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 $V_{\text{max}}$ and $K_m$ parameters for five hydrolytic enzymes: $\alpha$-glucosidase, $\beta$-glucosidase, $\beta$-xylosidase, cellobiohydrolase, and N-acetyl-glucosaminidase. Temperature sensitivities of $V_{\text{max}}$ 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 $V_{\text{max}}$ values and lower $K_m$ values; and (2) both $V_{\text{max}}$ 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 $V_{\text{max}}$ 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 $V_{\text{max}}$ and $K_m$ were temperature sensitive, with $Q_{10}$ values ranging from 1.64–2.27 for enzyme $V_{\text{max}}$ and 1.04–1.93 for enzyme $K_m$. No enzyme showed a significant interaction between N and temperature sensitivity for $V_{\text{max}}$, and only $\beta$-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 $V_{\text{max}}$ relative to $K_m$ imply that substrate degradation will increase with temperature. In addition, the $V_{\text{max}}$ 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 ($S$):

$$V = \frac{V_{\text{max}}S}{K_m + |S|}$$

where $V_{\text{max}}$ is the maximum potential rate of activity and $K_m$ is the half saturation constant, or the substrate concentration at $V_{\text{max}}$ (Fig. 1). At low substrate
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 $V_{\text{max}}$. 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 $V_{\text{max}}$ and $K_m$ parameters (Fig. 2a). If increased N availability causes increased microbial enzyme production, $V_{\text{max}}$ 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 concentrations.
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 trade-offs. 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). 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$_4$)$_2$SO$_4$ 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 Haplorhods) 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

<table>
<thead>
<tr>
<th>Site</th>
<th>Location &quot;N, W</th>
<th>MAT °C</th>
<th>MAP mm yr⁻¹</th>
<th>Dominant soil type</th>
<th>Wet N deposition rate kg N ha⁻¹ yr⁻¹</th>
<th>Fertilization rate kg N ha⁻¹ yr⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bear Brook, Maine</td>
<td>44°52'N, 68°06'W</td>
<td>4.9</td>
<td>1320</td>
<td>Spodosols</td>
<td>3.1</td>
<td>25.2</td>
</tr>
<tr>
<td>Fernow, West Virginia</td>
<td>39°03'N, 79°49'W</td>
<td>8.8</td>
<td>1458</td>
<td>Inceptisols</td>
<td>6.8</td>
<td>35</td>
</tr>
</tbody>
</table>

1Adams et al., 2006.
2Fernandez et al., 2010.
3SanClements et al., 2010.

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

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

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 (NH₄)₂SO₄ 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 (Quercus 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 m² area at Bear Brook and a 10 m² 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
Fluorimetric enzyme assays

We measured the activities of five hydrolytic enzymes: \(\alpha\)-glucosidase, \(\beta\)-glucosidase, \(\beta\)-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). \(V_{\text{max}}\) 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 \(V_{\text{max}}\) 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 \(V_{\text{max}}\) values and temperature sensitivities per unit soil mass in Table S1 and Fig. S1.

**Results**

\(V_{\text{max}}\) response to temperature and nitrogen

The \(V_{\text{max}}\) values increased significantly with temperature for all enzymes at both sites (Fig. 3). The magnitude of the temperature response of ln (\(V_{\text{max}}\)) 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 \(V_{\text{max}}\) did not vary with site or N treatment.

