NK	3	4.17 (0.94)	0.16 (0.03)	40.02 (11.88)	1.54 (0.40)

	PK	2	2.67 (0.18)	0.13 (0.01)	23.05 (6.30)	1.13 (0.28)
	NPK	3	3.63 (0.72)	0.16 (0.02)	32.64 (1.49)	1.47 (0.09)
Kansas	Control	2	6.42 (0.58)	0.38 (0.06)	98.50 (0.93)	5.85 (0.26)
	N	3	6.14 (0.77)	0.43 (0.06)	92.51 (39.32)	6.43 (2.82)
	P	3	9.85 (0.50)	0.50 (0.09)	135.44 (28.68)	7.12 (2.33)
	K	3	5.59 (0.65)	0.34 (0.04)	116.90 (37.27)	6.98 (1.98)
	NP	3	7.56 (2.19)	0.54 (0.19)	75.83 (13.56)	5.33 (1.32)
	NK	3	3.49 (0.91)	0.25 (0.06)	38.17 (13.98)	2.70 (0.95)
	PK	3	7.39 (1.27)	0.45 (0.08)	101.56 (29.98)	6.25 (1.89)
	NPK	3	9.29 (1.98)	0.70 (0.15)	76.41 (15.60)	5.73 (1.18)
Colorado	Control	3	4.32 (0.94)	0.14 (0.02)	36.40 (8.99)	1.14 (0.11)
	N	3	3.05 (0.61)	0.17 (0.03)	18.32 (2.25)	1.02 (0.14)
	P	3	3.44 (1.02)	0.13 (0.03)	26.52 (7.92)	0.99 (0.24)
	K	3	3.48 (0.59)	0.15 (0.03)	19.30 (3.19)	0.85 (0.16)
	NP	3	2.86 (0.48)	0.17 (0.02)	24.55 (5.25)	1.42 (0.12)
	NK	3	3.53 (0.58)	0.17 (0.02)	26.91 (5.68)	1.33 (0.22)
	PK	3	4.56 (0.75)	0.21 (0.03)	29.30 (3.20)	1.33 (0.13)
	NPK	3	2.60 (0.41)	0.17 (0.01)	22.54 (2.54)	1.49 (0.08)

Site	Treatment	n	BX activity (nmol mg^{-1} soil C hr^{-1})	BX activity (nmol μg^{-1} microbial C hr^{-1})	AP activity (nmol mg^{-1} soil C hr^{-1})	AP activity (nmol μg^{-1} microbial C hr^{-1})

Nebraska	Control	3	11.36 (2.68)	0.32 (0.02)	105.69 (20.94)	3.03 (0.34)
	N	3	13.24 (1.34)	0.59 (0.07)	121.08 (4.45)	5.42 (0.35)
	P	3	13.59 (1.88)	0.60 (0.12)	141.96 (18.50)	6.34 (1.36)
	K	3	12.14 (3.22)	0.57 (0.15)	137.89 (34.14)	6.51 (1.54)
	NP	2	17.28 (2.47)	0.72 (0.24)	140.35 (3.67)	5.68 (1.26)
	NK	3	15.94 (3.41)	0.62 (0.11)	149.29 (30.46)	5.82 (1.03)
	PK	2	10.20 (1.30)	0.50 (0.05)	132.01 (38.42)	6.46 (1.72)
	NPK	3	14.68 (0.45)	0.66 (0.04)	135.95 (6.47)	6.13 (0.43)
Kansas	Control	2	29.72 (2.14)	1.77 (0.22)	364.91 (37.61)	21.82 (3.41)
	N	3	32.17 (5.30)	2.24 (0.37)	385.15 (69.69)	26.63 (4.41)
	P	3	34.70 (3.66)	1.83 (0.46)	329.02 (13.21)	16.94 (3.43)
	K	3	23.11 (2.86)	1.40 (0.14)	273.33 (26.15)	16.55 (1.04)
	NP	3	28.54 (9.60)	2.06 (0.81)	240.06 (55.44)	17.03 (5.07)
	NK	3	15.41 (3.42)	1.09 (0.21)	218.37 (27.36)	15.62 (1.67)
	PK	3	31.52 (3.87)	1.93 (0.25)	363.94 (70.26)	22.27 (4.28)
	NPK	3	40.56 (6.98)	3.04 (0.52)	335.79 (82.62)	25.08 (6.12)
Colorado	Control	3	19.14 (7.04)	0.57 (0.07)	162.01 (36.44)	5.22 (0.35)
	N	3	8.74 (0.54)	0.48 (0.05)	113.09 (13.93)	6.22 (0.70)
	P	3	12.37 (1.81)	0.47 (0.05)	137.86 (19.77)	5.26 (0.47)
	K	3	11.51 (1.17)	0.50 (0.07)	138.92 (26.74)	6.09 (1.32)
	NP	3	10.53 (2.90)	0.60 (0.08)	116.22 (18.72)	6.83 (0.29)

