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

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ABSTRACT

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

1. 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. 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 nutrient availability may feed back on the global C cycle remains unknown (Wieder et al., 2015a). 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 its 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. Leaf litter and soil studies conducted across biomes have found that N enrichment often decreases microbial decomposition and respiration (Knorr et al., 2005; Ramirez et al., 2012; Zhou et al., 2014), especially for the leaf litter or SOM pools that cycle slowly (Berg and Matzner, 1997; Hobbie et al., 2012; Riggs et al., 2015). This negative response is particularly paradoxical since N enrichment often *increases* the input of C belowground via plant litter and root exudates (Liu and Greaver, 2010), which should increase C availability to microbes as well as the decomposition of more slowly cycling SOM (e.g., the priming effect). Although observed decreases in respiration and decomposition of slowly cycling SOM are in accordance with predictions from theoretical models of microbial activity following N addition (Ågren et al., 2001; Moorhead and Sinsabaugh, 2006; Perveen et al., 2014; Schimel and Weintraub, 2003), the biological and chemical mechanisms that underlie the response of SOM decomposition to added N remain uncertain. Are the inhibitory effects of N on SOM decomposition – and slowly cycling SOM decomposition in particular – due to plant biomass, microbial, or soil chemistry-mediated changes that occur in response to N addition? Identifying these mechanisms is key for elucidating how N enrichment will influence soil C sequestration, soil CO_2 emissions, and the global C cycle.

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Microbial decomposition of SOM is influenced by the activity of the microbial decomposer community, as well as the chemistry (or "decomposability") of the substrates being oxidized. Although microbial community composition shifts in response to N enrichment have been documented in multiple systems (Fierer et al., 2011; Leff et al., 2015; Ramirez et al., 2012), the extent to which they cause significant changes in microbial function – and, importantly, what those changes are – is unknown. A number of microbial mechanisms have been proposed to explain why microbial respiration decreases in response to N addition and are the focus of the study reported here (Fig. 1a). Microbial respiration associated with organic matter decomposition is influenced by decomposer carbon use efficiency (CUE; i.e., C allocation to anabolism (e.g., microbial growth) or catabolism (e.g., decomposition); Fig. 1a, Mechanism 1), the activity of exo-enzymes produced by those decomposers (Fig. 1a, Mechanism 2), as well as the biomass of decomposing microorganisms (Fig. 1a, Mechanisms 3).

First, N might decrease microbial respiration by increasing microbial CUE (Fig. 1a, 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 overflow respiration and extracellular enzymes, leading to increased microbial efficiency and reduced respiration following N addition. This could occur either because of shifts in CUE within organisms or, alternately, because of shifts in microbial community composition towards dominance by organisms that acquire C more effectively through increased CUE, leading to a community-wide shift in CUE. A few empirical studies have 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 results from measurements of microbial CUE along natural gradients of soil organic N and found that microbial CUE increased with increasing ambient N concentration; however, experimental N addition had the opposite effect, reducing microbial CUE.

Second, N addition might reduce decomposition because N directly inhibits oxidative enzymes, which decompose more complex C substrates such as lignin (Fig. 1a, Mechanism 2) (Fog, 1988). Researchers have suggested that lignin degradation may be inhibited by added N if lignin degradation is a mechanism of N acquisition or "N mining" from molecules physically protected by lignin (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 (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, such as grasslands, where there is less lignin and lignin-degrading microbes (Sinsabaugh, 2010).

Finally, N addition might reduce respiration by decreasing the biomass of the decomposing microbes (Fig. 1a, Mechanism 3). It is well established that N addition tends to decrease microbial biomass (Liu and Greaver, 2010; Treseder, 2008). Nitrogen addition leads to soil acidification (decreasing pH), 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) and microbial biomass may be lower in more acidic soils due to the direct effects of decreased pH on microbial physiology, base cation limitation (Treseder, 2008), or 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, decreased microbial biomass, 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 biomass and decomposition (Mueller et al., 2012).

