Soil Biology & Biochemistry 76 (2014) 193–200

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

Differential effects of pH on temperature sensitivity of organic carbon and nitrogen decay

Kyungjin Min, Christoph A. Lehmeier [1], Ford Ballantyne [2], Anna Tatarko, Sharon A. Billings*

Department of Ecology and Evolutionary Biology, Kansas Biological Survey, University of Kansas, 2101 Constant Ave., Lawrence KS 66047, USA

ARTICLE INFO

Article history:
Received 27 January 2014
Received in revised form
18 April 2014
Accepted 16 May 2014
Available online 28 May 2014

Keywords:
Extracellular enzymes
Soil organic matter
Decomposition
Warming
Nutrient availability
C:N flow ratio

ABSTRACT

Soil microorganisms release extracellular enzymes into the soil matrix to access carbon (C) and nitrogen (N) from soil organic matter (SOM). Temperature and pH are major factors governing the rates at which these enzymes decay SOM, hence influencing the availability of C and N for microbial assimilation. As temperature increases, the rate of decomposition is also expected to increase. Recent advances provide estimates of intrinsic temperature sensitivities of key decay reactions at one, circum-neutral pH, but how temperature sensitivity of enzymatic SOM degradation is influenced by pH remains unclear. Here we expand on recent work by determining specific activities of C-acquiring (β-glucosidase; BGase) and C- and N-acquiring (*N*-Acetyl-Glucosaminidase; NAGase) enzymes with purified, fluorescently labeled organic substrate at temperatures from 5 to 25 °C (5 °C steps) and at pH values from 3.5 to 8.5 (1 pH unit steps). Using specific activity data, we quantified temperature sensitivities of the reactions with estimates of activation energy (E_a) at each pH value. We then used E_a estimates to compute temperature-induced changes in the C:N flow ratio, which is defined as the ratio of enzymatic liberation rates of C to N from the substrates. Across all temperatures, BGase activity was generally high in the pH range of 5.5–8.5, while NAGase exhibited a relatively narrow optimum between pH 5.5–6.5. Temperature sensitivity of BGase differed significantly among pH values; the strongest temperature responses were observed at pH 4.5. NAGase, in contrast, did not exhibit any significant pH-dependent changes in temperature sensitivity. The temperature increase from 5 to 25 °C induced changes in the C:N flow ratio, with direction and magnitude strongly dependent on the pH. We observed a large, temperature-induced increase in C:N flow ratio at pH 4.5 and decreases in C:N flow ratio at pH > 5.5 that were most pronounced at pH 7.5. Our data show that pH can induce differential effects on reaction rates and temperature sensitivity of organic C and N liberation, with consequences for changes in the relative availabilities of C and N for microbial assimilation.

1. Introduction

The mean surface temperature of the Earth is projected to increase by 0.3–4.8 °C during the 21st century, primarily due to the increasing CO_2 concentration in the atmosphere (IPCC, 2013). Soil respiration (total CO_2 efflux from soil surface) represents as much as half the total biogenic CO_2 production (Schimel, 1995; Trumbore, 2006), much of which is produced by the activity of heterotrophic microorganisms (Högberg et al., 2001). Rates of heterotrophic soil respiration often increase with temperature (MacDonald et al., 1995; Mikan et al., 2002; Fierer et al., 2006; Zhou et al., 2009), generating a positive feedback to increasing concentrations of atmospheric CO_2. However, variable, unexplained responses of heterotrophic soil respiration to temperature have been observed across ecosystems (Zhou et al., 2009; Suseela et al., 2012), highlighting our limited understanding of the drivers of this flux. Thus, a more detailed knowledge of the mechanisms governing the response of heterotrophic soil respiration to temperature is indispensable for predicting the Earth's future atmospheric CO_2 concentrations.

* Corresponding author. Tel.: +1 785 864 1560.
E-mail addresses: kjmin.21@gmail.com (K. Min), christoph.lehmeier@gmail.com (C.A. Lehmeier), fb4@uga.edu (F. Ballantyne), atatarko@ku.edu (A. Tatarko), sharonb@ku.edu (S.A. Billings).
[1] Present address: Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK.
[2] Present address: Odum School of Ecology, University of Georgia, 140 E. Green St., Athens, GA 30602, USA.

http://dx.doi.org/10.1016/j.soilbio.2014.05.021
0038-0717/

Decomposition of SOM is a necessary precursor for sustaining heterotrophic soil respiration. Soil microorganisms exude enzymes into the soil matrix (extracellular enzymes) to cleave macromolecular compounds into smaller, assimilable resources. The effects of temperature on the rates of those enzymatic reactions, and ultimately heterotrophic soil CO_2 efflux, can be modeled using the biochemical and thermodynamic foundation established by van't Hoff and Arrhenius (van't Hoff, 1884; Arrhenius, 1889; Knorr et al., 2005; Davidson and Janssens, 2006).

$$K = A \cdot e^{\frac{-E_a}{RT}} \quad (1)$$

The model describes how reaction rates (K) vary as a function of temperature (T, in Kelvin) and is parameterized by the activation energy (E_a) required for the reaction to proceed, a pre-exponential factor (A) which represents the likelihood that molecules collide in the proper orientation, and R, the ideal gas constant.

When the reaction proceeds in unconstrained conditions, unlimited by substrate, E_a reflects the intrinsic temperature sensitivity of the reaction. Relative increases in reaction rates with temperature are higher for reactions with higher E_a than for reactions with lower E_a. However, myriad environmental factors, including chemical (e.g., redox potential), physical (e.g., adsorption/desorption of extracellular enzymes and substrates on soil particles, diffusion of extracellular enzymes), and microbial (e.g., biomass, extracellular enzyme production) variables, can induce deviations from the underlying van't Hoff–Arrhenius temperature sensitivity of enzymatic decay in soils (Davidson et al., 2006; Conant et al., 2011). To quantitatively determine the influence of such factors on SOM decay, we must first measure intrinsic temperature sensitivities (*sensu* van't Hoff–Arrhenius) for distinct enzyme-substrate pairings as reference values.

An important edaphic variable that influences SOM decomposition dynamics is the pH of the soil solution. Soil pH governs the ionization of functional groups of organic molecules, the conformation of substrates and enzymes, the degree to which extracellular enzymes adsorb to soil particles, and the solubility of cofactors essential for the enzymatic reactions (Tipton et al., 1979; Frankenberger and Johanson, 1982; Tabatabai, 1994; Brady and Weil, 2007). Significant temporal and spatial variation in pH observed in the field (Zoltan, 2008) is linked to variations in extracellular enzyme activities in diverse ecosystems (Sinsabaugh et al., 2008). Numerous studies have explored the influence of pH on extracellular enzyme kinetics, but the pH effect is often determined at a single temperature, and temperature sensitivities typically are quantified at a single pH (Deng and Tabatabai, 1994; Parham and Deng, 2000; Turner, 2010; Lehmeier et al., 2013). As a result, it is unclear how pH influences extracellular enzyme activities across a range of temperatures.