NK	3	11.43 (1.17)	0.60 (0.05)	132.49 (3.78)	6.66 (0.58)
PK	3	14.63 (1.81)	0.67 (0.07)	139.60 (6.36)	6.37 (0.18)
NPK	3	7.36 (0.95)	0.48 (0.03)	98.96 (13.50)	6.52 (0.45)

[a] Enzyme codes: AG = α-glucosidase; AP = acid phosphatase; BG = β-glucosidase; BX = β-xylosidase; CBH = cellobiohydrolase; NAG = N-acetyl-β-D-glucosaminidase; PO = phenol oxidase; PX = peroxidase.

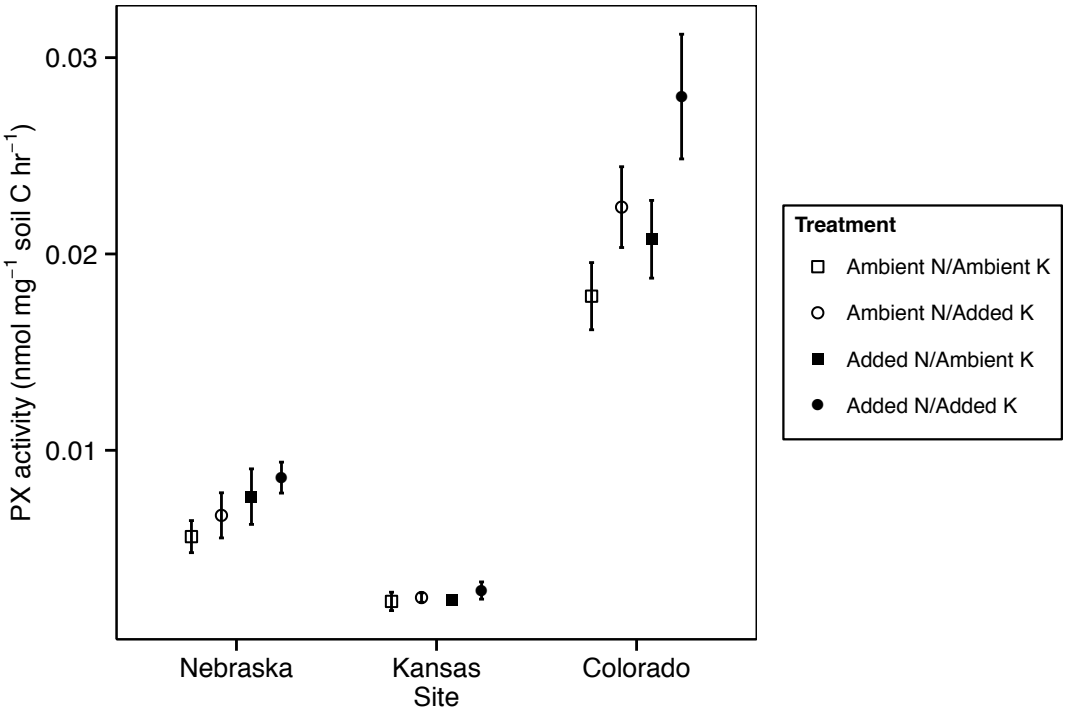

Figure S3.6 – Nitrogen and potassium treatment effects on peroxidase (PX) activity (nmol mg^{-1} soil C h^{-1}). Figure shows mean plus/minus one standard error. Treatment codes: open symbols = ambient nitrogen; shaded symbols = added nitrogen; squares = ambient potassium; circles = added potassium.