Our objective was to evaluate the microbial mechanisms by which N addition leads to decreased microbial respiration of SOM in grasslands. Specifically, we examined whether N addition decreased microbial respiration and decomposition by 1) increasing microbial CUE, 2) decreasing microbial oxidative enzyme activity, or 3) decreasing microbial biomass. Grassland

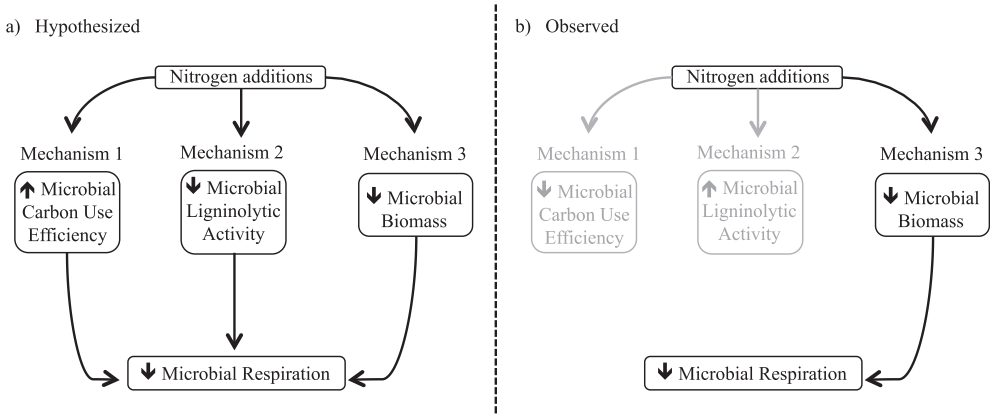

Fig. 1. Hypothesized (a) and observed (b) mechanisms of nitrogen addition effects on microbial respiration. (a) Hypothesized microbial mechanisms by which N might reduce respiration include increased carbon use efficiency (Mechanism 1), decreased ligninolytic enzyme activity (Mechanism 2), and decreased microbial biomass (Mechanism 3). (b) The observed effects of N addition on microbial carbon use efficiency and microbial ligninolytic enzyme activity were opposite to those necessary to explain the decrease in microbial respiration in response to N addition (Panel b, Mechanisms 1 and 2, grayed out). Instead, the negative effects of N addition on microbial biomass, possibly due to decreased substrate decomposability (e.g., higher root tissue N content) and/or effects of reduced soil pH on microbial physiology or Ca availability (but not on Al toxicity or physicochemical protection of SOM), likely explained the observed decrease in microbial respiration (Panel b, Mechanism 3).

ecosystems play a central role in the global C cycle and contain more soil C per unit area than the global average (Watson et al., 2000). We expected the unique plant and microbial community composition of grasslands to result in distinct mechanisms influencing microbial respiration and decomposition, compared to other biomes. To address our objective, we re-sampled soils from three sites of a long-term, continuous, grassland nutrient addition study (the Nutrient Network) where we previously observed inhibitory effects of N on microbial respiration and decomposition of slowly cycling SOM, despite increases in plant aboveground biomass and root biomass (Riggs et al., 2015). Furthermore, previous work conducted in the Nutrient Network established that nutrient addition caused significant microbial community composition shifts (Leff et al., 2015), including at the three sites studied here (J. Leff, personal communication). In particular, Leff and colleagues found that N addition increased the relative abundance of fast growing bacterial taxa (copiotrophs) and decreased the abundance of slow growing bacterial taxa (oligotrophs). However, how these shifts result in functionally significant differences in microbial decomposition is unknown.

2. Materials and methods

2.1. Soil sampling and processing

In August 2014, we collected soil samples from three multifactorial nutrient addition experiments in the U.S. Central Great Plains (Table 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 Riggs et al., 2015). 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} or 100 kg ha^{-1} 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 pentahydrate, 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). We previously determined via KCl extractions that, on average, inorganic N was 4×, 5.5×, and 17.3× greater in the N treatment plots relative to plots that did not receive N after 5 years of nutrient addition in Colorado, Nebraska, and Kansas, respectively (N main effect: $p < 0.0001$; Appendix – Table S1). Additionally, N mineralization rates were 5× greater in added N versus ambient N plots (Riggs et al., 2015). Consequently, this experiment provides an accurate measure of the effects of increasing available N on microbial decomposition.

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, sieved soil was subsampled for further analysis (see below). Air-dried, 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 pretreatment) and were excluded from all statistical analyses since we could not isolate biological sources of respired C from inorganic sources. 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 and straining with a 250 μm sieve, 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).

Table 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 (Riggs et al., 2015) 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 (Riggs et al., 2015). One core per block sampled at each site (n = 3).