Changes in enzymatic activities in response to temperature and pH may alter microbial resource availability and associated microbial feedbacks. Simulating the concurrent extracellular enzymatic degradation of cellulose (no N) and chitin (C:N of 8), Lehmeier et al. (2013) demonstrated that changes in temperature can affect relative rates of C and N liberation (termed the C:N flow ratio, Billings and Ballantyne, 2013) solely through the different temperature sensitivities of cellulose- and chitin-cleaving reactions. Because soil microorganisms experience stoichiometric constraints (Sterner and Elser, 2002; Cleveland and Liptzin, 2007), changing relative availability of C and N may directly affect microbial growth and respiration (Frey et al., 2004; Treseder, 2008; Min et al., 2011). Shifting relative availability of C and N may prompt microbes to adjust relative production and exudation rates of C- and N-acquiring extracellular enzymes to balance the bioavailability of C and N (Billings and Ballantyne, 2013). Given the roles of pH and temperature as fundamental determinants of reaction rates, quantifying any interactive effects of pH and temperature on extracellular enzyme activities is critical for predicting microbial feedbacks to soil respiration across diverse ecosystems.

Here, we quantify the temperature sensitivity of enzyme-catalyzed reactions using purified enzymes and organic substrates analogous to cellulose and chitin across an ecologically relevant pH range, from 3.5 to 8.5. We performed the experiments at five distinct temperatures, from 5 to 25 °C with 5 °C steps, and determined the temperature sensitivity of the reactions over this temperature range. We then used estimates of temperature sensitivity to assess how pH may likely influence the relative rates of C and N liberation from cellulose and chitin decay as temperature changes. We chose analog substrates for cellulose and chitin because they are two of the most abundant and globally ubiquitous substrates in soils and serve as important resources of C (cellulose) and N and C (chitin) for microbes. Our approach enables us to determine enzyme activity per unit enzyme mass with sufficient single substrate, thereby elucidating intrinsic, temperature-dependent changes in decay rates at a fundamental, biochemical level, unfettered by other confounding factors present in soils.

2. Materials and methods

2.1. Measuring reaction rates using purified extracellular enzymes and MUB-labeled substrates

We measured the specific activity of two pairs of extracellular enzymes and corresponding substrates in buffer solution at temperatures ranging from 5 to 25 °C (5 °C steps) and pH values from 3.5 to 8.5 (1 pH unit steps): BGase (EC 3.2.1.21; Megazyme, Ireland) with 4-Methylumbelliferyl β-D-cellobioside (MUB-BG; Sigma–Aldrich, USA) and NAGase (EC 3.2.1.52; New England Biolabs, USA) with 4-Methylumbelliferyl *N*-Acetyl-Glucosaminide (MUB-NAG; Sigma–Aldrich, USA). When a MUB label is cleaved from a substrate by the activity of the specific enzyme, it emits a fluorescence signal upon excitation by light (Mead et al., 1955). Aliquots of crystalline MUB-BG (273 μM), MUB-NAG (400 μM), and a MUB standard (10 μM; Sigma–Aldrich, USA) were dissolved in deionized water. Extracellular enzymes were dissolved in 0.2 M of sodium acetate buffer adjusted to pH 3.5, 4.5, 5.5, 6.5, 7.5 or 8.5. The reported pH, therefore, refers to the buffer pH instead of the pH of the final reaction solution; the pH of the final reaction solution is, however, close to the pH of the buffer, given that more than 80% of the solution volume in each well was buffer and that the buffer itself inherently minimizes changes in solution pH. The amount of BGase and NAGase in one individual well of a 96-well plate was 0.024 and 0.16 units, respectively.

To perform each enzyme activity assay, we pipetted 50 μl of dissolved substrates and 200 μl of enzyme solutions into 16 wells of 96-well black microplates (Costar®, USA). In each microplate,

Table 1
The mixture of the assay solutions. At each pH, three different controls (enzyme, quench, and substrate) and MUB standard are assayed in the same plates for correcting and calibrating the fluorescence signal in sample wells.

Category		
Control	Enzyme	50 μl of 0.2 M NaAc Buffer + 200 μl of enzyme in buffer
	Quench	50 μl of 10 μM MUB + 200 μl of enzyme in buffer
	Substrate	50 μl of 273 μM MUB-BG or 400 μM MUB-NAG + 200 μl of 0.2 M NaAc Buffer
Standard		50 μl of 10 μM MUB + 200 μl of 0.2 M NaAc Buffer
Sample		50 μl of 273 μM MUB-BG or 400 μM MUB-NAG + 200 μl of enzyme in buffer

designated wells were filled with 250 μl of three different controls (enzyme, quench, and substrate control) as well as MUB standard (Table 1). After pipetting, we transferred each plate to a Synergy™ HT microplate reader (BioTek Instruments, Inc., USA) and recorded the evolution of fluorescence every minute for the BGase/MUB-BG (Fig. 1a). Because NAGase/MUB-NAG reactions generally proceed more slowly than BGase/MUB-BG reactions, MUB fluorescence generation by NAGase was recorded every two minutes instead of every minute. Fluorescence was measured for sufficient duration to quantify the initial linear increase in total fluorescence (i.e. the accumulation of MUB upon enzymatic cleavage), which then served to calculate specific enzyme activity rates.

Fluorescence of MUB-labeled substrate (substrate control) as well as of MUB standard can be influenced by solution age (the time since dissolving MUB-labeled substrate or MUB standard in deionized water) and by the buffer pH in the reaction solution (Niemi and Vepsäläinen, 2005; DeForest, 2009). To account for this, we generated fresh substrate control for every experiment, mixed the control with buffer at the same pH value as buffer used in the respective enzyme/substrate reactions, and determined the fluorescence exhibited by these solutions. Measuring the fluorescence of substrate control permitted us to correct enzymatic reactions for any fluorescence generated by phenomena other than the enzyme-specific MUB release (Table 1). After correcting reaction fluorescence with control values, the measurements of the fresh MUB standard were used to calibrate fluorescence of reactions to obtain the molar amounts of MUB-labeled substrate cleaved by the enzymes per unit time (DeForest, 2009).