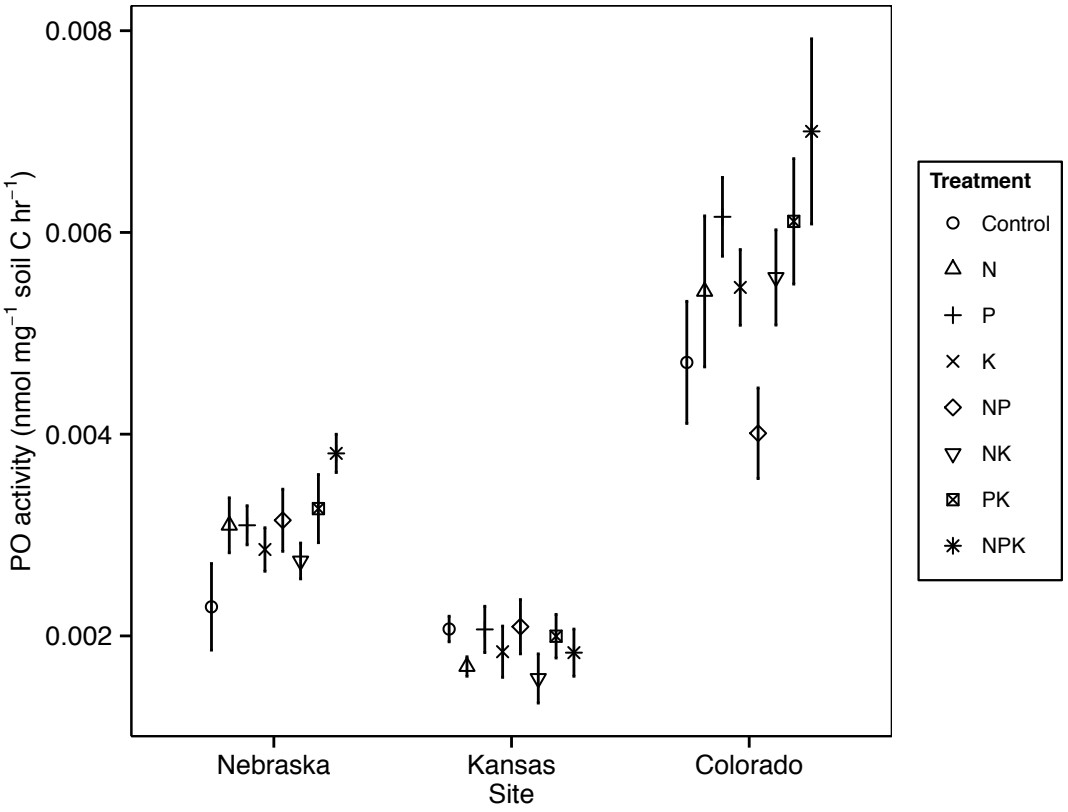

Figure S3.7 – Treatment effects on phenol oxidase (PO) activity (nmol mg^{-1} soil C h^{-1}). Figure shows mean plus/minus one standard error. Treatment codes: Control = control plots; N = nitrogen addition plots; P = phosphorus addition plots; K = potassium addition plots; NP = nitrogen and phosphorus addition plots; NK = nitrogen and potassium addition plots; PK = phosphorus and potassium addition plots; NPK = nitrogen, phosphorus, and potassium addition plots.