2.2. 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 0.05 M K_2SO_4 and filtered. A replicate soil sample was fumigated with chloroform in a vacuum for 72 h, extracted with 0.05 M K_2SO_4 and filtered (Whatman No. 42; 2.5 μm pore size). Filtered extracts were analyzed for total dissolved organic C and total dissolved 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 (Beck et al., 1997; Brookes et al., 1985). Additionally, we used the concentration of C in the unfumigated extract as a measure of dissolved organic carbon (DOC) concentration in each sample.

2.3. Microbial respiration and decomposition parameters

We measured microbial respiration and decomposition rates during a long-term laboratory incubation. Before we initiated the laboratory incubation, a 50 g subsample of fresh, sieved soil from each plot was adjusted to 70% field capacity and pre-incubated for 6 h 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 CO_2–C g^{-1} soil day^{-1}) was calculated by measuring the accumulation of CO_2 in airtight 1 L Mason jars during 24–48 h 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. We measured CO_2 concentration inside the jars at the start and end of each interval by analyzing air samples collected via syringe with an infrared gas analyzer (LICOR 7000). When not being measured, soil samples were covered with gas-permeable, low-density polyethylene film and kept at 20 °C in the dark. Soil samples were maintained at 70% field capacity throughout the incubation. One sample was contaminated with excess water during the long-term respiration incubation and excluded from the analysis.

We calculated cumulative C respired (mg CO_2–C 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. In addition, we estimated decay rate and pool size parameters using maximum likelihood estimation (MLE; bbmle package in R). Specifically, we fit our daily respiration rate data (Appendix – Figure S1) 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 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 – Supplement S1 and Figure S2 for equifinality evaluation methods and results).

In addition to fitting our respiration rate data to a two-pool model, we compared the fit against a one-pool model (one-pool model not shown). We used the Akaike Information Criterion (AIC) – which penalizes for the number of parameters estimated – as a measure of model fit. The two-pool model was always the best model fit (see Section 3, Results), and consequently we report parameter estimates from the two-pool model only.

2.4. 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, 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 1 L Mason jars and respired CO_2 was sampled at 0 and 24 h using an infrared gas analyzer (LICOR 7000). 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. Carbon dioxide, 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 evaporated for >48 h in a 60 °C forced-air drying oven and the remaining solid 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. (2004). Briefly, 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).

2.5. 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). In addition, 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 were prepared for analysis by adding 1 g equivalent dry mass soil (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 in a 1.8 L blender. 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. During initial tests, we selected final substrate concentrations so that substrate amount did not limit activity (200 μM for CBH, BG, AG, and NAG; 400 μM for BX and AP). Additionally, incubation time was assayed for linear release of MUB. Blank wells contained 200 μl homogenate and 50 μl buffer. Standard wells contained 50 μl MUB standard ranging from 0.05 to 5 μM (final concentration), along with either 200 μl homogenate (quenched curve) or 200 μl of buffer (unquenched curve). Plates were incubated at 20 °C for 2 h, 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 h, centrifuged the tubes at 3600 rpm for 5 min, pipetted the supernatant into 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 0.35 ml L-DOPA. The peroxidase assay contained 1.4 ml homogenate, 0.35 ml L-DOPA, and 0.07 ml 0.3% H_2O_2. Blanks contained 1.4 ml homogenate and 0.35 ml buffer. During initial tests, we selected the final substrate concentration of L-DOPA (1 mM L-DOPA) so that substrate amount did not limit activity; additionally, we assayed incubation time for reaction linearity.

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.

2.6. Additional variables

The amount of root material collected in 2014 was small and insufficient for additional analyses of root chemistry beyond root C and N. Consequently, we supplemented our root dataset with chemical analyses of roots collected from control and N addition plots at the same sites in 2012 (after five years of nutrient addition and two years before the present study; root sampling methods detailed in Riggs et al., 2015). 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 and three years before the present study). 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.

2.7. 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). 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. 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.

3. Results

3.1. 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 – Tables S2 and S3 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 × K interaction: $p = 0.03$; site × N interaction: $p = 0.01$; Fig. 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 marginally 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 × K interaction: $p = 0.02$; site × N interaction: $p = 0.03$): N decreased microbial N by 25% on average in the