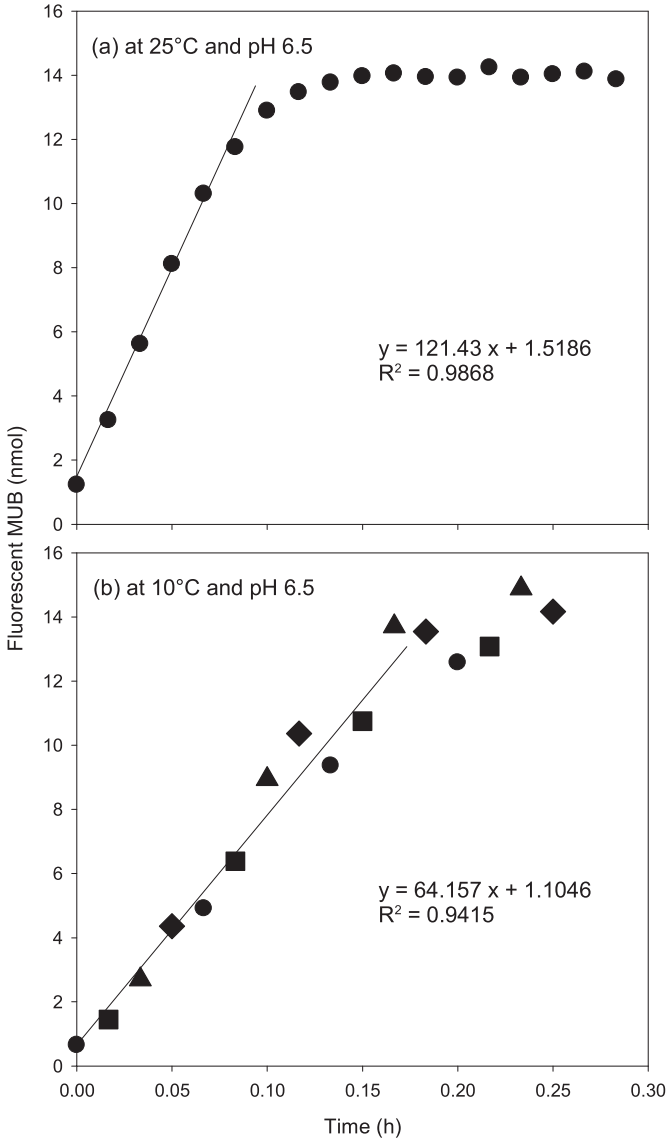

Fig. 1. Two examples of fluorescent MUB generation over incubation time as a result of BGase/MUB-BG reactions at pH 6.5 at either 25 °C (a) or 10 °C (b). Linear regression lines were fitted to the initial linear stage of MUB accumulation to derive BGase-mediated MUB release from MUB-BG in nmol h^{-1}. While the data points in (a) are from one 96-well plate, a total of four identical plates were alternated in (b) during the incubation period to represent one replicate (see Section 2.2 for a detailed description; Circle, plate 1; square, plate 2; triangle, plate 3; diamond, plate 4).

2.2. Controlling reaction temperatures

Because the microplate reader cannot reduce the plate temperature below ambient temperature, we performed measurements at 20 °C and below differently from those at 25 °C. For assays at 25 °C, one plate at each pH was placed in the microplate reader. Using a default kinetic measurement mode, we recorded the accumulated fluorescence until we observed no more increase in fluorescence with time (Fig. 1a). For BGase/MUB-BG, the reaction temperature was 25 °C during the assay. The actual reaction temperature for the NAGase/MUB-NAG reaction was 26 °C.

For lower temperatures (5, 10, 15, and 20 °C), we modified protocols from Lehmeier et al. (2013). First, all the solutions, microplates, and pipettes were kept in an incubator (VWR low temperature incubator, USA) at the desired temperature. We pipetted the solutions in the designated wells of two identical microplates (see Section 2.1; Table 1), put one in the incubator, measured the fluorescence of the other plate immediately, and put this plate back into the incubator. This measurement represents t0 (Fig. 1b). For BGase/MUB-BG reactions, one minute after the solutions had been pipetted into the two microplates, the second incubating plate was measured (t1) and returned to the incubator. We continued alternating two plates to ensure that no reaction temperature was higher than that intended due to excessive time outside of the incubator. Thus, plate 1 was measured at t0, t4, t8, and t12 (i.e., 0, 4, 8, and 12 min after the solutions were pipetted into the plate), and plate 2 at t1, t5, t9 and t13. After the measurements of the first two plates, the solutions were pipetted into the other two plates and they were measured in the same, alternating way, with plate 3 measured at t2, t6, t10 and t14 and plate 4 at t3, t7, t11 and t15. For NAGase/MUB-NAG reactions, we followed a similar protocol except we conducted the measurements at two-minute intervals, as this was better suited for documenting the initial linear phase of fluorescence evolution with time for this reaction. Thus, for each BGase/MUB-BG and NAGase/MUB-NAG reaction, four microplates were used to generate one time course at temperatures of 10, 15, and 20 °C at each pH, and were treated as one replicate (Fig. 1b). For the reactions at 5 °C, we expanded this method and used eight microplates, instead of four, each measured only two times. High R^2 of the initial linear phase of fluorescence accumulation across all the individual plates for one replicate time course demonstrates the suitability of this protocol.

2.3. Calculating specific enzyme activities

All enzyme assays across the whole range of pH and temperature presented in this study were performed with the same concentrations of substrates in the reaction wells: 273 μM MUB-BG and 400 μM MUB-NAG (Table 1). Preliminary experiments conducted at pH 6.5 and 25 °C for MUB-BG and pH 5.5 and 26 °C for MUB-NAG demonstrated that these concentrations were sufficient to

saturate enzymes in these conditions (data not shown). We assumed that saturation concentrations are not higher at lower temperatures (Somero, 2004; Wallenstein et al., 2010), an assumption supported by the enzyme activity data (Fig. 2).

Our calculations of enzymatic reaction rates follow DeForest (2009), but because we knew the amount of enzyme present in one well, we were able to obtain specific enzyme activity per unit enzyme mass. We equate specific enzyme activities for BGase and NAGase with intrinsic specific enzyme activities at any given temperature and pH, given the absence of confounding factors often present in soil matrices such as substrate limitation or protection from decay (Davidson et al., 2006; Conant et al., 2011).

2.4. Statistical analysis and estimating intrinsic temperature sensitivities of reactions

After log-transformation of specific enzyme activities to meet the assumptions of normality and equal variance, we determined the effects of temperature and pH on ln (specific enzyme activity) for each enzyme using two-way ANOVA. For each enzyme, we first fit a full model that included the interaction between temperature and pH, and then we used the Akaike Information Criterion (AIC) to select the most parsimonious model, which guided our comparisons of specific activity across pH and temperature. We employed Bonferroni-corrected *p*-values to conduct post-hoc, multiple comparisons of specific enzyme activities.

To assess the influence of pH on the relative temperature sensitivity of enzymatic reactions, i.e. E_a, we fit linear models with Arrhenius transformed specific activity (ln (specific enzyme activity)*R, see Eq. (1)) as the response, pH and enzyme type as categorical predictors, and 1/T (in Kelvin) as a covariate. Using the Arrhenius transformation as the response is standard practice when estimating E_a because slope estimates are identical to estimates of E_a. As for the analysis of specific enzyme activities, we first fit a full model and subsequently performed model selection using AIC to arrive at a reduced model, but our main interest was a direct comparison of E_a estimates across pH and enzyme type. Because we had *priori* knowledge of specific tests of ecological interest – that E_a values differ across pH for each enzyme and that E_a values differ between enzymes at each pH value -, we used pre-specified linear contrasts (Christensen, 1996). We tested for pairwise differences between slopes (E_a) for each enzyme separately (each pH value generated a different slope estimate for each enzyme) and test for slope (E_a) differences between BGase and NAGase at each pH. These comparisons were made using single-step adjusted *p*-values to adjust for experiment-wide Type I error. All statistical analyses were performed using R v. 2.12.2 (R Core Team 2013) and SPSS (IBM SPSS Statistics ver.20), and results were considered significant when $p < 0.05$.