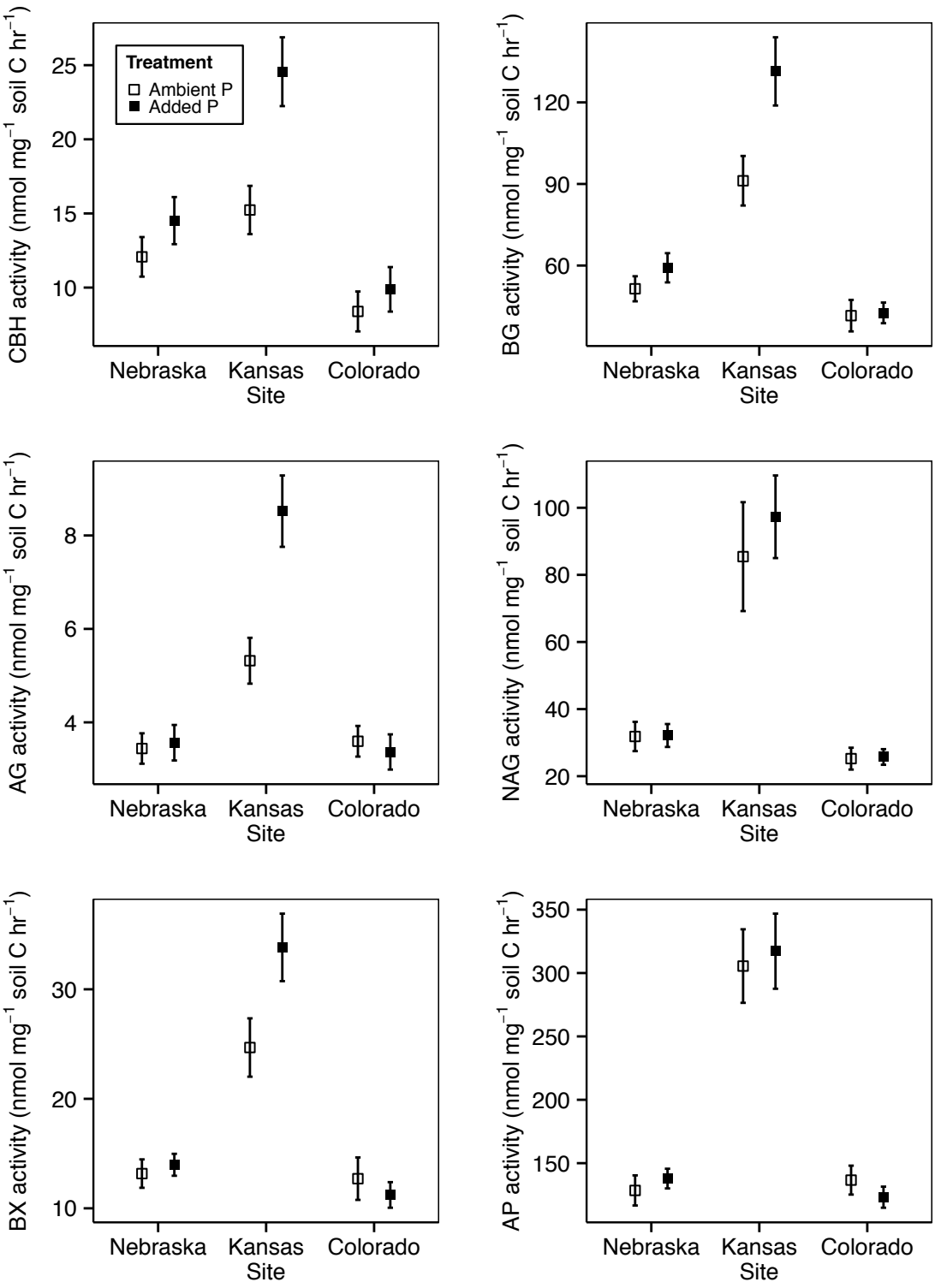

Figure S3.8 – Phosphorus treatment effects on microbial hydrolytic enzyme activity (nmol mg^{-1} soil C h^{-1}). All panels show mean plus/minus one standard error. Enzyme codes: AG = α-glucosidase; AP = acid phosphatase; BG = β-glucosidase; BX = β-xylosidase; CBH = cellobiohydrolase; NAG = N-acetyl-β-D-

glucosaminidase. Treatment codes: open symbols = ambient phosphorus; shaded symbols = added phosphorus.