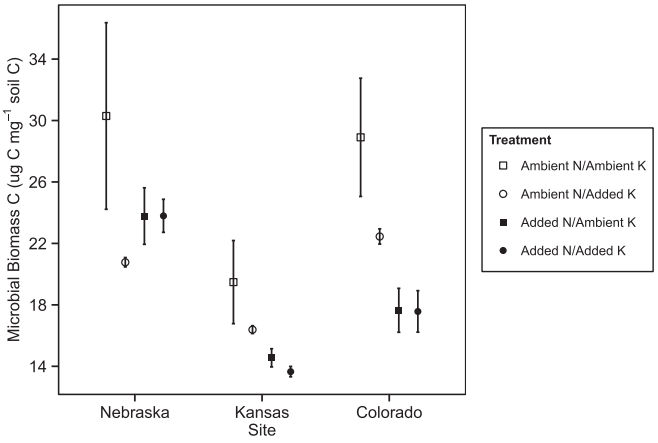

Fig. 2. Treatment effects on microbial biomass carbon per mass soil carbon. Figure shows mean plus/minus one standard error. Treatment codes: open symbols = ambient N; shaded symbols = added N; squares = ambient K; circles = added K.

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.

Nitrogen 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 – Figure S3). The effects of N on DOC concentration were the same whether analyzed per mass soil or per mass soil C.

3.2. Microbial respiration and decomposition parameters

Nitrogen addition decreased the cumulative C respired per mass soil C by 21% on average (N main effect: $p = 0.0001$; Fig. 3; see Appendix – Tables S4 and S5 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 cumulative CO_2 produced per microbial biomass C (μg CO_2–C respired μg^{-1} microbial C) were site-specific (site × N interaction: $p = 0.05$): N significantly decreased mass-specific cumulative microbial respiration in Kansas (post-hoc comparison: $p = 0.02$) and had no effect on mass-specific cumulative respiration in Nebraska and Colorado (post-hoc comparison: $p > 0.1$; Fig. 4).

There was moderately significant interactive effect of N x P x K on microbial mass-specific cumulative respiration and a significant positive effect of K on microbial mass-specific cumulative respiration: K addition increased cumulative mass-specific respiration (μg CO_2–C respired μg^{-1} microbial C) by 16.6% on average (K main effect: $p = 0.0008$; N x P × K interaction: $p = 0.07$; Appendix – Figure S4). 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 to 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 × 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 (Fig. 5a). Similarly, N increased the size of the fast pool in Kansas only (site × N interaction: $p = 0.005$; post-hoc comparison (Kansas): $p < 0.001$; Fig. 5b). By contrast, the effects of N of the slow pool were consistent across sites. Nitrogen decreased the decay rate of the slow pool by 31% (N main effect: $p < 0.0001$; Fig. 5c) and had no effect on the size of the slow pool (N main effect: $p > 0.1$; Fig. 5d). Other nutrient treatments had no effect on the decay rates of the fast and slow pools.

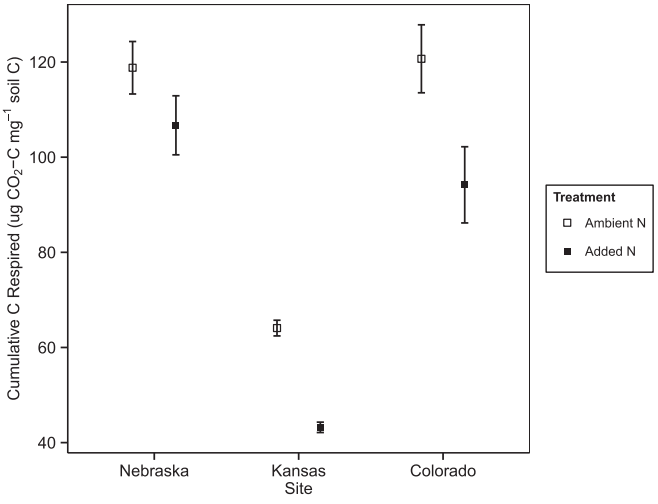

Fig. 3. Nitrogen treatment effects on cumulative carbon respired per mass soil carbon. Figure shows mean plus/minus one standard error. Treatment codes: open symbols = ambient N; shaded symbols = added N.