2.5. Computing the C:N flow ratio of liberated resources

For the substrates used in this study, the C:N flow ratio (Billings and Ballantyne, 2013) describes the relative release rates of C and N atoms upon enzymatic cleavage of the MUB-labeled substrates. As MUB-BG and MUB-NAG serve as proxies for cellulose (polymer of glucose) and chitin (polymer of NAG), respectively, the BGase-catalyzed liberation of one MUB molecule from MUB-BG is comparable to the release of one glucose molecule from cellulose (Sinsabaugh et al., 2008; German et al., 2012). Analogously, the NAGase-catalyzed MUB liberation from MUB-NAG is comparable to the release of one NAG molecule from chitin (Sinsabaugh et al., 2008; DeForest, 2009; German et al., 2012). Because the same MUB fluorophore is liberated from both substrates, units of fluorescence can be directly converted into numbers of C and N atoms liberated (Lehmeier et al., 2013); glucose liberation generates 6 assimilable C atoms and no N, and NAG liberation generates 8C atoms and 1 N atom. Thus, the C:N flow ratio from simultaneous decay of MUB-BG and MUB-NAG can be calculated as

$$\frac{dC}{dN} = \frac{A_{BG}}{A_{NAG}} \cdot e^{\frac{(E_{aNAG} - E_{aBG})}{RT}} \cdot 6 + 8 \qquad (2)$$

where E_{aBG}, A_{BG}, E_{aNAG}, and A_{NAG} are estimated using the general linear model with Arrhenius transformed activity as a function of 1/T and pH for the BGase/MUB-BG and the NAGase/MUB-NAG reactions, respectively (Lehmeier et al., 2013; see Section 2.4). We used estimates of E_a, which specify relative temperature sensitivity of enzymatic MUB-BG and MUB-NAG decay across the temperature range studied here, to compute the C:N flow ratio across the

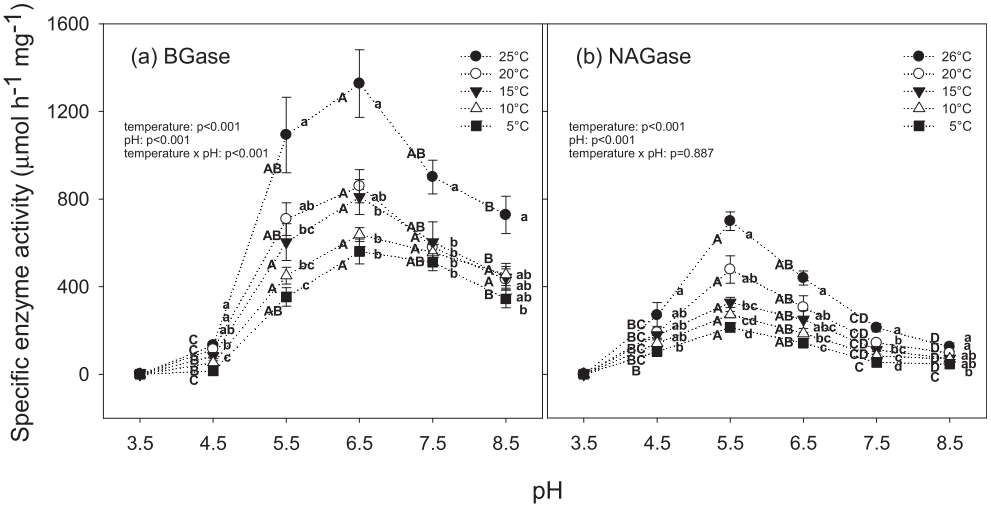

Fig. 2. Specific activities of (a) BGase and (b) NAGase (µmol h^{-1} enzyme mg^{-1}) at different temperatures as a function of pH. Error bars represent ±1 standard error ($n = 4–8$ for BGase/MUB-BG and $n = 3–4$ for NAGase/MUB-NAG). Results from two-way ANOVA with temperature and pH as independent variables are included in each panel. Multiple comparisons were made across temperature within pH values, lower case letters, and across pH for particular temperature, upper case letters. We used Bonferroni corrected *p*-values to determine statistically significant comparisons at $p < 0.05$ level. Note that indicators of statistical significance reflect analyses performed using log-transformed specific activities to satisfy the assumptions of normality and equal variance.

specified pH range. A C:N flow ratio was considered significantly different from one if E_a of both reactions, at the same pH value, were significantly different from each other at the $p < 0.05$ level.

3. Results

3.1. Effects of temperature and pH on specific enzyme activities

We observed significant effects of temperature ($p < 0.001$) and pH ($p < 0.001$) for both log-transformed specific activities of BGase and log-transformed specific activities of NAGase, but a significant interaction ($p < 0.001$) only for BGase. Within each temperature, log-transformed specific activities of BGase were higher at pH 5.5–8.5 than those at 4.5 (see upper case letters in Fig. 2a). At a given pH, temperature-induced increases in log-transformed specific BGase activities varied across pH value (see lower case letters in Fig. 2a). Within each temperature, NAGase activity was highest at pH 5.5 and 6.5, indicating a well-defined, optimal pH for this reaction (see Fig. 2b).

3.2. Temperature sensitivity of reactions

In this study, temperature sensitivities were defined by E_a (see Eq. (1)), which was computed using the entire temperature range of 5–25 °C, and dictates how changes in temperature over this range, alter rates of reaction. The E_a of the BGase/MUB-BG reactions was significantly influenced by pH (Fig. 3a, Table 2, and Supplementary Table 1). The E_a of BGase was highest at pH 4.5 and was significantly different from E_a values at all other pH values. The second highest E_a of BGase occurred at pH 5.5, which was significantly greater than the E_a at pH 7.5. In contrast, none of the E_a values computed for NAGase/MUB-NAG reactions were significantly different from each other among pH values (Fig. 3b, Table 2, and Supplementary Table 1). This is reflected in the near parallel slopes (E_a) of log-transformed specific activities of NAGase (Fig. 3b).

We also observed significantly higher E_a of BGase compared to NAGase at pH 4.5, and significantly lower E_a of BGase compared to NAGase at pH 7.5 (Table 2 and Supplementary Table 1). Apparent differences in E_a between BGase and NAGase at pH of 5.5, 6.5 and 8.5, although often pronounced, were not statistically significant.

Table 2
Activation energies (E_a; in kJ mol^{-1}) of BGase/MUB-BG and NAGase/MUB-NAG reactions, estimated using slopes in Fig. 3. Using pre-specified linear contrasts, we tested for differences among E_a across pH and enzyme at the $p < 0.05$ level (see Section 2.4). Different upper case letters denote significant differences in E_a across pH values for a given enzyme (comparing slopes within one of the panels in Fig. 3); different lower case letters denote significant differences in E_a between two enzymes at a given pH (comparing slopes across panels for a given pH in Fig. 3).

	3.5	4.5	5.5	6.5	7.5	8.5
BGase/MUB-BG						
E_a	NA	65.1	37.2	28.0	16.9	22.2
Significance		A, a	B, a	BC, a	C, a	BC, a
NAGase/MUB-NAG						
E_a	NA	28.9	39.4	36.7	45.2	33.4
Significance		A, b	A, a	A, a	A, b	A, a

NA, not applicable.

