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Temperature sensitivity of soil enzyme kinetics under N-fertilization in two temperate forests

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Abstract

Soil microbes produce extracellular enzymes that degrade carbon (C)-containing polymers in soil organic matter. Because extracellular enzyme activities may be sensitive to both increased nitrogen (N) and temperature change, we measured the effect of long-term N addition and short-term temperature variation on enzyme kinetics in soils from hardwood forests at Bear Brook, Maine, and Fernow Forest, West Virginia. We determined the V_{max} and K_m parameters for five hydrolytic enzymes: α-glucosidase, β-glucosidase, β-xylosidase, cellobiohydrolase, and *N*-acetyl-glucosaminidase. Temperature sensitivities of V_{max} and K_m were assessed within soil samples subjected to a range of temperatures. We hypothesized that (1) N additions would cause microbial C limitation, leading to higher enzyme V_{max} values and lower K_m values; and (2) both V_{max} and K_m would increase at higher temperatures. Finally, we tested whether or not temperature sensitivity of enzyme kinetics is mediated by N addition. Nitrogen addition significantly or marginally significantly increased V_{max} values for all enzymes, particularly at Fernow. Nitrogen fertilization led to significantly lower K_m values for all enzymes at Bear Brook, but variable K_m responses at Fernow Forest. Both V_{max} and K_m were temperature sensitive, with Q_{10} values ranging from 1.64–2.27 for enzyme V_{max} and 1.04–1.93 for enzyme K_m. No enzyme showed a significant interaction between N and temperature sensitivity for V_{max}, and only β-xylosidase showed a significant interaction between N and temperature sensitivity for K_m. Our study is the first to experimentally demonstrate a positive relationship between K_m and temperature for soil enzymes. Higher temperature sensitivities for V_{max} relative to K_m imply that substrate degradation will increase with temperature. In addition, the V_{max} and K_m responses to N indicate greater substrate degradation under N addition. Our results suggest that increasing temperatures and N availability in forests of the northeastern US will lead to increased hydrolytic enzyme activity, despite the positive temperature sensitivity of K_m.

Keywords: carbon cycle, decomposition, enzyme kinetics, extracellular enzyme, microbe, nitrogen fertilization, soil warming

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Introduction

Microbial extracellular enzymes catalyze the rate-limiting step in soil organic matter decomposition and dissolved organic matter production (Sinsabaugh, 1994; Allison & Vitousek, 2005). Therefore, the activities of these enzymes can regulate ecosystem responses to changes in nutrient availability, temperature, and substrate quality (Schimel & Bennett, 2004; Sinsabaugh *et al*., 2005; Allison *et al*., 2010). Since human activities have doubled global inputs of fixed N since the mid 20th century (Vitousek *et al*., 1997; Galloway *et al*., 2003, 2004, 2008), and global temperatures are predicted to rise by 4–7 °C by 2100 (IPCC, 2007), an understanding of how microbial enzyme kinetics respond to N and temperature is important for predicting the future release of soil C to the atmosphere. However, the complex linkages between C, N, and temperature have not been resolved on an enzymatic level.

Hydrolytic enzymes control the decomposition of many biological macromolecules abundant in plant litter and soil such as cellulose, hemicellulose, chitin, and protein (Allison *et al*., 2007a). Hydrolytic enzyme activity (V) can be described by the Michaelis–Menten relationship, which is a saturating function of substrate concentration (S):

$$V = V_{max}[S]/(K_m + [S])$$

where V_{max} is the maximum potential rate of activity and K_m is the half saturation constant, or the substrate concentration at ½V_{max} (Fig. 1). At low substrate

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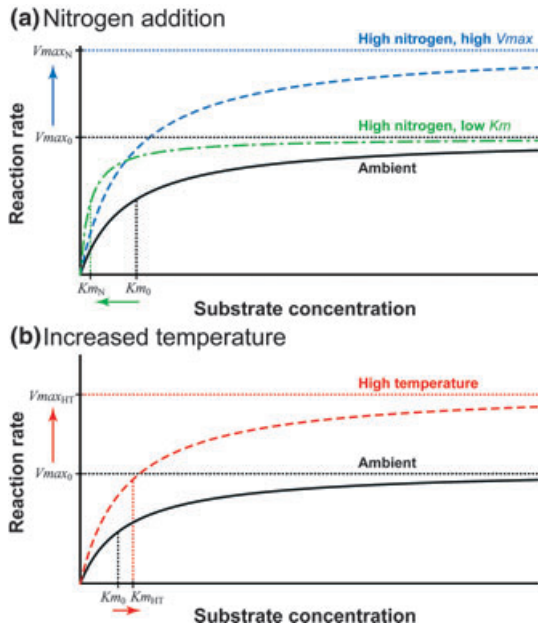

Fig. 1 Conceptual model of enzyme kinetic responses to nitrogen and temperature. (a) Michaelis–Menten curves depicting enzyme activity under ambient (solid black curve) and high-nitrogen (dashed blue and dash-dot green curves) conditions. Two non-mutually exclusive kinetic responses to increased nitrogen availability are shown: (1) High N, High V_{max}: N addition could increase microbial allocation to enzyme production, leading to higher V_{max} values. (2) High N, Low K_m: Microbial community shifts due to long-term N addition could result in enzymes with higher efficiencies and lower K_m values. (b) Michaelis–Menten curves under ambient (black curve) and high temperature (dashed red curve) conditions. $V_{max_0} = V_{max}$ under ambient conditions; $V_{max_N} = V_{max}$ under high N conditions; $V_{max_{HT}} = V_{max}$ at high temperature; $K_{m_0} = K_m$ under ambient conditions; $K_{m_N} = K_m$ under high N conditions; $K_{m_{HT}} = K_m$ at high temperature. Arrows show direction of kinetic parameter change at high temperature or nitrogen.

concentrations, enzymes decompose substrates with efficiencies directly related to their K_m value (Marx *et al.*, 2005; Davidson *et al.*, 2006). However, most studies of soil enzyme activity have aimed to measure only V_{max}. Measuring activity at substrate saturation has the advantage of reducing competitive inhibition by naturally occurring substrates within the soil matrix. Moreover, measuring K_m is challenging due to uncertainty regarding the relative contributions of artificial substrates and naturally occurring substrates under non-saturating conditions. Nonetheless, enzymes may operate under non-saturating conditions in soils, making K_m an important parameter that merits increased attention (Davidson *et al.*, 2006; German *et al.*, 2011).