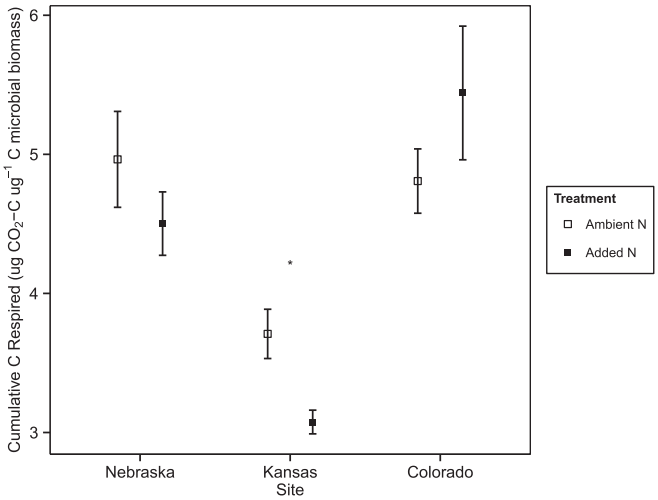

Fig. 4. Nitrogen treatment effects on cumulative carbon respired per mass microbial carbon. Figure shows mean plus/minus one standard error. Stars indicate significance from post-hoc pairwise comparisons: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. Treatment codes: open symbols = ambient N; shaded symbols = added N.

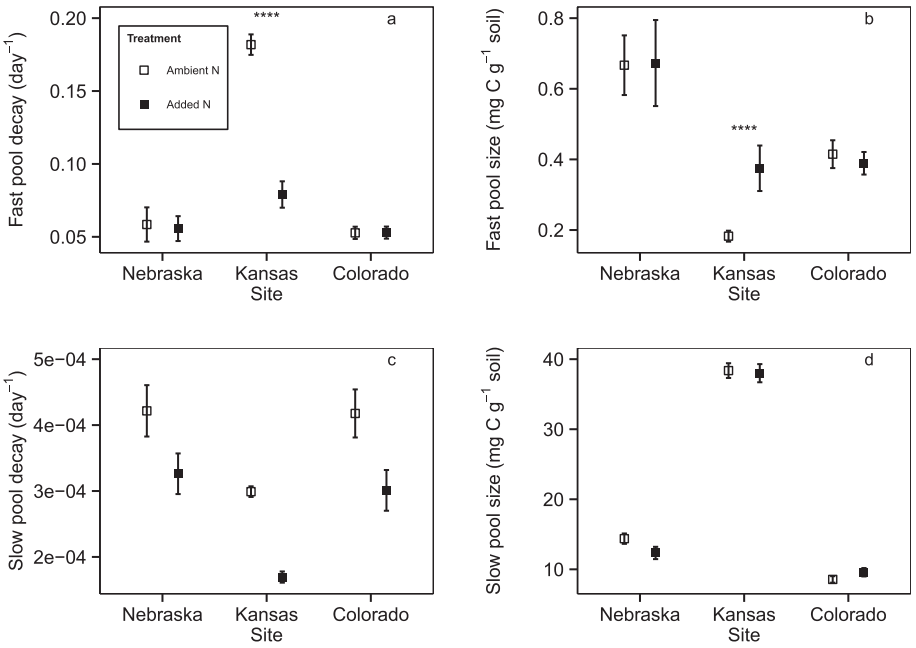

Fig. 5. Nitrogen treatment effects on the decay rate (a) and size (b) of the fast decomposing C pool, decay rate (c) and size (d) of the slow decomposing C pool measured with a long-term microbial respiration incubation. All panels show mean plus/minus one standard error. Stars indicate significance from post-hoc pairwise comparisons: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. Treatment codes are the same across all panels. Treatment codes: open symbols = ambient N; shaded symbols = added N.

3.3. 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; Fig. 6). The effects of N on CUE varied with substrate type and site (see Appendix – Tables S6 and S7 for microbial CUE ANOVA and data tables). The CUE of ^{13}C glucose decreased with N addition in Nebraska, but not Kansas (site × 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 – Figure S5).

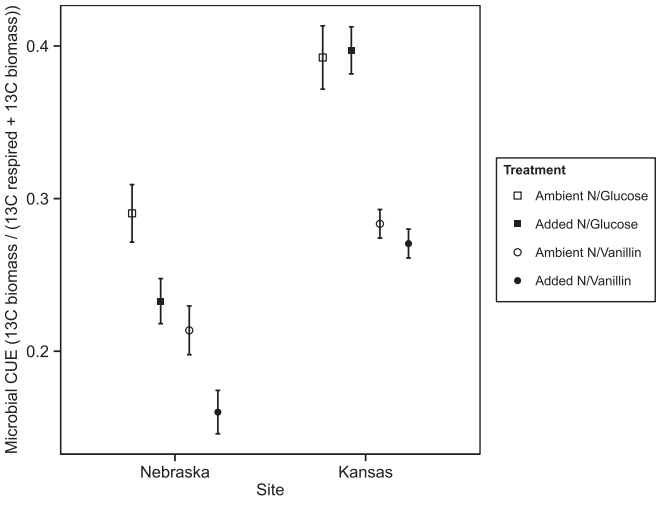

Fig. 6. Nitrogen treatment effects on microbial carbon use efficiency of ^{13}C glucose and ^{13}C vanillin from soil collected in Nebraska and Kansas. Figure shows mean plus/minus one standard error. CUE = carbon use efficiency. Treatment codes: open symbols = ambient N; shaded symbols = added N; squares = ^{13}C glucose; circles = ^{13}C vanillin.