3.3. Estimated C:N flow ratio

Solution pH affected the C:N flow ratio resulting from the BGase/MUB-BG and NAGase/MUB-NAG reactions. Across the temperatures and pH values tested, the C:N flow ratio was lowest at pH 4.5 and 5 °C (~10:1), and exhibited a maximum value of approximately ~40:1 at pH 6.5 and 5 °C (Table 3). Except at pH 5.5, where the C:N flow ratio was approximately 30:1 regardless of temperature, temperature changes induced variability in the C:N flow ratio (Table 3 and Fig. 4). At pH 4.5, the C:N flow ratio significantly

Table 3
Estimated C:N flow ratios in simultaneously proceeding BGase/MUB-BG and NAGase/MUB-NAG reactions at each temperature and pH. By definition, the C:N flow ratio is the ratio of C to N atoms liberated upon substrate decay (see Section 2.5). Highlighted columns represent C:N flow ratios when E_a values of BGase/MUB-BG and NAGase/MUB-NAG reactions at the same pH were significantly different from each other at the $p < 0.05$ level (see Table 2).

Temperature (°C)	pH 3.5	pH 4.5	pH 5.5	pH 6.5	pH 7.5	pH 8.5
5	NA	9.6	29.3	41.3	38.5	28.9
10	NA	9.9	29.4	39.2	34.2	26.7
15	NA	10.4	29.4	37.3	30.7	24.8
20	NA	10.9	29.5	35.5	27.7	23.2
25	NA	11.6	29.6	33.9	25.2	21.7

NA, not available.

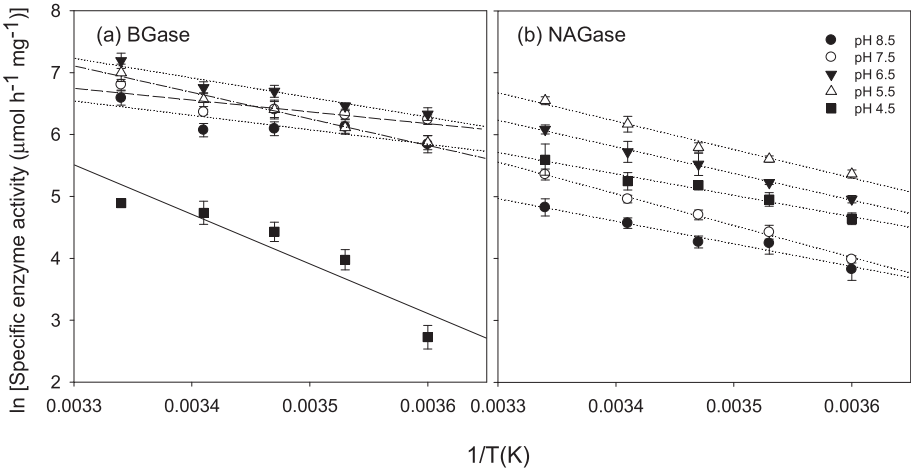

Fig. 3. Temperature sensitivities (E_a) of (a) BGase/MUB-BG and (b) NAGase/MUB-NAG reactions. To assess E_a of the reactions as detailed in Eq. (1), we plot ln-transformed specific enzyme activities against 1/temperature (in K). The slopes of the linear regressions are considered equivalent to the E_a of each relevant reaction; the intercepts are values for ln (A) (see Eq. (1)). In (a), the slope of the solid line (pH 4.5; E_a = 65.1 kJ mol^{-1}) is significantly higher than those of broken lines (see Table 2 and Supplementary Table 1 for more information). Among broken lines, the slope of the dash–dot line (—·—, pH 5.5; E_a = 37.2 kJ mol^{-1}) is significantly greater than the dash–dash line (——, pH 7.5; E_a = 16.9 kJ mol^{-1}; $p = 0.028$). The slopes of NAGase/MUB-NAG reactions in (b) were not significantly different across pH values.

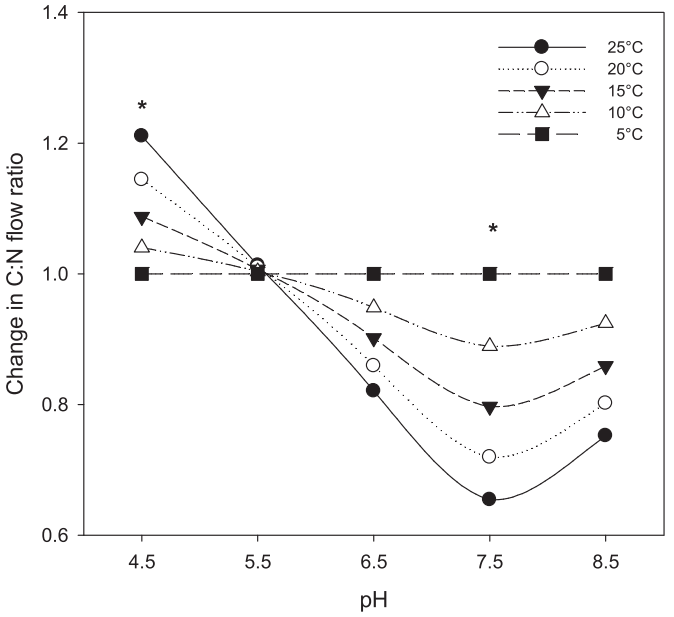

Fig. 4. C:N flow ratio of resources liberated during BGase/MUB-BG and NAGase/NAG reactions proceeding at 10, 15, 20, and 25 °C relative to that at 5 °C at specified pH values. This normalization represents how the relative amount of C and N potentially assimilable to microbes during decay changes as temperature increases from 5 °C. See Section 2.5 for a complete description of C:N flow ratio. Asterisks at pH 4.5 and 7.5 denote statistically significant changes in the C:N flow ratio as temperature increases from 5 to 25 °C, as determined by significantly different E_a values of both reactions at the same pH ($p < 0.05$).

increased by 21% when temperature increased from 5 to 25 °C ($p < 0.05$ for the linear contrast comparing $E_{a\mathrm{BG}}$ and $E_{a\mathrm{NAG}}$; Fig. 4). In contrast, the C:N flow ratio decreased with temperature above pH 5.5, and exhibited a significant decline with increasing temperature from 5 to 25 °C at pH 7.5 (as determined by $p < 0.05$ for the linear contrast comparing $E_{a\mathrm{BG}}$ and $E_{a\mathrm{NAG}}$; Fig. 4). At pH 6.5 and 8.5, the temperature-related decreases in the C:N flow ratio were not statistically significant despite their large magnitude.

4. Discussion

In this study, we quantified specific activities of two extracellular enzyme/substrate reactions representative of microbially-mediated decay of two important SOM compounds, cellulose and chitin, and estimated the intrinsic temperature sensitivity of these reactions. We assessed how the temperature responses of these reactions are influenced by the pH in the reaction medium, over the naturally occurring range of soil pH. As a result, we generated baseline values of intrinsic sensitivities of BGase and NAGase reactions to which measurements of apparent temperature sensitivities of cellulose and chitin decay in soils can be compared quantitatively. Thus, our work provides a means of estimating the contribution of potentially changing chemical, physical, and microbial variables with temperature to observed temperature responses of cellulose and chitin decomposition and microbial CO_2 efflux from soils.