Conceptual models linking N availability to extracellular enzyme activity have been developing for several decades (Sinsabaugh & Moorhead, 1994; Schimel & Weintraub, 2003, Allison *et al.* 2007a). The emerging principle behind these models is an economic framework based on the allocation of limited resources. Soil microbes exist in a competitive environment, and will preferentially allocate energy toward the acquisition of whatever resource is most limiting their growth. Enzymes are N-rich molecules, and as N availability increases it follows that microbes will allocate more resources toward the production of enzymes used to acquire energy or other nutrients (Sinsabaugh & Moorhead, 1994, Allison *et al.* 2007a). Moreover, due to the stoichiometric constraints regulating microbial C and N demand (Sinsabaugh *et al.*, 2008), increased N availability may increase microbial C limitation and thus cause a heightened need for C-acquiring enzymes (Allison & Vitousek, 2005).

Mechanistically, changes in hydrolytic enzyme activity due to N addition may be due to changes in both enzyme V_{max} and K_m parameters (Fig. 2a). If increased N availability causes increased microbial enzyme production, V_{max} will likewise increase. In addition, long-term increases in available N often result in microbial community shifts (e.g., Frey *et al.*, 2004; Wallenstein *et al.*, 2006; Allison *et al.*, 2007b; Allison & Martiny, 2008; Nemergut *et al.*, 2008). Changes in community composition may in turn lead to the production of different isoforms of enzymes with altered active site

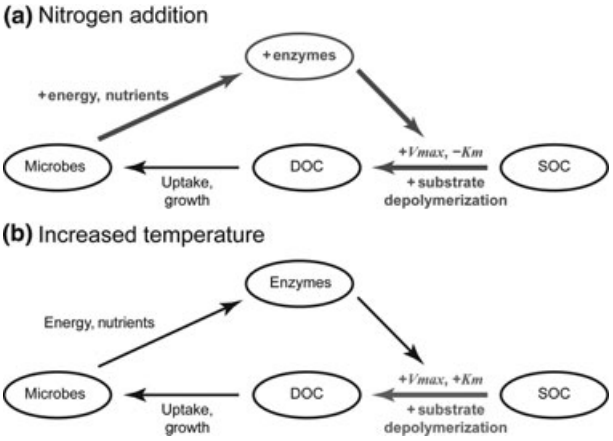

Fig. 2 Conceptual model linking microbial pools, extracellular enzymes, and organic carbon pools in the soil matrix. Colors represent predicted changes in enzymatic processes under added nitrogen (blue) or increased temperature (red). (a) Under high nitrogen conditions, microbial resource allocation toward C-degrading enzyme production increases. Changes in enzyme kinetic parameters increase the rate of conversion of soil organic carbon (SOC) to dissolved organic carbon (DOC). (b) Higher temperatures directly affect the kinetic behavior of extracellular enzymes such that SOC is depolymerized at a higher rate. However, net changes in SOC and DOC pool sizes will depend on the relative changes in both V_{max} and K_m parameters.

properties that enhance substrate-binding affinity (Somero, 1978). These shifts could thus result in an enzyme pool with higher intrinsic efficiencies (lower K_m values) such that metabolic needs can be met at a lower cost. However, decreasing K_m to reduce energy expenditures and increase per capita C acquisition may have tradeoffs. Lower K_m values are generally associated with lower V_{max} values, as well as a loss of regulatory sensitivity (Somero, 1978).

A better mechanistic understanding of extracellular enzyme kinetics could help resolve uncertainty surrounding ecosystem responses to added N. Both decomposition studies and microbial enzyme studies have reported positive, negative, and neutral responses to N fertilization (Knorr *et al.*, 2005; Janssens *et al.*, 2010; Weiss, 2011). N addition can increase the production of hydrolytic enzymes when N is a limiting nutrient (Carreiro *et al.*, 2000; Saiya-Cork *et al.*, 2002; Sinsabaugh *et al.*, 2005, Allison *et al.*, 2007a, Keeler *et al.*, 2009). However, N addition has also been found to reduce oxidase enzyme activity (Carreiro *et al.*, 2000; Saiya-Cork *et al.*, 2002; Waldrop *et al.*, 2004; Sinsabaugh *et al.*, 2005), possibly due to suppression of basidiomycete fungi or increased recalcitrance of lignin compounds with added N (Fog, 1988; Frey *et al.*, 2004; Waldrop *et al.*, 2004; Sinsabaugh, 2010). For decomposition processes involving hydrolytic enzymes, changes in K_m could represent an unrecognized mechanism controlling microbial decomposition responses to N that might have been overlooked due to an exclusive focus on V_{max}.

As with N, empirical studies of extracellular enzyme responses to temperature have focused on V_{max} rather than K_m (e.g. Trasar-Cepeda *et al.*, 2007). Biochemical theory predicts that enzyme V_{max} responds positively to temperature up to some optimum value, beyond which the enzyme begins to denature (Davidson & Janssens, 2006). In addition, the increased kinetic energy of both enzymes and substrates with increased temperature could result in a destabilization of the enzyme–substrate complex, thereby reducing enzyme-binding affinity and raising K_m (Davidson & Janssens, 2006; Davidson *et al.*, 2006). Model simulations indicate that increased K_m could offset the positive effect of temperature on V_{max}, especially at low substrate concentrations where K_m has a large influence on enzyme activity rates (Davidson & Janssens, 2006; Davidson *et al.*, 2006). Although K_m temperature sensitivity has not been measured in soils, the K_m values of some enzymes from animals are known to increase with temperature (Somero, 1978; Hochachka & Somero, 2002; Huestis *et al.*, 2009).