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: $p > 0.1$). Phosphorus addition also 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, in contrast to N, 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 × 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.

3.4. 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 – Tables S8 and S9 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: $p < 0.0001$; Fig. 7; Appendix – Figure S6). 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

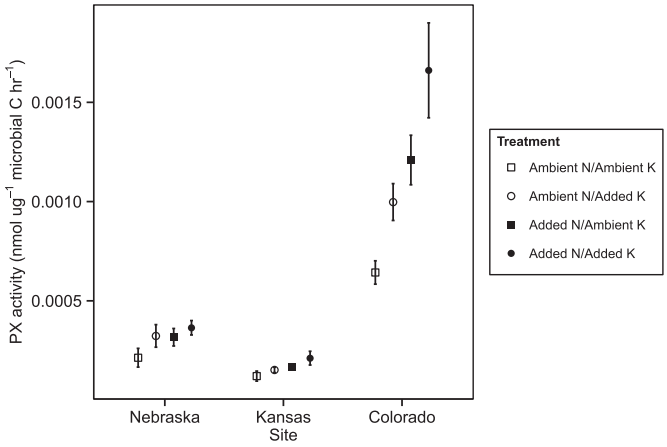

Fig. 7. Treatment effects on peroxidase activity per mass microbial carbon. Figure shows mean plus/minus one standard error. PX = peroxidase. Treatment codes: open symbols = ambient N; shaded symbols = added N; squares = ambient K; circles = added K.

effect: $p = 0.0002$). There was a significant interactive effect of N, P, and K on PO activity per mass soil C (N × P × K interaction: $p = 0.04$) and per mass microbial C (N × P × K interaction: $p = 0.01$; Fig. 8; Appendix – Figure S7). 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 ~1.2× 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 – Figures S8 and S9). Phosphorus addition significantly increased CBH activity per mass soil C (P main effect: $p = 0.01$) and per mass microbial C (P main effect: $p = 0.0005$);

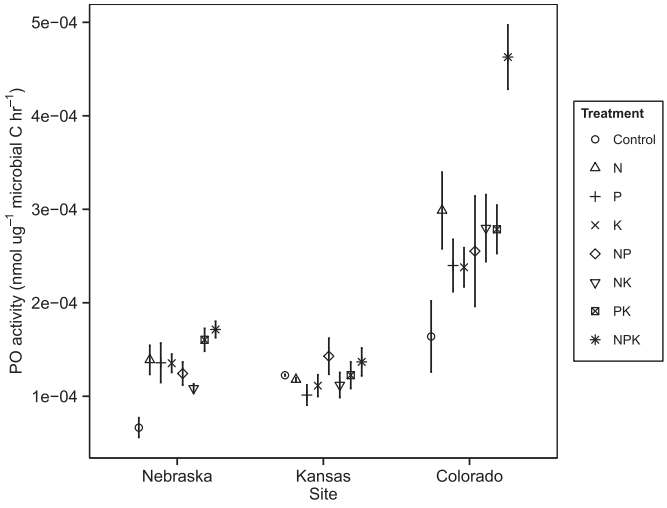

Fig. 8. Treatment effects on phenol oxidase activity per mass microbial carbon. Figure shows mean plus/minus one standard error. PO = phenol oxidase. Treatment codes: Control = control plots; N = nitrogen (N) addition plots; P = phosphorus (P) addition plots; K = potassium (K) addition plots; NP = nitrogen and phosphorus addition plots; NK = nitrogen and potassium addition plots; PK = phosphorus and potassium addition plots; NPK = nitrogen, phosphorus, and potassium addition plots.

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 × 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).