4.1. Specific enzyme activities of BGase and NAGase

Robust activity of BGase in variable environments (Fig. 2a) is consistent with the pivotal role it plays for microbial C metabolism in cleaving a disaccharide into two hexoses. Because glucose promotes the highest yield of adenosine triphosphate and dry cell mass per molecule consumed compared to other simple C-containing compounds (Bauchop and Elsden, 1960), producing BGase that remains viable across a wide range of environmental conditions may be an important feature for some microbial decomposition strategies. This is consistent with BGase activity remaining relatively constant in environmental samples across seasons with wide pH and temperature ranges (Rastin et al., 1988; Bandick and Dick, 1999; Bell and Henry, 2011), apparently regardless of cellulose content in soils (German et al., 2011).

In contrast, NAGase activity declined as reaction conditions deviated from pH 5.5–6.5, at all temperatures (Fig. 2b). We know of no study reporting on the stability of NAGase across soils of different pH and temperature; further study is necessary to clarify why pH 5.5–6.5 represents an apparently optimum range for NAGase activity. However, the lower estimates of intrinsic NAGase activity relative to BGase activity reported here and elsewhere (Lehmeier et al., 2013) are not surprising, given the more critical role of BGase to central metabolism. Carbon derived from any NAG taken up by a cell must eventually be transformed to glucose intracellularly for its use in glycolysis or the tricarboxylic acid or pentose phosphate cycles, increasing the metabolic cost of its use. Nitrogen derived from NAG cleavage can be an important source of microbial N (Sinsabaugh et al., 2008), but N can also be obtained via uptake of amino acids, inorganic N, or nitrogenous monomers released via other decay reactions (Lipson and Monson, 1998; Nordin et al., 2004; Schimel and Bennett, 2004; Tiemann and Billings, 2012).

4.2. Effects of pH on the intrinsic temperature sensitivity

Variations of temperature sensitivities (E_a) of BGase and NAGase across pH highlight how soil pH may influence SOM decay dynamics in different ecosystems. For example, despite an apparently narrow optimum at pH 5.5–6.5 (Fig. 2b), the NAGase/MUB-NAG reaction did not exhibit significantly varying temperature sensitivity across the wide range of pH values studied here (similar slopes in Fig. 3b). This suggests that temperature exerts a relatively similar, positive influence on chitin decay in soils regardless of soil pH. Thus, all else being equal, we might predict that NAGase activity in forest and grassland soils, which typically have a pH between 4.5 and 6.5 (IGBP-DIS, 1998), may experience the same degree of stimulation with increasing temperature as NAGase in desert soils, which typically have a more alkaline pH (IGBP-DIS, 1998).

In contrast, the significant influence of pH on BGase temperature sensitivity, as defined by E_a, suggests that ecosystems with different soil pH may experience relatively distinct changes in the rate of BGase-mediated cellulose decay in response to temperature. For example, the lower E_a of BGase at pH 6.5, 7.5, and 8.5 compared to other pH values (Table 2 and Supplementary Table 1) and the relatively high specific activities of BGase at those pH values (Fig. 2a) may have important implications for SOM dynamics in relatively alkaline desert soils. The data suggest that BGase exuded by microbial communities in alkaline desert soils may exhibit a relatively large fraction of its maximum potential activity even with declines in temperature from relatively high day-time maximums (Whitford, 2002). Traditionally, relatively low accumulation of SOM in deserts is explained by low net primary production. Recently, Stursova and Sinsabaugh (2008) argued that thermally stable oxidative enzymes, along with their apparently high optimal pH, may promote SOM decomposition in desert soils. Here, our finding suggests that the maintenance of BGase activity in alkaline conditions (Fig. 2a) across a large gradient of temperature may also limit SOM accumulation in those soils. Furthermore, the relative temperature sensitivity of BGase/MUB-BG reactions at pH 4.5 was higher than those at all other pH values (Fig. 3a, Table 2, and

Supplementary Table 1). This suggests that in many of the world's forest soils, which tend to exhibit relatively low pH values, BGase-driven cellulose decay exhibits greater relative temperature sensitivity than that in more alkaline soils. Given the approximately 341 Pg C residing in such soils (estimated from Batjes, 2012), our work underscores the relative vulnerability of SOC stocks residing in soils with pH of ~4.5 and subjected to decay via BGase.

4.3. Linking temperature and pH to microbially available C and N

Differential effects of pH on the temperature sensitivity of C- and N-acquiring enzymes' reactions have ecological implications for soil microbe resource availability. The C:N flow ratio provides insight into how resources assimilable to microbial communities change with temperature and pH. This concept assumes that all of the C and N atoms liberated during the simulated MUB-BG and MUB-NAG decay, our proxies for cellulose and chitin decay in soils, are readily available for microbial assimilation. As such, changes in the C:N flow ratio may prompt soil microbes to alter their resource investment in extracellular enzymes, in an effort to balance C and N demand with changing relative availability of these elements in the environment. Our study design precludes us from discerning temperature-related changes in microbial biomass or composition, substrate targets, concentration of isozymes of extracellular enzymes at different pH values, or temperature sensitivity of enzymes other than BGase and NAGase. Instead, this study illuminates how two ecologically relevant extracellular enzymes may function in different chemical environments *post* microbial exudation.

We observed variable patterns of C:N flow ratio in response to temperature from 5 to 25 °C across pH values (Table 3 and Fig. 4), largely driven by significant differences in the temperature sensitivity of BGase among pH values. The significant increase in C:N flow ratio at pH 4.5 with increasing temperature implies that C becomes relatively more available than N as temperature increases. Across the world, highly acidic soil (≤pH 4.5) is usually found in tundra systems, boreal forests, high-latitude peatlands, tropical forests, and tropical peatlands (FAO, 2013). Among these biomes, those at high-latitude are expected to experience substantially greater SOM decay in the future, relative to systems at lower latitudes, due to the magnitude of warming projected in this region (IPCC, 2013) and BGase adapted to cold temperatures (German et al., 2012). Here, we propose another explanation for why these acidic ecosystems are highly likely to undergo significant SOM decay as temperature increases: at pH of 4.5, preferentially stimulated BGase activity with temperature, relative to NAGase, could result in increasing C:N flow ratio. All else being equal, an increase in C availability tends to promote declines in microbial C use efficiency, enhancing relatively high respiratory C losses (del Giorgio and Cole, 1998; Manzoni et al., 2012). This argument is supported, indirectly, by studies reporting higher temperature sensitivity of C mineralization compared to N mineralization in alpine soil slurries at pH_{CaCl_2} of 3.9–4.3 and tussock tundra soil slurries at pH 4.9 (Koch et al., 2007; Wallenstein et al., 2009).