The goal of this study was to examine N responses and temperature sensitivities of kinetic parameters of hydrolytic enzymes in soils from two hardwood forests of the northeastern US with long-term N fertilization experiments. We chose to focus on hydrolytic enzymes because their kinetic parameters can be readily measured, whereas methods for the study of oxidative enzyme kinetics require further development (Sinsabaugh, 2010; German *et al.*, 2011). However, we acknowledge that accurately predicting soil C responses to global change will require studies on the temperature sensitivities and N responses of oxidative enzymes as well, given their dominant role in decomposition in many soils (Sinsabaugh, 2010). Based on microbial allocation theory, we hypothesized that V_{max} values would increase with long-term N fertilization. Furthermore, we hypothesized that increased microbial demand for C would result in the production of more efficient enzymes with lower K_m values (Fig. 2a). Based on biochemical theory (Davidson & Janssens, 2006; Davidson *et al.*, 2006) and prior studies on digestive enzymes (Somero, 1978, 2004; Hochachka & Somero, 2002), we hypothesized that V_{max} and K_m values of soil microbial enzymes would increase with temperature in the laboratory (Fig. 2b). Finally, we sought to discern whether or not N fertilization would alter the temperature sensitivity of soil enzyme V_{max} and K_m parameters (see Fig. 1 for visualization of predicted changes to Michaelis–Menten curves with N addition and temperature change).

Methods

Site descriptions

This study was conducted using forest floor material collected from hardwood forests at the Bear Brook Watersheds in Maine and the Fernow Experimental Forest in West Virginia (Table 1). These sites fall near opposite ends of an N deposition gradient spanning the northeastern US. While Fernow receives 6.8 kg N ha^{-1} yr^{-1}, Bear Brook receives only 3.1 kg N ha^{-1} yr^{-1} of estimated wet deposition (Fernandez *et al.*, 2010). The sites differ in mean annual temperature, with Fernow averaging 3.9 °C warmer than Bear Brook (Table 1). Fernow also has less than half of the forest floor C stock and has markedly lower C : N ratios relative to Bear Brook (Table 2; Weiss, 2011).

Bear Brook watersheds. Bear Brook is located in eastern Maine, USA (44°52′N, 68°06′W). Mean annual temperature is 4.9 °C (Fernandez *et al.*, 2010). Its long-term fertilization experiment consists of two approximately 11 ha watersheds: a reference watershed (East Bear) and a treated watershed (West Bear) that has received additions of $(NH_4)_2SO_4$ since 1989 at an annual rate of 25.2 kg N ha^{-1} yr^{-1}. Soils in the Bear Brook Watersheds are Spodosols, (coarse-loamy and loamy Haplorthods) and Inceptisols (loamy, mixed, active Aeric Endoaquepts).

Erratum: The reference watershed at Fernow that is mentioned below was in fact WS13 and the fertilized watershed was WS3.

Table 1 Study site characteristics

Site	Location[*†]	MAT[†] (°C)	MAP[‡] (mm yr^{-1})	Dominant soil type[§]	Wet N deposition rate (kg N ha^{-1} yr^{-1})[†]	Fertilization rate[*†] (kg N ha^{-1} yr^{-1})
Bear Brook, Maine	44°52′N, 68°06′W	4.9	1320	Spodosols	3.1	25.2
Fernow, West Virginia	39°03′N, 79°49′W	8.8	1458	Inceptisols	6.8	35

[*]Adams *et al.*, 2006.
[†]Fernandez *et al.*, 2010.
[‡]Pan *et al.*, 1997.
[§]SanClements *et al.*, 2010.

Table 2 Forest floor properties of treated and reference catchments for the two field sites

Site	Treatment	Horizon	%N	%C	C : N Ratio	Soil pH
Bear Brook	Reference	O	1.99 ± 0.10	44.50 ± 1.93	22.43 ± 0.86	5.0
	+N	O	1.85 ± 0.15	41.03 ± 4.20	21.97 ± 0.54	5.0
Fernow	Reference	O/A	0.63 ± 0.04	10.58 ± 0.55	17.05 ± 1.56	5.0
	+N	O/A	0.50 ± 0.05	7.11 ± 0.71	14.32 ± 0.59	5.0

These soils average approximately 1 m in depth across the site. Bedrock is primarily quartzite and gneiss with granitic intrusions (Norton *et al.*, 1999). Vegetation in both watersheds at Bear Brook is dominated at high elevations by red spruce (*Picea rubens* Sarg.) and balsam fir (*Abies balsamea* L.). At lower elevations, northern hardwoods dominate, particularly American beech (*Fagus grandifolia*), sugar maple (*Acer saccharum*), and red maple (*Acer rubrum*) (SanClements *et al.*, 2010).

Fernow Experimental Forest. Fernow Forest is located within the Allegheny Mountain section of the unglaciated Allegheny Plateau in West Virginia (39°03′N, 79°49′W). Mean annual temperature is 8.8 °C (Fernandez *et al.*, 2010). Similar to Bear Brook, Fernow includes a paired watershed experiment with a reference watershed (WS3) and an experimental watershed (WS13) which have received additions of $(NH_4)_2SO_4$ since 1989 at an annual rate of 35 kg N ha^{-1} (Adams *et al.*, 2006). Soils of the study watersheds are coarse-textured Inceptisols (loamy-skeletal, mixed mesic Typic Dystrochrept) of the Calvin series, and average about 1 m in depth. These generally sandy loams are derived from acidic sandstones and shales (Kochenderfer, 2006). Tree species are generally similar across watersheds, although early-successional species, such as black birch (*Betula lenta* L.), black cherry (*Prunus serotina* Ehrh.), and yellow-poplar (*Liriodendron tulipifera* L.) dominate on the treated watershed, whereas late-successional species, such as sugar maple (*Acer saccharum* Marshall) and northern red oak (*Q. rubra* L.) dominate on the reference watershed (Adams *et al.*, 2006, 2007; Kochenderfer, 2006).

Soil sampling and analyses

Both the reference and N-fertilized watersheds were sampled at Bear Brook and Fernow. In each watershed, three composites of five soil samples each were collected using a 3 cm core diameter at Bear Brook and a 2 cm core diameter at Fernow. Each composite was collected over a 15 m^2 area at Bear Brook and a 10 m^2 area at Fernow. Sampling regions were located throughout each watershed and chosen to represent the edaphic variation within each watershed to ensure that we captured a similar amount of variability between treated and reference watersheds. This sampling scheme increases our confidence that any differences we observe are due to N addition rather than random variation in soil properties. A variable-depth sampling scheme was used to obtain the entire O-horizon at each site. Owing to the shallowness of the O-horizon at Fernow, the upper 2.5 cm of the mineral horizon (A) was also sampled. Samples were collected in late November, 2009, at Bear Brook and in early May, 2010, at Fernow. Mean monthly air temperatures around the sampling times were approximately 3 °C for Bear Brook and (I.J. Fernandez, unpublished data) and 12 °C for Fernow (Adams *et al.*, 1994).