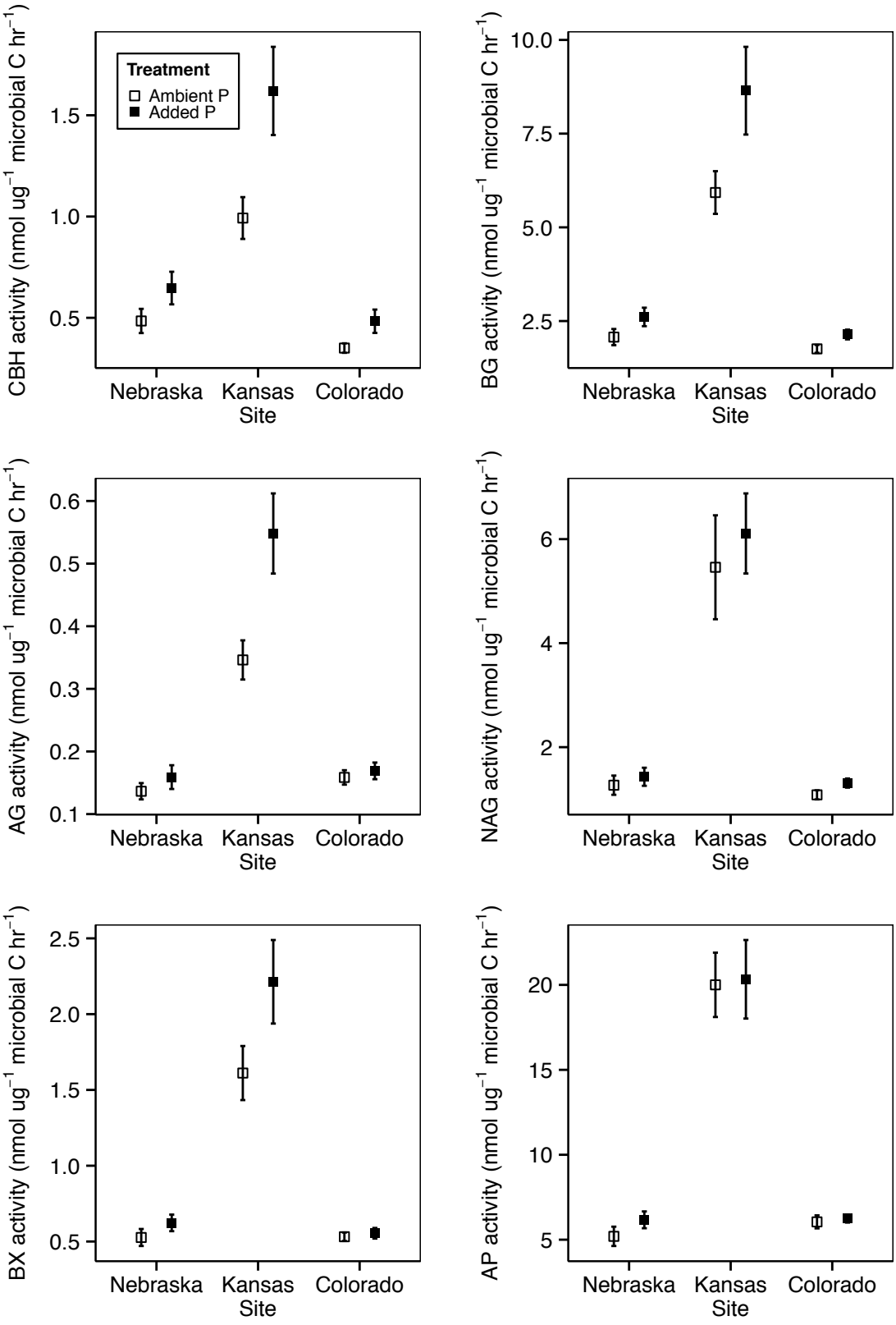
Treatment
Ambient P
Added P
CBH activity (nmol ug^{-1} microbial C hr^{-1})
BG activity (nmol ug^{-1} microbial C hr^{-1})
AG activity (nmol ug^{-1} microbial C hr^{-1})
NAG activity (nmol ug^{-1} microbial C hr^{-1})
BX activity (nmol ug^{-1} microbial C hr^{-1})
AP activity (nmol ug^{-1} microbial C hr^{-1})
Nebraska
Kansas
Colorado
Site

Figure S3.9 – Phosphorus treatment effects on microbial hydrolytic enzyme activity (nmol μg^{-1} microbial biomass C h^{-1}). All panels show mean plus/minus one standard error. Enzyme codes: AG = α-glucosidase; AP = acid phosphatase; BG = β-glucosidase; BX = β-xylosidase; CBH = cellobiohydrolase; NAG = N-acetyl-β-D-glucosaminidase. Treatment codes: open symbols = ambient phosphorus; shaded symbols = added phosphorus.