For pH values higher than 4.5, we observed both qualitative and quantitative shifts in the C:N flow ratio. At pH 5.5, the C:N flow ratio exhibited no significant change with temperature (Table 3; Fig. 4), consistent with reports of unvarying C:N mineralization with temperature in incubated soils at pH 5.9 (Nadelhoffer et al., 1991) and similar Q_{10} values of BGase and NAGase reactions at pH 5.8 (German et al., 2012). At pH 7.5, C:N flow ratios decreased significantly with temperature, with similar, but non-significant trends also evident at pH 6.5 and 8.5. These data are consistent with Lehmeier et al. (2013), who demonstrated a decreasing C:N flow ratio from 41 to 34 at pH 6.5 as temperature increased from 7 to 25 °C. Our results also are congruent with studies of arctic soils at pH of 6.1–7.0, in which the ratio of C:N mineralization declined with increasing temperature (Nadelhoffer et al., 1991). Analogous with potentially increasing C losses with temperature increases at pH 4.5, observations in relatively alkaline conditions highlight the potential for enhanced N loss in soils with higher pH as temperature increases. As N availability increases to a greater extent than C availability, losses may occur via ammonia volatilization and leaching of dissolved organic N (Aber et al., 1995; Bussink and Oenema, 1998).

5. Conclusions

By quantifying intrinsic temperature sensitivities of BGase/MUB-BG and NAGase/MUB-NAG reactions at multiple pH values, we provide the baseline values describing how liberation rates of C and N from cellulose and chitin in different soils will respond to changing environments. We thus provide a point of comparison for inferring the drivers of apparent temperature sensitivities of decay, and the flows of C and N emanating from them. Our work demonstrates that pH can exert differential influences on the temperature sensitivities of BGase and NAGase. As a result, relative flows of assimilable C and N during cellulose and chitin decay may vary with pH as temperature changes. Although we do not know how C- and N-acquiring enzymes other than BGase and NAGase will influence relative C and N availability with changing temperature and pH, our study demonstrates that disparate responses of BGase and NAGase to pH and temperature dictate fluctuations in the relative availabilities of essential microbial resources, presenting a temperature-driven feedback to which microorganisms may respond by altering their decomposition strategies.

Acknowledgments

This study was supported by National Science Foundation grant DEB-0950095. We are grateful to Anuradha Roy for use of the fluorescence plate reader at the High Throughput Screening Lab at the University of Kansas, Chao Song for help with data analysis, and Nicole Niehues for help with experimental preparation. We also thank two anonymous reviewers for their helpful comments.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2014.05.021.

References

Aber, J.D., Magill, A., Mcnulty, S.G., Boone, R.D., Nadelhoffer, K.J., Downs, M., Hallett, R., 1995. Forest biogeochemistry and primary production altered by nitrogen saturation. Water Air and Soil Pollution 85, 1665–1670.
Arrhenius, S., 1889. Über die Reaktionsgeschwindigkeit bei der Inversion von Rohrzucker durch Säuren. Zeitschrift fur Physik Chemique 4, 226–248.
Bandick, A.K., Dick, R.P., 1999. Field management effects on soil enzyme activities. Soil Biology & Biochemistry 31, 1471–1479.
Batjes, N.H., 2012. ISRIC-WISE Derived Soil Properties on a 5 by 5 Acr-Minutes Global Grid (Ver. 1.2). ISRIC Report 2012/01, Wageningen.
Bauchop, T., Elsden, S., 1960. The growth of micro-organisms in relation to their energy supply. Journal of General Microbiology 23, 457–469.
Bell, T.H., Henry, H.A., 2011. Fine scale variability in soil extracellular enzyme activity is insensitive to rain events and temperature in a mesic system. Pedobiologia 54, 141–146.
Billings, S.A., Ballantyne, F., 2013. How interactions between microbial resource demands, soil organic matter stoichiometry, and substrate reactivity determine the direction and magnitude of soil respiratory responses to warming. Global Change Biology 19, 90–102.
Brady, N., Weil, R., 2007. Nature and Properties of Soils, 14th ed. Prentice Hall.
Bussink, D., Oenema, O., 1998. Ammonia volatilization from dairy farming systems in temperate areas: a review. Nutrient Cycling in Agroecosystems 51, 19–33.
Christensen, R., 1996. Plane Answers to Complex Questions: the Theory of Linear Models. Springer.

Conant, R.T., Ryan, M.G., Ågren, G.I., Birge, H.E., Davidson, E.A., Eliasson, P.E., Evans, S.E., Frey, S.D., Giardina, C.P., Hopkins, F.M., Hyvönen, R., Kirschbaum, M.U.F., Lavallee, J.M., Leifeld, J., Parton, W.J., Steinweg, J.M., Wallenstein, M.D., Wetterstedt, J.Å.M., Bradford, M., 2011. Temperature and soil organic matter decomposition rates–synthesis of current knowledge and a way forward. Global Change Biology 17, 3392–3404.

Cleveland, C., Liptzin, D., 2007. C: N: P stoichiometry in soil: is there a "Redfield ratio" for the microbial biomass? Biogeochemistry 85, 235–252.

Davidson, E.A., Janssens, I.A., 2006. Temperature sensitivity of soil carbon decomposition and feedbacks to climate change. Nature 440, 165–173.

Davidson, E.A., Janssens, I.A., Luo, Y., 2006. On the variability of respiration in terrestrial ecosystems: moving beyond Q_{10}. Global Change Biology 12, 154–164.

DeForest, J.L., 2009. The influence of time, storage temperature, and substrate age on potential soil enzyme activity in acidic forest soils using MUB-linked substrates and L-DOPA. Soil Biology & Biochemistry 41, 1180–1186.

Deng, S.P., Tabatabai, M.A., 1994. Cellulase activity of soils. Soil Biology & Biochemistry 26, 1347–1354.

FAO, 2013. Land Resources: pH (topsoil). Retrieved 6/3/2013 from. http://www.fao.org/nr/land/soils/en/.

Fierer, N., Coman, B.P., Schimel, J.P., Jackson, R.B., 2006. Predicting the temperature dependence of microbial respiration in soil: a continental-scale analysis. Global Biogeochemical Cycles 20, GB3026. http://dx.doi.org/10.1029/2005GB002644.

Frankenberger, W., Johanson, J., 1982. Effect of pH on enzyme stability in soils. Soil Biology & Biochemistry 14, 433–437.

Frey, S.D., Knorr, M., Parrent, J.L., Simpson, R.T., 2004. Chronic nitrogen enrichment affects the structure and function of the soil microbial community in temperate hardwood and pine forests. Forest Ecology and Management 196, 159–171.

German, D.P., Chacon, S.S., Allison, S.D., 2011. Substrate concentration and enzyme allocation can affect rates of microbial decomposition. Ecology 92, 1471–1480.

German, D.P., Marcelo, K.R., Stone, M.M., Allison, S.D., 2012. The Michaelis–Menten kinetics of soil extracellular enzymes in response to temperature: a cross-latitudinal study. Global Change Biology 18, 1468–1479.

del Giorgio, P.A., Cole, J.J., 1998. Bacterial growth efficiency in natural aquatic systems. Annual Review of Ecology and Systematics 29, 503–541.

Högberg, P., Nordgren, A., Buchmann, N., Taylor, A.F., Ekblad, A., Högberg, M.N., Nyberg, G., Ottosson-Löfvenius, M., Read, D.J., 2001. Large-scale forest girdling shows that current photosynthesis drives soil respiration. Nature 411, 789–792.

IGBP-DIS, 1998. SoilData(V.0): a Program for Creating Global Soil-Property Databases. IGBP Global Soils Data Task, France.