Once collected, samples were hand-mixed to composite and transported on ice to Cornell University. In the lab, 2 g subsamples (field moist) of each soil composite were frozen at −20 °C for enzyme analysis. Freezing was intended to arrest microbial activity and minimize change in the enzymatic community during storage (Lee *et al.*, 2007; Keeler *et al.*, 2009). We acknowledge that freezing may have some effect on soil enzyme activities as has been seen in other studies (e.g. Lee *et al.*, 2007; DeForest, 2009), but since all our soils were subjected to freezing at the same temperature we felt it was unlikely that this treatment would alter our experimental outcome.

For each soil composite, three replicate subsamples of approximately 1 g each were dried at 110 °C for 1 day to determine soil moisture content. Soil pH was measured using

a 1 : 10 solution of soil to deionized water. For both reference and N-fertilized watersheds at each site, soil pH values were approximately 5.0, which are somewhat higher than published values for the O-horizon at Bear Brook (pH 4.0) and Fernow (pH 3.8–4.1) (Weiss, 2011). We therefore used a sodium acetate solution buffered to pH 5 for all enzyme assays. A subsample of each soil composite was dried for a minimum of 24 h at 50 °C, ground into a fine powder using a ball mill, and analyzed for C and N content with an elemental analyzer (Elementar vario EL-III, Hanau, Germany).

Fluorimetric enzyme assays

We measured the activities of five hydrolytic enzymes: α-glucosidase, β-glucosidase, β-xylosidase, cellobiohydrolase, and *N*-acetyl-glucosaminidase (Table 3) according to the fluorimetric protocol of Saiya-Cork *et al.* (2002) with modifications by Allison *et al.* (2009), DeForest (2009) and German *et al.* (2011). Soils were thawed for analysis, and each enzyme was assayed in each soil at 4, 7, 16, 22, 28, 34, and 40 °C for 1 h. Briefly, 0.25 g soil (dry weight equivalent) was homogenized in 125 ml of sodium acetate buffer using a hand blender. A quantity of 200 μl of this soil homogenate was added to two replicate columns in a 96 well microplate. Fluorescent substrate proxies specific to each enzyme were added to the assay wells in concentrations of 1–400 μM (Table 3). Assays were run with two standard columns containing soil homogenate and methylumbelliferone (MUB), the fluorescent tag attached to each substrate proxy. Each assay microplate also contained two columns of blanks for measuring background fluorescence in the substrate, one column of soil homogenate blanks, and one column of acetate buffer blanks. After incubation, 10 μl of 1 M NaOH was added to each well to terminate enzyme activity. Following termination of each reaction, we used a fluorometer set at 365 nm excitation and 450 nm emission to measure fluorescence. From these fluorescence values, we calculated enzyme activity as the rate of substrate converted in nmol g^{-1}dry soil h^{-1} (DeForest, 2009). V_{max} and K_m values were calculated for each soil composite by fitting the Michaelis–Menten equation to the enzyme activity data with nonlinear regression in the statistics program R (R Development Core Team, 2006). Because soil C concentrations varied with site and N addition (Table 2), we present V_{max} in units of nmol g^{-1} soil C h^{-1}. This normalization factors out variations in soil C concentration that are expected to relate positively to microbial biomass and enzyme production (Sinsabaugh *et al.*, 2008; Fierer *et al.*, 2009). However, we also report V_{max} values and temperature sensitivities per unit soil mass in Table S1 and Fig. S1.

Statistical analysis

The V_{max} and K_m parameters for each enzyme were log-transformed because they increased exponentially with temperature. We examined the effects of site, N treatment, temperature, and their interactions on V_{max} and K_m using an analysis of covariance (ANCOVA) with temperature as the covariate. Since enzyme activity measurements across temperatures within soil cores are nonindependent, we treated soil composite as a random factor in the ANCOVA and tested for significance using a linear mixed-effects model and the R package nlme (Pinheiro & Bates, 2000). Regression coefficients (slopes and intercepts) were plotted as linear relationships along with the data for each N treatment × site combination. Slopes represent the temperature sensitivities of each enzyme kinetic parameter and were also expressed as Q_{10} values according to:

$$Q_{10} = \exp(\text{slope} \times 10)$$

Results

V_{max} response to temperature and nitrogen

The V_{max} values increased significantly with temperature for all enzymes at both sites (Fig. 3). The magnitude of the temperature response of ln (V_{max}) varied across enzymes, ranging from 0.050 to 0.082 $°C^{-1}$, which corresponds to Q_{10} values of 1.64 to 2.27 (Table 4). We found no statistically significant interactions between temperature and site or N addition, meaning that the temperature sensitivity of V_{max} did not vary with site or N treatment.

The V_{max} values for all enzymes, except *N*-acetyl-glucosaminidase, increased significantly ($P < 0.05$) with N addition, but the increases were always more pronounced at Fernow (Fig. 3). This interaction between N

Table 3 Summary of enzymes studied, their broad ecological functions, and substrate proxies used for laboratory assays. All substrate proxies used are methylumbelliferone (MUB)-linked. The appropriate concentration ranges were determined in a preliminary experiment (German *et al.*, 2011)

Enzyme	Enzyme function	Substrate proxy	Substrate concentration gradient
α-glucosidase	Releases glucose from starch	4-MUB α-D-glucopyranoside	2–200 μM
β-glucosidase	Releases glucose from cellulose	4-MUB β-D-glucopyranoside	2–200 μM
β-xylosidase	Releases xylose from hemicellulose	4-MUB β-D-xylopyranoside	2–200 μM
Cellobiohydrolase	Releases disaccharides from cellulose	4-MUB-β-D-cellobioside	1–100 μM
N-acetyl glucosaminidase	Releases *N*-acetyl glucosamine from chitin	4-MUB *N*-acetyl-β-D-glucosaminide	4–400 μM