3.5. Plant substrate chemistry

Nutrient addition significantly increased the concentration of N in plant roots at all three sites (site × N interaction: $p = 0.0001$; see Appendix – Tables S10–13 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 × 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 × N interaction: $p = 0.01$; post-hoc comparisons: $p < 0.0001$ (Nebraska); $p < 0.0001$ (Kansas); $p = 0.006$ (Colorado); Appendix – Figure S10).

3.6. Soil chemistry

Nutrient treatments significantly altered soil pH and soil extractable micronutrient concentration (see Appendix – Tables S14 and S15 for soil chemistry ANOVA and data tables). Nitrogen addition significantly decreased soil pH (N × site interaction: $p = 0.005$; Appendix – Figure S11) from an average of 6.7 in ambient N plots to 6.3 in added N plots in Nebraska (post-hoc comparison: $p < 0.0001$), 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: $p < 0.0001$). 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 × 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 × 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 × 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 × 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: $p = 0.0003$).

4. Discussion

Nitrogen addition decreased the decomposition rate of the slowly cycling soil C pool by 31% and microbial respiration by 21% on average at three grassland sites in the U.S. Central Great Plains region. These results are in line with our previous research (Riggs et al., 2015), as well as those observed in both grassland and non-grassland systems by others. For example, in a meta-analysis of

soil respiration studies, Zhou et al. (2014) found that N addition decreased microbial heterotrophic respiration by approximately 18% in grasslands, and by nearly 13% across biomes, on average. 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 (Fig. 1b). Instead, we found that N addition decreased microbial biomass by 31% on average, although the mechanistic explanation for this response is still unclear. 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 (Treseder, 2008).

4.1. Nitrogen addition and substrate complexity decreased microbial CUE

In contrast to our hypothesis, N addition did not significantly increase microbial CUE, and in fact decreased CUE in most instances (Fig. 1b, Mechanism 1). The result was surprising since theoretical models predict that CUE will increase as the availability of nutrients increases (Ågren et al., 2001; Schimel and Weintraub, 2003). 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 relative to that of C). 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, as found in this study.

Why would CUE decrease in response to N addition? Carbon use efficiency 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 tend to have lower C to nutrient biomass requirements and lower CUE compared to fungi (Keiblinger et al., 2010; but see Thiet 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). Additionally, although uncommon, other studies of grassland microbial community responses to N addition have shown reduced fungal biomass and reduced fungal activity relative to bacterial activity (e.g., Denef et al., 2009; de Vries et al., 2007). Additionally, fast-growing organisms are predicted to have lower growth yields (or growth efficiency) compared to slow growing organisms (Fierer et al., 2007). Consequently, an increase in dominance by fast growing bacterial taxa (copiotrophs) in response to N addition, could lead to lower CUE. In summary, an increase in bacteria relative to fungi under N enrichment, as well as an increase in fast growing relative to slow growing bacteria, may explain the observed decrease in CUE in response to N addition.

A previous study by Leff et al. (2015) characterized the microbial community across 25 Nutrient Network sites including those sampled here (at the three sites sampled here, treatment effects matched those observed for the broader set of sites sampled, J Leff, personal communication), allowing us to further evaluate these different possibilities. Leff et al. (2015) documented declines in Glomeromycota, but increases in Ascomycota; similarly, some bacterial and archaeal phyla increased while others decreased. Thus it is unclear whether there was a net change in fungi:bacteria abundance given potentially offsetting increases and decreases among different phyla. However, bacterial phyla that are considered more copiotrophic (Alphaproteobacteria, Actinobacteria) increased with nutrient addition, while those considered more oligotrophic (Acidobacteria) decreased, perhaps explaining lower CUE in response to N addition (Fierer et al., 2007).

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, which will lead to decreased microbial growth efficiency (Brant et al., 2006). Since N addition can change the chemistry and input rate of plant tissues (Yuan and Chen, 2012), overall microbial CUE (and consequently C respired) could change due to microbial responses to changing substrate chemistry with added N. While N addition generally increases plant N concentrations, the effects of added N on plant C chemistry have been poorly characterized (Hobbie, 2015). Further, the consequences of greater litter N concentrations for SOM decomposability are unclear, making it difficult to predict whether CUE might increase or decrease because of changes in overall substrate chemistry with added N. We found that N decreased the decomposition rate of the slowly cycling C, but did not change its pool size. However, our method of estimating SOM pool size and decay rates from respiration rate cannot distinguish whether N decreased the decay rate of slower cycling C because N reduced the inherent decomposability of that pool of C versus because N increased the inherent CUE of the decomposers. However, given our direct measurements of CUE, that latter seems unlikely.