Table S3.9 – Root nitrogen concentration: ANOVA table.

Effect	Root N %
Site	***
N	****
P	
K	
N x P	
N x K	
P x K	*
N x P x K	
Site x N	***
Marginal R^2 [a]	0.81
Conditional R^2 [b]	0.85

† p ≤0.10, * p ≤0.05, ** p ≤0.01, *** p ≤0.001, **** p ≤0.0001, NA = non-significant interaction term removed from model.

[a] Marginal R^2 represents the variance that is explained by fixed effects only.

[b] Conditional R^2 represents the variance that is explained by both fixed and random effects.

Table S3.10 – Root carbon fractions: ANOVA tables.

	Root LR %	Root CELL %	Root HBP %	Root SCC %
Site	***	*	***	*
N				
Site x N	*	NA	NA	NA
Marginal R^2 [a]	0.86	0.47	0.91	0.49
Conditional R^2 [b]	0.86	0.47	0.91	0.49

† p ≤0.10, * p ≤0.05, ** p ≤0.01, *** p ≤0.001, **** p ≤0.0001, NA = non-significant interaction term removed from model.

[a] Marginal R^2 represents the variance that is explained by fixed effects only.

[b] Conditional R^2 represents the variance that is explained by both fixed and random effects.

Table S3.11 – Root nitrogen concentration: data table. Values are mean (standard error in parentheses).

Site	Treatment	n	Root N %
Nebraska	Control	3	0.86 (0.10)
	N	3	1.00 (0.11)
	P	3	0.81 (0.09)
	K	3	0.85 (0.04)
	NP	3	0.96 (0.18)
	NK	3	0.98 (0.14)
	PK	3	0.96 (0.07)
	NPK	3	1.00 (0.03)
Kansas	Control	3	0.52 (0.01)
	N	3	1.02 (0.06)
	P	3	0.52 (0.03)
	K	3	0.62 (0.02)
	NP	3	1.01 (0.12)
	NK	3	0.99 (0.04)
	PK	3	0.61 (0.03)
	NPK	3	0.97 (0.02)
Colorado	Control	3	1.32 (0.08)
	N	3	1.49 (0.07)
	P	3	1.16 (0.14)
	K	3	1.18 (0.04)
	NP	3	1.32 (0.11)
	NK	3	1.29 (0.07)
	PK	3	1.20 (0.06)
	NPK	3	1.46 (0.06)

Table S3.12 – Root carbon fractions: data table. Values are mean (standard error in parentheses). [a]

Site	Treatment	n	Root LR %	Root CELL %	Root HBP %	Root SCC %
Nebraska	Control	3	33.8 (1.4)	37.3 (1.3)	9.0 (1.6)	19.8 (1.6)
	N	3	30.9 (1.1)	32.3 (1.0)	12.2 (0.8)	24.6 (1.4)
Kansas	Control	3	19.9 (1.3)	30.8 (1.0)	26.4 (0.7)	22.8 (1.9)
	N	3	24.7 (1.8)	32.3 (1.1)	23.1 (1.9)	19.9 (0.5)
Colorado	Control	3	28.9 (1.4)	29.9 (1.7)	25.0 (1.2)	16.2 (2.0)
	N	3	27.1 (0.6)	30.2 (0.7)	25.3 (0.4)	17.3 (0.4)

[a] Root carbon fraction codes: LR = lignin plus recalcitrants; CELL = cellulose; HBP = hemicellulose plus bound proteins; SCC = soluble cell contents.