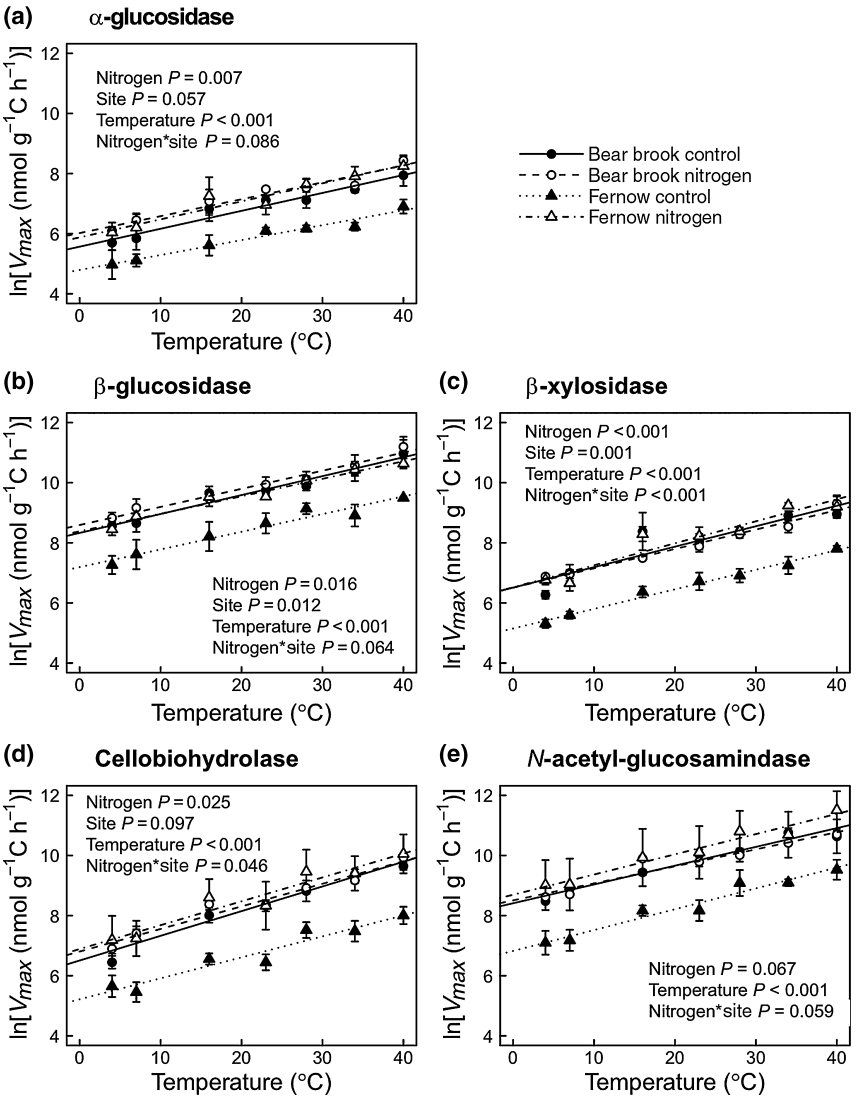

Fig. 3 Temperature sensitivities of V_{max} for (a) α-glucosidase, (b) β-glucosidase, (c) β-xylosidase, (d) cellobiohydrolase, and (e) *N*-acetyl-glucosaminidase by site and nitrogen treatment. V_{max} is expressed as nmol g^{-1} C h^{-1}. Symbols represent the mean (± SE) V_{max} for three replicate composites at a given temperature. Regression lines are shown for each combination of site and treatment. Effects significant at $P < 0.10$ are listed from a full factorial ANCOVA with nitrogen treatment and site as main effects and laboratory incubation temperature as the covariate.

and site was significant for β-xylosidase ($P < 0.001$) and cellobiohydrolase ($P < 0.05$), and marginally significant for the other enzymes ($P < 0.10$). For *N*-acetyl-glucosaminidase, the main effect of N addition was also marginally significant ($P = 0.067$). Overall, V_{max} values were significantly greater at Bear Brook for β-glucosidase ($P < 0.05$) and β-xylosidase ($P < 0.001$) and marginally significantly greater for α-glucosidase ($P = 0.057$) and cellobiohydrolase ($P = 0.097$). However, these site differences were only observed in unfertilized soils, consistent with the N × site interactions (Fig. 3). When expressed per soil mass, V_{max} values were markedly higher at Bear Brook regardless of N treatment because Bear Brook soils contained more C (Fig. S1, Table S1).

K_m response to temperature and nitrogen

As with V_{max}, we observed a significant positive response of K_m to increasing temperature for all enzymes (Fig. 4). The magnitude of the temperature response of ln (K_m) varied across enzymes, ranging from 0.004 to 0.066 $°C^{-1}$ (Table 5). These temperature sensitivities correspond to Q_{10} values of 1.04 to 1.93 and are lower than the corresponding V_{max} Q_{10} values for all enzymes. β-xylosidase was the only enzyme for

Table 4 Regression intercepts and slopes for log (V_{max}) in nmol g^{-1} soil C h^{-1} as a function of temperature (n = 21)

Enzyme	Site	Treatment	Intercept	± SE	Slope	± SE	Q_{10}
α-glucosidase	Bear Brook	Control	5.6	0.2	0.060	0.008	1.81
		Nitrogen	6.0	0.2	0.056	0.007	1.75
	Fernow	Control	4.8	0.2	0.050	0.007	1.64
		Nitrogen	5.9	0.3	0.060	0.011	1.82
β-glucosidase	Bear Brook	Control	8.3	0.2	0.063	0.008	1.88
		Nitrogen	8.6	0.2	0.060	0.008	1.83
	Fernow	Control	7.2	0.2	0.059	0.010	1.80
		Nitrogen	8.4	0.1	0.058	0.005	1.79
β-xylosidase	Bear Brook	Control	6.5	0.3	0.068	0.010	1.98
		Nitrogen	6.5	0.1	0.064	0.005	1.91
	Fernow	Control	5.1	0.1	0.065	0.005	1.92
		Nitrogen	6.5	0.2	0.073	0.008	2.08
Cellobiohydrolase	Bear Brook	Control	6.5	0.2	0.082	0.007	2.27
		Nitrogen	6.8	0.1	0.075	0.005	2.12
	Fernow	Control	5.2	0.2	0.070	0.009	2.01
		Nitrogen	6.9	0.5	0.079	0.019	2.21
N-acetyl-glucosaminidase	Bear Brook	Control	8.4	0.2	0.063	0.007	1.87
		Nitrogen	8.5	0.1	0.057	0.005	1.76
	Fernow	Control	6.8	0.2	0.069	0.009	2.00
		Nitrogen	8.7	0.5	0.067	0.021	1.96

which the K_m temperature response showed a significant dependence on site ($P < 0.05$) and N treatment ($P < 0.05$). These interactions were driven by higher temperature sensitivities for K_m at Fernow and in the N treatment (Table 5).