4.2. 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 (Fig. 1b, Mechanism 2). 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, this N effect may be system-specific (Sinsabaugh, 2010). For example, in a grassland N addition study, Zeglin et al. (2007) reported no effects of N addition on oxidative enzyme activity after 2, 17, or 55 years of nutrient addition. The negative effects of N on oxidative enzymes may be minimal or non-existent in grassland systems because Glomeromycota and Ascomycota (as opposed to Basidomycota) dominate and lignin content of the plant community is less relative to forests (Sinsabaugh, 2010). Indeed, Ascomycota are relatively more abundant than Basidiomycota across the Nutrient Network sites characterized by Leff et al. (2015). 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, low 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., Hobbie et al., 2012), although we did not observe such an increase here. Alternatively, 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., Bach et al., 2013; Sinsabaugh, 2010). Nitrogen addition could increase oxidative activity of the soil indirectly through changing soil chemistry (e.g., pH) that changes the availability of reactive minerals.

4.3. 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 (Fig. 1b, Mechanism 3). Negative effects of N addition on microbial biomass are well documented and several plant-, soil-, and microbe-mediated hypotheses could explain this response (Treseder, 2008). First, N addition could decrease microbial biomass if N addition leads to a decrease in substrate "decomposability" (e.g., an increase in chemically complex or otherwise slow to degrade substrates) and/or quantity. We found that N addition decreased the root lignin:N ratio and increased root N concentration, consistent with other studies that report increased aboveground plant N concentration in response to N addition (Hobbie, 2015). These tissue chemistry responses should lead to *greater* microbial access to C and biomass production, since the decay rate of litter tends to increase as the lignin:N ratio decreases (Melillo et al., 1982; Talbot and Treseder, 2011). However, increased N concentration can also slow decomposition in its later stages by inhibiting microbial decomposition activity (Berg and Matzner, 1997; Knorr et al., 2005). Since we did not observe negative effects of N on enzyme activity, the mechanism by which higher tissue N concentration would decrease microbial decomposition activity in this study is unknown. Consequently, it seems unlikely that microbial biomass decreased due to reduced substrate "decomposability."

Likewise, negative effects of N on microbial biomass via plant substrate quantity seem unlikely since we expect N addition to increase total C inputs belowground in grasslands. 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 (Riggs et al., 2015). 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 quantity explain the decrease in microbial biomass observed following N addition.

Second, N addition could decrease microbial biomass via soil acidification, which can lead to microbial physiology and community composition change (Rousk et al., 2010), base cation limitation (Treseder, 2008), Al toxicity to microbes (Flis et al., 1993), and/or physicochemical protection of C (Mueller et al., 2012). This would be consistent with results from a 12 year N addition experiment conducted in a Mongolian grassland where soil acidification best explained the decreases microbial biomass and microbial respiration (Chen et al., 2015). We found that N addition decreased soil pH but 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 (extractable Al was not measured). Lower Ca availability could limit microbial growth under more acidic pH, leading to decreased microbial biomass (Treseder, 2008).

Finally, N-mediated shifts in microbial community composition (Leff et al., 2015) could explain the decrease in microbial biomass. It is hypothesized that fast growing bacterial taxa (copiotrophs) have a smaller standing biomass pool relative to slow growing bacterial taxa (oligotrophs) (Fierer et al., 2007). Although empirical studies are needed to address the robustness of this hypothesis, a microbial-explicit carbon decomposition model did demonstrate that increasing the relative abundance of copiotrophs relative to oligotrophs decreased the microbial biomass pool due to the higher turnover rates of copiotrophs (Wieder et al., 2015b). 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 observed in Kansas: at that site, N enrichment significantly decreased mass-specific respiration (cumulative respiration per unit microbial biomass) 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) (Riggs et al., 2015). Additionally, previously we found that N addition had a modest positive effect on aggregation at these grassland sites, particularly for large macro-aggregates (Riggs et al., 2015). Increased aggregate occlusion of C in response to N addition may have reduced the C available to microbes, leading to decreased respiration of the Kansas soil samples in addition to the decreased respiration due to lower microbial biomass.

4.4. 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 (Riggs et al., 2015). 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 (Bradford et al., 2008). 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.

5. Conclusion

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. Although we did observe significant effects of N addition on soil pH and root tissue chemistry, the exact mechanism by which N addition led to decreased microbial biomass remains uncertain.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2016.04.023.

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