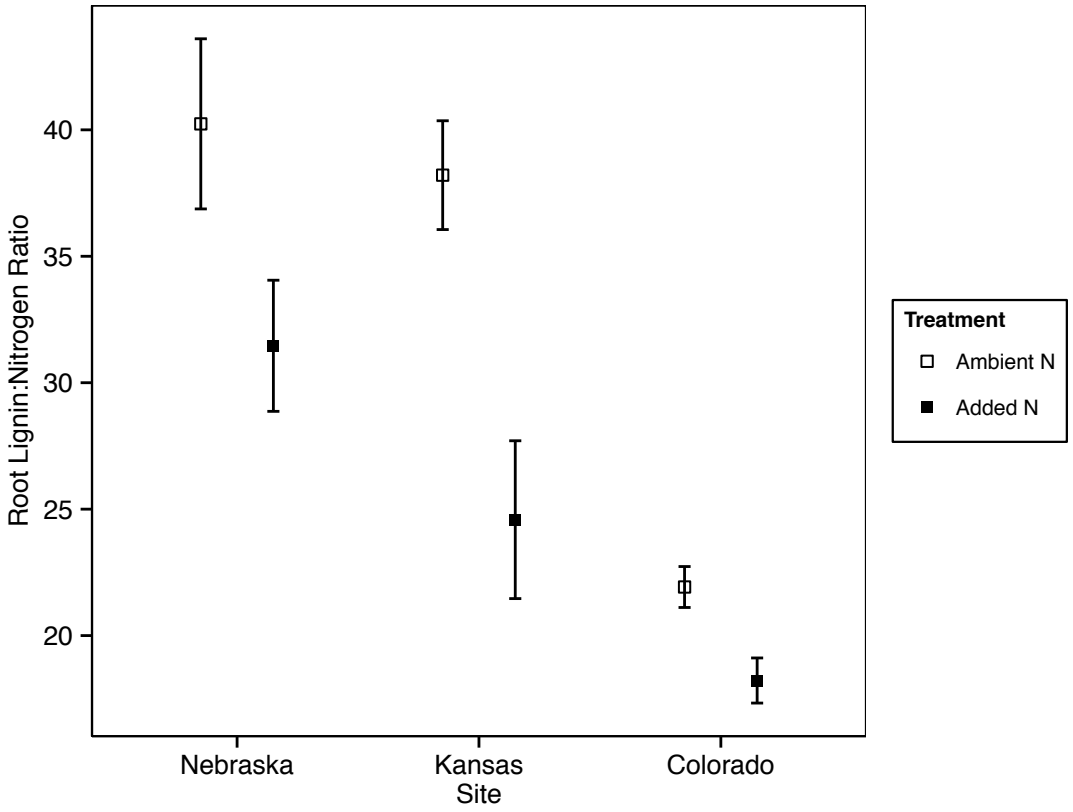

Figure S3.10 – Nitrogen treatment effects on root lignin:N ratio. Figure shows mean plus/minus one standard error. Treatment codes: open squares = ambient nitrogen; closed squares = added nitrogen.

Table S3.13 – Soil chemistry: ANOVA tables.

Effect	Soil pH	Soil extractable Ca (ppm)	Soil extractable Mg (ppm)	Soil extractable B (ppm)	Soil extractable Cu (ppm)	Soil extractable Mn (ppm)	Soil extractable P (ppm)	Soil extractable K (ppm)	Soil extractable Fe (ppm)
Site	****	****	****	**		**	***		**
N	****	***			†				
P	†	†	**				****		
K				**	***	***		****	
N x P					*				
N x K					†				
P x K									*
N x P x K				†	†				†
Site x N	**	NA	*	NA	NA	NA	NA	NA	NA
Site x P	NA	NA	*	NA	NA	NA	***	NA	NA
Site x K	NA	NA	NA	NA	NA	NA	NA	*	NA
Marginal R^2 [a]	0.92	0.87	0.82	0.54	0.42	0.53	0.85	0.62	0.46
Conditional R^2 [b]	0.92	0.87	0.82	0.54	0.42	0.53	0.85	0.62	0.50

† $p \leq 0.10$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$, NA = non-significant interaction term removed from model.

[a] Marginal R^2 represents the variance that is explained by fixed effects only.
[b] Conditional R^2 represents the variance that is explained by both fixed and random effects.

Table S3.14 – Soil pH: data table. Values are mean (standard error in parentheses).