For all enzymes except *N*-acetyl-glucosaminidase, the K_m response to N was site dependent ($P < 0.05$ for N × site interactions). For *N*-acetyl-glucosaminidase, K_m values were significantly lower with N fertilization across both sites ($P < 0.05$). For the other enzymes, N fertilization generally reduced K_m at Bear Brook, whereas the K_m response to N fertilization was more variable at Fernow (Fig. 4). There, the K_m of α-glucosidase responded positively to N, while β-glucosidase, β-xylosidase, and cellobiohydrolase K_m values were not significantly affected. Across N treatments, all enzymes except *N*-acetyl-glucosaminidase showed significantly ($P < 0.05$) greater K_m values at Bear Brook than at Fernow (Fig. 4).

Discussion

We tested the hypothesis that V_{max} values would increase with N fertilization due to enhanced production of C-degrading enzymes by microbes relieved of N limitation (Fig. 2a). Sampling in the O and upper mineral horizons allowed us to target the fraction of the soil with the most hydrolysable C substrate, which is where we would expect the strongest response of hydrolytic enzymes to N addition. Particularly at Fernow, the N treatment effect we observed for α-glucosidase, β-glucosidase, β-xylosidase, and cellobiohydrolase supports this hypothesis, and indicates that N fertilization stimulates microbes in these soils to produce enzymes that degrade starch, cellulose, and hemicellulose. *N*-acetyl-glucosaminidase may have responded weakly to N because this enzyme releases N from organic matter in addition to C. Chitin is one of the dominant sources of organic N to soils, and *N*-acetyl-glucosaminidase releases small, N-containing amino sugars from chitin (Olander & Vitousek, 2000). If N is not limiting, it may be more energetically efficient for microbes to produce enzymes that mainly target C (Olander & Vitousek, 2000, Allison *et al.*, 2007a).

The response of V_{max} to N was greater at Fernow for all enzymes, though the N × site interaction was only significant for β-xylosidase and cellobiohydrolase. This trend suggests that N addition may have resulted in more severe C limitation to microbes in Fernow soils, which contained less C, a lower C : N ratio, and more mineral-associated soil organic matter than the Bear Brook soils (Table 2). Physical and biological differences between the sites contribute to the lower C stocks at Fernow, where the O-horizon is very shallow. As a result, the entire O-horizon and the upper 2.5 cm of the mineral horizon (A) were sampled for this study, whereas Bear Brook samples contained only O-horizon material, which is more C-rich. Since enzymes are

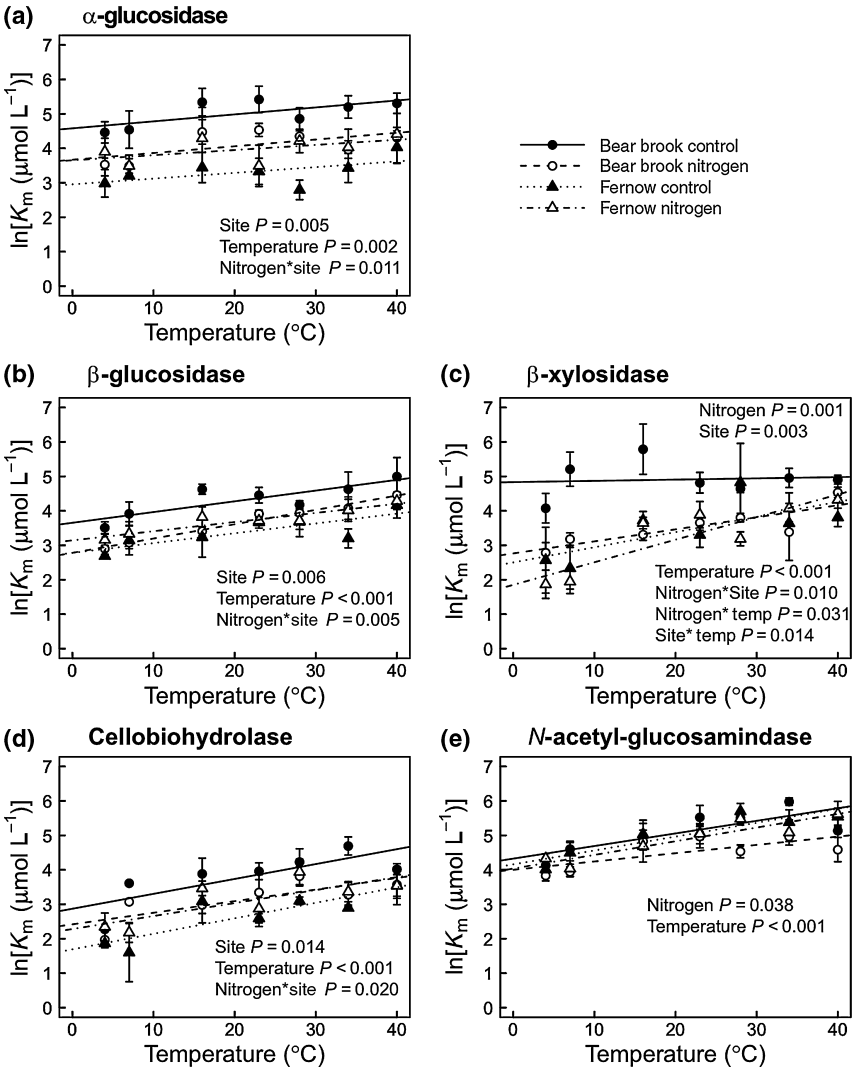

Fig. 4 Temperature sensitivities of K_m for (a) α-glucosidase, (b) β-glucosidase, (c) β-xylosidase, (d) cellobiohydrolase, and (e) *N*-acetyl-glucosaminidase by site and nitrogen treatment. K_m is expressed as μmol L^{-1}. Symbols represent the mean (± SE) K_m for three replicate composites at a given temperature. Regression lines are shown for each combination of site and treatment. Effects significant at $P < 0.10$ are listed from a full factorial ANCOVA with nitrogen treatment and site as main effects and laboratory incubation temperature as the covariate.