The \(V_{\text{max}}\) 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

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**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)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme function</th>
<th>Substrate proxy</th>
<th>Substrate concentration gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha)-glucosidase</td>
<td>Releases glucose from starch</td>
<td>4-MUB (\alpha)-d-glucopyranoside</td>
<td>2–200 μM</td>
</tr>
<tr>
<td>(\beta)-glucosidase</td>
<td>Releases glucose from cellulose</td>
<td>4-MUB (\beta)-d-glucopyranoside</td>
<td>2–200 μM</td>
</tr>
<tr>
<td>(\beta)-xylosidase</td>
<td>Releases xylose from hemicellulose</td>
<td>4-MUB (\beta)-d-xylpyranoside</td>
<td>2–200 μM</td>
</tr>
<tr>
<td>Cellobiohydrolase</td>
<td>Releases disaccharides from cellulose</td>
<td>4-MUB (\beta)-d-cellobioside</td>
<td>1–100 μM</td>
</tr>
<tr>
<td>(N)-acetyl glucosaminidase</td>
<td>Releases (N)-acetyl glucosamine from chitin</td>
<td>4-MUB (N)-acetyl-(\beta)-d-glucosaminide</td>
<td>4–400 μM</td>
</tr>
</tbody>
</table>
and site was significant for \( \beta \)-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, \(V_{\text{max}}\) values were significantly greater at Bear Brook for \(\beta\)-glucosidase \((P < 0.05)\) and \(\beta\)-xylosidase \((P < 0.001)\) and marginally significantly greater for \(\alpha\)-glucosidase \((P = 0.057)\) and cellobiohydrolase \((P = 0.097)\). However, these site differences were only observed in unfertilized soils, consistent with the \(N \times \) site interactions (Fig. 3). When expressed per soil mass, \(V_{\text{max}}\) 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 \(V_{\text{max}}\), 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 \(V_{\text{max}} Q_{10}\) values for all enzymes. \(\beta\)-xylosidase was the only enzyme for

![Fig. 3 Temperature sensitivities of \(V_{\text{max}}\) for (a) \(\alpha\)-glucosidase, (b) \(\beta\)-glucosidase, (c) \(\beta\)-xylosidase, (d) cellobiohydrolase, and (e) \(N\)-acetyl-glucosaminidase by site and nitrogen treatment. \(V_{\text{max}}\) is expressed as nmol g\(^{-1}\) C h\(^{-1}\). Symbols represent the mean (± SE) \(V_{\text{max}}\) 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.](image-url)
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 $\alpha$-glucosidase responded positively to N, while $\beta$-glucosidase, $\beta$-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 $\alpha$-glucosidase, $\beta$-glucosidase, $\beta$-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 $\beta$-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

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

N-rich proteins, microbes may have responded to low C availability and high N availability at Fernow by allo-
cating 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 atmos-
pheric 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 $V_{\text{max}}$ 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 $V_{\text{max}}$ 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

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Fig. 4 Temperature sensitivities of $K_m$ for (a) $\alpha$-glucosidase, (b) $\beta$-glucosidase, (c) $\beta$-xylosidase, (d) cellobiohydrolase, and (e) N-acetyl-

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Please note that the diagram and table are not pasted in the text.
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 $V_{\text{max}}$ and $K_m$ parameters would respond positively to temperature (Fig. 2b, Davidson & Janssens, 2006; Davidson et al., 2006). Consistent with this hypothesis, both $V_{\text{max}}$ 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 $V_{\text{max}}$ 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