Site	Treatment	n	pH
Nebraska	Control	3	6.7 (0.0)
	N	3	6.2 (0.1)
	P	3	6.6 (0.1)
	K	3	6.8 (0.1)
	NP	2	6.3 (0.1)
	NK	3	6.4 (0.1)
	PK	2	6.8 (0.1)
	NPK	3	6.4 (0.1)
Kansas	Control	1	6.2 (NA)
	N	3	6.0 (0.0)
	P	3	6.2 (0.0)
	K	3	6.1 (0.0)
	NP	3	5.9 (0.0)
	NK	2	6.0 (0.0)
	PK	3	6.1 (0.0)
	NPK	3	6.0 (0.0)
Colorado	Control	3	6.0 (0.0)
	N	3	5.7 (0.0)
	P	3	5.7 (0.0)
	K	3	5.8 (0.1)
	NP	3	5.5 (0.1)
	NK	3	5.7 (0.1)
	PK	3	5.8 (0.0)
	NPK	3	5.6 (0.1)

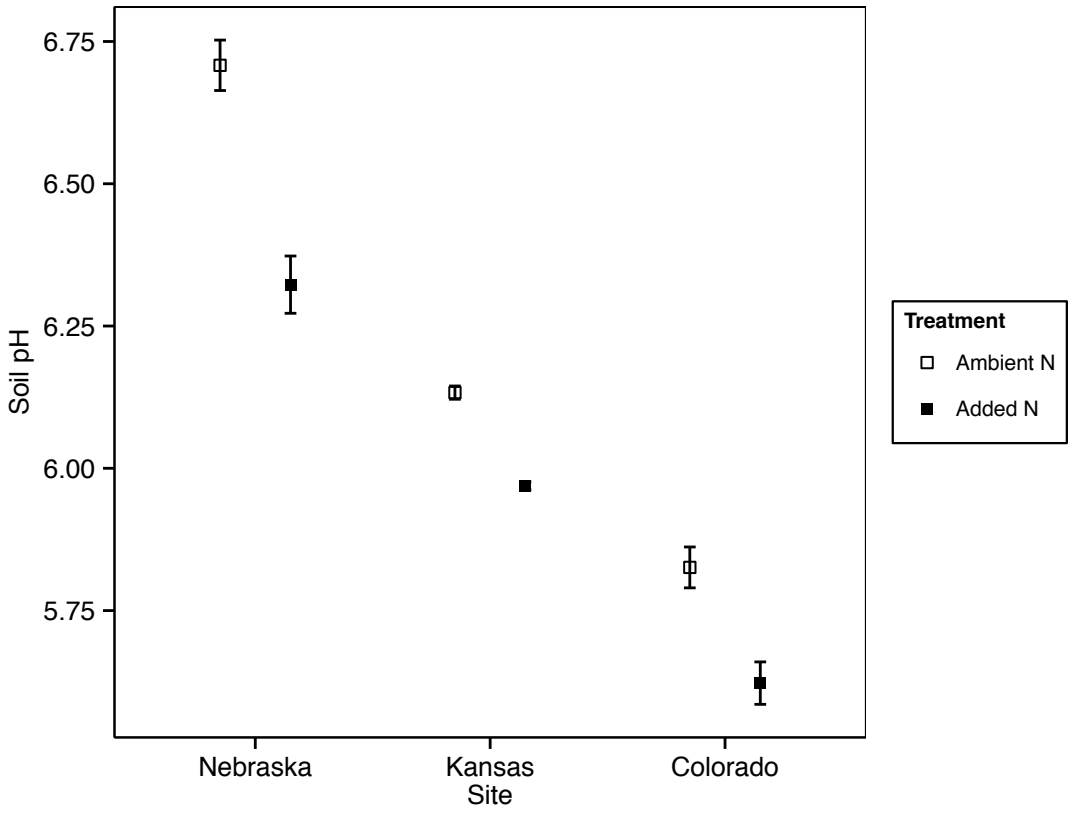

Figure S3.11 – Nitrogen treatment effects on soil pH. Figure shows mean plus/minus one standard error. Treatment codes: open squares = ambient nitrogen; closed squares = added nitrogen.