IPCC, 2013. Climate change 2013: the physical science basis. In: Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge and New York.

Knorr, W., Prentice, I., House, J., Holland, E., 2005. Long-term sensitivity of soil carbon turnover to warming. Nature 433, 298–301.

Koch, O., Tscherko, D., Kandeler, E., 2007. Temperature sensitivity of microbial respiration, nitrogen mineralization, and potential soil enzyme activities in organic alpine soils. Global Biogeochemical Cycles 21, GB4017. http://dx.doi.org/10.1029/2007BG002983.

Lehmeier, C.A., Min, K., Niehues, N.D., Ballantyne, F., Billings, S.A., 2013. Temperature-mediated changes of exoenzyme-substrate reaction rates and their consequences for the carbon to nitrogen flow ratio of liberated resources. Soil Biology & Biochemistry 57, 374–382.

Lipson, D.A., Monson, R.K., 1998. Plant-microbe competition for soil amino acids in the alpine tundra: effects of freeze-thaw and dry-rewet events. Oecologia 113, 406–414.

MacDonald, N.W., Zak, D.R., Pregitzer, K.S., 1995. Temperature effects on kinetics of microbial respiration and net nitrogen and sulfur mineralization. Soil Science Society of America Journal 59, 233–240.

Manzoni, S., Taylor, P., Richter, A., Porporato, A., Ågren, G.I., 2012. Environmental and stoichiometric controls on microbial carbon-use efficiency in soils. New Phytologist 196, 79–91.

Mead, J.A.R., Smith, J.N., Williams, R.T., 1955. Studies in detoxication. 67. Biosynthesis of the glucuronides of umbelliferone and 4-methylumbelliferone and their use in fluorimetric determination of beta-glucuronidase. Biochemical Journal 61, 569–574.

Mikan, C.J., Schimel, J.P., Doyle, A.P., 2002. Temperature controls of microbial respiration in arctic tundra soils above and below freezing. Soil Biology & Biochemistry 34, 1785–1795.

Min, K., Kang, H., Lee, D., 2011. Effects of ammonium and nitrate additions on carbon mineralization in wetland soils. Soil Biology & Biochemistry 43, 2461–2469.

Nadelhoffer, K.J., Giblin, A.E., Shaver, G.R., Laundre, J.A., 1991. Effects of temperature and substrate quality on element mineralization in six Arctic soils. Ecology 72, 242–253.

Niemi, R., Vepsäläinen, M., 2005. Stability of the fluorogenic enzyme substrates and pH optima of enzyme activities in different Finnish soils. Journal of Microbiological Methods 60, 195–205.

Nordin, A., Schmidt, I.K., Shaver, G.R., 2004. Nitrogen uptake by arctic soil microbes and plants in relation to soil nitrogen supply. Ecology 85, 955–962.

Parham, J., Deng, S., 2000. Detection, quantification and characterization of β-glucosaminidase activity in soil. Soil Biology & Biochemistry 32, 1183–1190.

Rastin, N., Rosenplänter, K., Hüttermann, A., 1988. Seasonal variation of enzyme activity and their dependence on certain soil factors in a beech forest soil. Soil Biology & Biochemistry 20, 637–642.

Schimel, D.S., 1995. Terrestrial ecosystems and the carbon cycle. Global Change Biology 1, 77–91.

Schimel, J.P., Bennett, J., 2004. Nitrogen mineralization: challenges of a changing paradigm. Ecology 85, 591–602.

Sinsabaugh, R.L., Lauber, C.L., Weintraub, M.N., Ahmed, B., Allison, S.D., Crenshaw, C., Contosta, A.R., Cusack, D., Frey, S.D., Gallo, M.E., Gartner, T.B., Hobbie, S.E., Holland, K., Keeler, B.L., Powers, J.S., Stursova, M., Takacs-Vesbach, C., Waldrop, M.P., Wallenstein, M.D., Zak, D.R., Zeglin, L.H., 2008. Stoichiometry of soil enzyme activity at global scale. Ecology Letters 11, 1252–1264.

Sterner, R.W., Elser, J.J., 2002. Ecological Stoichiometry: the Biology of Elements from Molecules to the Biosphere. Princeton University Press, Princeton.

Somero, G.N., 2004. Adaptation of enzymes to temperature: searching for basic "strategies". Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 139, 321–333.

Stursova, M., Sinsabaugh, R.L., 2008. Stabilization of oxidative enzymes in desert soil may limit organic matter accumulation. Soil Biology & Biochemistry 40, 550–553.

Suseela, V., Conant, R.T., Wallenstein, M.D., Dukes, J.S., 2012. Effects of soil moisture on the temperature sensitivity of heterotrophic respiration vary seasonally in an old-field climate change experiment. Global Change Biology 18, 336–348.

Tabatabai, M.A., 1994. Soil enzymes. In: Weaver, W., Angle, J.S., Bottomley, P.S. (Eds.), Methods of Soil Analysis Part 2. Microbiological and Biological Properties. Soil Science Society of America, Wisconsin, pp. 775–833.

Tiemann, L.K., Billings, S.A., 2012. Tracking C and N flows through microbial biomass with increased soil moisture variability. Soil Biology & Biochemistry 49, 11–22.

Tipton, K.F., McDonald, A., Dixon, H., 1979. Effects of pH on enzymes. In: Purich, D.L. (Ed.), Contemporary Enzyme Kinetics and Mechanism. Academic Press, pp. 124–172.

Treseder, K., 2008. Nitrogen additions and microbial biomass: a meta-analysis of ecosystem studies. Ecology Letters 11, 1111–1120.

Trumbore, S., 2006. Carbon respired by terrestrial ecosystems – recent progress and challenges. Global Change Biology 12, 141–153.

Turner, B.L., 2010. Variation in pH optima of hydrolytic enzyme activities in tropical rain forest soils. Applied and Environmental Microbiology 76, 6485–6493.

van't Hoff, J.H., 1884. Etudes de dynamique chemique. Frederik Muller & Co., Amsterdam.

Wallenstein, M.D., McMahon, S.K., Schimel, J.P., 2009. Seasonal variation in enzyme activities and temperature sensitivities in Arctic tundra soils. Global Change Biology 15, 1631–1639.

Wallenstein, M.D., Allison, S.D., Ernakovich, J.E., Steinweg, J.M., Sinsabaugh, R.L., 2010. Controls on the temperature sensitivity of soil enzymes: a key driver of in situ enzyme activity rates. In: Shukla, G., Varma, A. (Eds.), Soil Enzymology. Springer, Berlin, pp. 245–258.

Whitford, W.G., 2002. Ecology of Desert Systems. Academic Press.

Zoltan, S., 2008. Spatial and temporal pattern of soil pH and Eh and their impact on solute iron content in a wetland (Transdanubia, Hungary). Acta Geographica Debrecina. Landscape and Environment Series 2, 34–45.

Zhou, T., Shi, P., Hui, D., Luo, Y., 2009. Global pattern of temperature sensitivity of soil heterotrophic respiration (Q_{10}) and its implications for carbon-climate feedback. Journal of Geophysical Research 114, GO2016. http://dx.doi.org/10.1029/2008JG000850.