N-rich proteins, microbes may have responded to low C availability and high N availability at Fernow by allocating N to the production of enzymes that acquire C (Sinsabaugh & Moorhead, 1994; Allison & Vitousek, 2005). Another characteristic of Fernow consistent with its positive enzymatic response to N is that this site has experienced decades of elevated N inputs from atmospheric deposition (Peterjohn *et al.*, 1996). In addition, fertilized soils at Fernow have received approximately 40% more added N than at Bear Brook (Table 1). Higher rates of N deposition may have led to reduced proteolytic activity at Fernow relative to Bear Brook, as observed in other studies (e.g., Allison & Vitousek, 2005; Allison *et al.*, 2008). Less proteolytic activity could reduce enzyme turnover rates, resulting in a larger pool of enzymes and higher V_{max} values. Finally, differences in tree species composition between the fertilized and reference watersheds at Fernow could have contributed to different enzymatic responses. Early-successional tree species were more abundant in the N-fertilized watershed at Fernow, and associated differences in litter inputs or root exudation may have contributed to increased V_{max} values.

Particularly when substrate concentrations are low, reducing K_m may be an effective strategy for microbes to increase enzymatic efficiency and alleviate C limitation that may arise in the presence of excess N (Fig. 2a). We therefore hypothesized that N fertilization would

Table 5 Regression intercepts and slopes for log (K_m) in μmol L^{-1} as a function of temperature (n = 21)

Enzyme	Site	Treatment	Intercept	± SE	Slope	± SE	Q_{10}
α-glucosidase	Bear Brook	Control	4.6	0.3	0.020	0.011	1.22
		Nitrogen	3.7	0.2	0.020	0.009	1.22
	Fernow	Control	3.0	0.3	0.017	0.011	1.18
		Nitrogen	3.7	0.4	0.015	0.016	1.16
β-glucosidase	Bear Brook	Control	3.7	0.2	0.031	0.010	1.37
		Nitrogen	2.8	0.2	0.042	0.006	1.52
	Fernow	Control	2.8	0.3	0.028	0.011	1.33
		Nitrogen	3.1	0.2	0.027	0.007	1.30
β-xylosidase	Bear Brook	Control	4.8	0.3	0.004	0.014	1.04
		Nitrogen	2.8	0.3	0.035	0.011	1.42
	Fernow	Control	2.5	0.5	0.044	0.019	1.55
		Nitrogen	1.9	0.3	0.066	0.013	1.93
Cellobiohydrolase	Bear Brook	Control	2.9	0.3	0.043	0.011	1.54
		Nitrogen	2.4	0.3	0.034	0.012	1.40
	Fernow	Control	1.7	0.3	0.045	0.011	1.57
		Nitrogen	2.3	0.3	0.038	0.012	1.46
N-acetyl-glucosaminidase	Bear Brook	Control	4.3	0.2	0.037	0.010	1.44
		Nitrogen	4.0	0.2	0.024	0.008	1.27
	Fernow	Control	4.2	0.2	0.040	0.009	1.50
		Nitrogen	4.0	0.2	0.040	0.009	1.49

lead to the production of enzymes with lower K_m values. We found support for this hypothesis at Bear Brook for all enzymes, and additional support across both sites for *N*-acetyl-glucosaminidase. Decreases in enzyme K_m could occur via changes in microbial community composition and selection for microbes that produce more efficient enzymes in response to added N or increased C limitation. However, molecular studies of community composition are warranted to verify whether or not communities in our N-fertilized soils are in fact genetically distinct, as observed in other ecosystems (e.g., Allison *et al*., 2007b, Allison & Martiny, 2008; Nemergut *et al*., 2008). Surprisingly, we did not observe a decline in K_m in response to N addition for most enzymes at Fernow, even though microbes were probably more C limited there. It is possible that increased enzyme production, rather than improved enzyme efficiency, was a preferred microbial strategy at Fernow because fertilization alleviated N limitation more effectively there than at Bear Brook (see C : N ratios, Table 2). Moreover, K_m values were already low at Fernow relative to Bear Brook (Fig. 4), and it is possible that there was less potential for additional reductions under N fertilization. However, the mechanism for the site-specific response of K_m to N remains unclear.

We hypothesized that both V_{max} and K_m parameters would respond positively to temperature (Fig. 2b, Davidson & Janssens, 2006; Davidson *et al*., 2006). Consistent with this hypothesis, both V_{max} and K_m exhibited significant positive responses to temperature for all enzymes, though the magnitude of this response varied across different enzymes. To our knowledge, no other studies have measured K_m temperature sensitivity of soil enzymes, although positive sensitivities have been observed in studies of enzyme thermal tolerance in animal systems (Somero, 1978; Hochachka & Somero, 2002; Huestis *et al*., 2009). These studies suggest that enzyme K_m tends to be relatively unresponsive to temperature in stable, intracellular environments, where selection for a precise metabolic function is high. However, digestive enzymes which experience a more heterogeneous gut environment tend to be more variable in their response to temperature and more temperature sensitive overall (Somero, 1978). Since soils also contain a heterogeneous mixture of organic substrates (Allison, 2006a), it is not surprising that soil K_m values are also temperature sensitive.

The finding that K_m responds positively to temperature for hydrolytic enzymes is important because higher K_m values could offset increases in V_{max} with increasing temperature at low substrate concentrations (Davidson *et al*., 2006). The heterogeneous nature of soil means that enzymes experience varied substrate concentrations across space and time. In biochemical literature it has been suggested that an enzyme's regulatory

sensitivity under non-saturating conditions may be more important than sheer catalytic power (Somero, 1978). This view may also be relevant to environmental systems. Substrate concentrations in the microsites where enzymes function are variable, and it is likely that concentrations are often low enough for the K_m parameter to affect enzyme activity (Davidson *et al.*, 2006; Lehmann *et al.*, 2008). Thus, the net effect of rising temperatures on decomposition rates may be weaker than expected from the V_{max} response if substrate concentrations are low. However, our data suggest that for all enzymes, the temperature sensitivity of K_m was lower than that of V_{max}. Processes such as soil respiration that depend on both enzymatic parameters are therefore likely to show a net positive response to temperature increase.