### Table 5: Regression intercepts and slopes for log ($K_m$) in $\mu$mol L$^{-1}$ as a function of temperature ($n = 21$)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Site</th>
<th>Treatment</th>
<th>Intercept $\pm$ SE</th>
<th>Slope $\pm$ SE</th>
<th>$Q_{10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-glucosidase</td>
<td>Bear Brook</td>
<td>Control</td>
<td>4.6 $\pm$ 0.3</td>
<td>0.020 $\pm$ 0.011</td>
<td>1.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nitrogen</td>
<td>3.7 $\pm$ 0.2</td>
<td>0.020 $\pm$ 0.009</td>
<td>1.22</td>
</tr>
<tr>
<td></td>
<td>Fernow</td>
<td>Control</td>
<td>3.0 $\pm$ 0.3</td>
<td>0.017 $\pm$ 0.011</td>
<td>1.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nitrogen</td>
<td>3.7 $\pm$ 0.4</td>
<td>0.015 $\pm$ 0.016</td>
<td>1.16</td>
</tr>
<tr>
<td>$\beta$-glucosidase</td>
<td>Bear Brook</td>
<td>Control</td>
<td>3.7 $\pm$ 0.2</td>
<td>0.031 $\pm$ 0.010</td>
<td>1.37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nitrogen</td>
<td>2.8 $\pm$ 0.2</td>
<td>0.042 $\pm$ 0.006</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>Fernow</td>
<td>Control</td>
<td>2.8 $\pm$ 0.3</td>
<td>0.028 $\pm$ 0.011</td>
<td>1.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nitrogen</td>
<td>3.1 $\pm$ 0.2</td>
<td>0.027 $\pm$ 0.007</td>
<td>1.30</td>
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<tr>
<td>$\beta$-xylosidase</td>
<td>Bear Brook</td>
<td>Control</td>
<td>4.8 $\pm$ 0.3</td>
<td>0.004 $\pm$ 0.014</td>
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<td></td>
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<td>Nitrogen</td>
<td>2.8 $\pm$ 0.3</td>
<td>0.035 $\pm$ 0.011</td>
<td>1.42</td>
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<tr>
<td></td>
<td>Fernow</td>
<td>Control</td>
<td>2.5 $\pm$ 0.5</td>
<td>0.044 $\pm$ 0.019</td>
<td>1.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nitrogen</td>
<td>1.9 $\pm$ 0.3</td>
<td>0.066 $\pm$ 0.013</td>
<td>1.93</td>
</tr>
<tr>
<td>Cellobiohydrolase</td>
<td>Bear Brook</td>
<td>Control</td>
<td>2.9 $\pm$ 0.3</td>
<td>0.043 $\pm$ 0.011</td>
<td>1.54</td>
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<tr>
<td></td>
<td></td>
<td>Nitrogen</td>
<td>2.4 $\pm$ 0.3</td>
<td>0.034 $\pm$ 0.012</td>
<td>1.40</td>
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<tr>
<td></td>
<td>Fernow</td>
<td>Control</td>
<td>1.7 $\pm$ 0.3</td>
<td>0.045 $\pm$ 0.011</td>
<td>1.57</td>
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<tr>
<td></td>
<td></td>
<td>Nitrogen</td>
<td>2.3 $\pm$ 0.3</td>
<td>0.038 $\pm$ 0.012</td>
<td>1.46</td>
</tr>
<tr>
<td>$N$-acetyl-glucosaminidase</td>
<td>Bear Brook</td>
<td>Control</td>
<td>4.3 $\pm$ 0.2</td>
<td>0.037 $\pm$ 0.010</td>
<td>1.44</td>
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<td>Nitrogen</td>
<td>4.0 $\pm$ 0.2</td>
<td>0.224 $\pm$ 0.008</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>Fernow</td>
<td>Control</td>
<td>4.2 $\pm$ 0.2</td>
<td>0.040 $\pm$ 0.009</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nitrogen</td>
<td>4.0 $\pm$ 0.2</td>
<td>0.040 $\pm$ 0.009</td>
<td>1.49</td>
</tr>
</tbody>
</table>
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 \( V_{\text{max}} \) 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 \( V_{\text{max}} \). 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; Štursova & 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 \( V_{\text{max}} \) with N addition for any enzyme studied, and \( \beta \)-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 \( V_{\text{max}} \) and \( K_m \) across N treatments argues against this mechanism for most of the enzymes we examined. In the case of \( \beta \)-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 \( \beta \)-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 \( V_{\text{max}} \) 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 \( V_{\text{max}} \) 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 \( V_{\text{max}} \) 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 \( V_{\text{max}} \) and \( K_m \) at our sites. Additional studies of both \( V_{\text{max}} \) 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 $V_{\text{max}}$ 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 $V_{\text{max}}$ and $K_m$ parameters. The finding that Fernow Forest enzymes tended to respond to N with higher $V_{\text{max}}$ values while Bear Brook enzymes tended to respond to N with lower $K_m$ values indicates the importance of site differences in driving enzyme $V_{\text{max}}$ 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 $V_{\text{max}}$ or $K_m$. However, we found that both $V_{\text{max}}$ 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 $V_{\text{max}}$. Our results also emphasize that future empirical studies should evaluate the dependence of $K_m$ on temperature in addition to $V_{\text{max}}$ to accurately characterize decomposition responses to temperature change (German et al., 2011).

Acknowledgements

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References


Supporting Information

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

**Figure S1.** Temperature sensitivities of \( V_{\text{max}} \) for (a) \( \alpha \)-glucosidase, (b) \( \beta \)-glucosidase, (c) \( \beta \)-xylosidase, (d) cellulohydrolase, and (e) \( \alpha \)-N-acetyl-glucosaminidase by site and nitrogen treatment. \( V_{\text{max}} \) is expressed as nmol g dry soil \( \text{h}^{-1} \). Symbols represent the mean (± SE) \( V_{\text{max}} \) 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 \( \log (V_{\text{max}}) \) in nmol g \( \text{dry soil h}^{-1} \) as a function of temperature (\( T = 21 \)).

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