An important consideration regarding our K_m measurements is that they can only be considered 'apparent' due to the confounding effects of many temperature-sensitive processes in the soil environment. As Davidson & Janssens (2006) note, temperature changes may alter the adsorption and desorption of organic compounds. In our reaction mixtures, substrate desorption at higher temperatures could result in greater concentrations of competing substrates, and thus an apparent loss of catalytic efficiency through competitive inhibition. Furthermore, adsorption reactions can also be temperature sensitive, particularly for molecules with high molecular weights, such as enzyme proteins (Thornley & Cannell, 2001). If enzyme adsorption increases at higher temperatures, more enzymes could become stabilized on mineral and other soil surfaces. Bound enzymes are thought to have low catalytic efficiencies relative to mobile enzymes due to the limited ability of immobilized enzyme molecules to access substrates and possible occlusion of enzyme active sites (Quiquampoix *et al.*, 2002; Allison, 2006b; Šťursová & Baldrian, 2010). These changes in environmental conditions with temperature increase could explain some or all of the K_m temperature sensitivity that we observed. However, uncertainty as to whether the measured temperature sensitivity of K_m is intrinsic or apparent does not diminish the importance of our findings for environmental systems.

We observed no changes in the temperature sensitivity of V_{max} with N addition for any enzyme studied, and β-xylosidase was the only enzyme for which N altered the temperature sensitivity of K_m. There is abundant evidence that microbial community composition is sensitive to environmental conditions such as N availability (Fierer & Jackson, 2006, Allison *et al.*, 2007b, Fierer *et al.*, 2007; Allison *et al.*, 2008). Different microbial communities may produce distinct enzyme isoforms, which could differ in their catalytic properties and their intrinsic K_m (Somero, 1978). However, the relatively consistent temperature sensitivity of V_{max} and K_m across N treatments argues against this mechanism for most of the enzymes we examined. In the case of β-xylosidase, K_m was more temperature sensitive in fertilized soils at both sites. This result suggests that hemicellulose degradation may be affected more by temperature change than the degradation of other C compounds. However, the main effect of N on β-xylosidase was to significantly reduce K_m, so the increase in K_m observed with rising temperature would only offset the N effect at very high temperatures. Therefore, the primary effect of N addition would still be an increase in the efficiency of hemicellulose degradation.

Although our enzyme results are relevant for predicting soil C responses to global change, we acknowledge that there are important limitations to our study. One limitation is that we did not measure the temperature sensitivity of oxidative enzymes that degrade recalcitrant compounds making up the bulk of organic C in many soils, and that have been found to be particularly responsive to N additions (Sinsabaugh, 2010). Measuring the temperature sensitivity of these enzymes is difficult because they employ free radical mechanisms that do not follow Michaelis–Menten kinetics (Claus, 2004). New approaches may be required to assess the temperature sensitivities of these reactions. Another limitation of our study is that we only considered the short-term biochemical response of enzymes to temperature. Short-term temperature manipulations ignore the effect of changes in enzyme turnover rates that may occur due to climate warming. Faster enzyme turnover in warmer soils could result in weaker responses of V_{max} to temperature than we observed in the laboratory. Moreover, if microbial communities and enzymes adapt to long-term changes in temperature as expected with global climate change, then the temperature sensitivity of enzyme kinetic parameters may be lower than reported herein (Bradford *et al.*, 2008; Allison *et al.*, 2010). It is notable that reductions in V_{max} through adaptive mechanisms would reduce rates of soil C decomposition, whereas reductions in K_m would tend to increase decomposition. Finally, our study only examined temperature sensitivities for V_{max} and K_m at single time point in two sites. However, the temperature sensitivities of microbial and enzymatic parameters are known to vary seasonally (Fenner *et al.*, 2005; Bradford *et al.*, 2008; Wallenstein *et al.*, 2009), and there could be seasonality in the temperature sensitivity of enzyme V_{max} and K_m at our sites. Additional studies of both V_{max} and K_m responses to long-term temperature change and climate gradients will be essential for accurately predicting soil C turnover in a changing climate.

Conclusions

Linkages between C and N cycling in soils have not been well studied on an enzymatic level, despite attempts to conceptually link microbial enzyme production with nutrient availability (Sinsabaugh & Moorhead, 1994, Allison *et al.*, 2007a). In a global change context, empirical studies constraining the relationship between enzyme kinetics and environmental variables are needed to parameterize models of the soil C cycle (Schimel & Weintraub, 2003; Fontaine & Barot, 2005; Allison *et al.*, 2010). Our study addresses this need in two hardwood forests, where we found that N fertilization increased the V_{max} values for most soil hydrolytic enzymes, especially at Fernow Forest. In accordance with microbial allocation theory, N fertilization may stimulate the microbial community to produce more C-acquiring enzymes. We also found that N fertilization can lead to the production of C-acquiring enzymes with higher catalytic efficiency (lower K_m values). Although this response was more important at Bear Brook, our results reveal that microbes may respond to N addition and C limitation through changes in both V_{max} and K_m parameters. The finding that Fernow Forest enzymes tended to respond to N with higher V_{max} values while Bear Brook enzymes tended to respond to N with lower K_m values indicates the importance of site differences in driving enzyme V_{max} and K_m responses to N.

Models of enzymatic decomposition may not need to account for interactions between N and temperature since we did not observe strong effects of N fertilization on the temperature sensitivity of V_{max} or K_m. However, we found that both V_{max} and K_m parameters responded positively to temperature across all treatments. These findings indicate that models of decomposition based on extracellular enzyme activities should account for substrate concentrations and the temperature dependence of both K_m and V_{max}. Our results also emphasize that future empirical studies should evaluate the dependence of K_m on temperature in addition to V_{max} to accurately characterize decomposition responses to temperature change (German *et al.*, 2011).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Temperature sensitivities of V_{max} for (a) α-glucosidase, (b) β-glucosidase, (c) β-xylosidase, (d) cellobiohydrolase, and (e) *N*-acetyl-glucosaminidase by site and nitrogen treatment. V_{max} is expressed as nmol g dry soil^{-1} h^{-1}. Symbols represent the mean (± SE) V_{max} for three replicate composites at a given temperature. Regression lines are shown for each combination of site and treatment. Effects significant at $P < 0.10$ are listed from a full factorial ANCOVA with nitrogen treatment and site as main effects and laboratory incubation temperature as the covariate.

Table S1. Regression intercepts and slopes for log (V_{max}) in nmol g^{-1} dry soil h^{-1} as a function of temperature (n = 21).

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