



## Temperature-mediated changes of exoenzyme–substrate reaction rates and their consequences for the carbon to nitrogen flow ratio of liberated resources

Christoph A. Lehmeier, Kyungjin Min, Nicole D. Niehues, Ford Ballantyne IV<sup>1</sup>, Sharon A. Billings\*

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

### ARTICLE INFO

#### Article history:

Received 7 August 2012

Received in revised form

22 October 2012

Accepted 25 October 2012

Available online 11 November 2012

#### Keywords:

Activation energy

Carbon

Climate change

Exoenzyme

Nitrogen

Soil organic matter

Soil warming

Temperature

### ABSTRACT

Soil microorganisms produce exoenzymes to access resources stored in soil organic matter. Knowledge about the effect of temperature on the rates at which exoenzymes degrade substrates is particularly important for understanding carbon and nitrogen cycling with warming, and possible feedbacks to climate change.

Here, we studied reaction rates of purified enzymes and substrates in controlled laboratory conditions at temperatures ranging from 5 °C to 27 °C. We employed three enzyme–substrate pairings representative of reactions common to soil profiles:  $\beta$ -glucosidase and  $\beta$ -D-cellobioside (BGase/BG),  $\beta$ -N-acetylglucosaminidase and N-acetyl- $\beta$ -D-glucosaminide (NAGase/NAG), and peroxidase and 3,4-Dihydroxy-L-phenylalanine (peroxidase/L-Dopa).

Across the entire temperature range studied, BGase showed the highest specific activity ( $V_{\max 27\text{ °C}} = 1338 \mu\text{mol h}^{-1} \text{mg}_{\text{BGase}}^{-1}$ ), followed by NAGase ( $V_{\max 25\text{ °C}} = 260 \mu\text{mol h}^{-1} \text{mg}_{\text{NAGase}}^{-1}$ ) and peroxidase ( $V_{\max 25\text{ °C}} = 36 \mu\text{mol h}^{-1} \text{mg}_{\text{peroxidase}}^{-1}$ ). From 7.5 °C to 25 °C, the specific activities of BGase, NAGase and peroxidase increased by 103%, 111% and 835%, respectively. The activation energy ( $E_a$ ) required for a reaction to proceed thus was highest for peroxidase/L-Dopa (99.8 kJ mol<sup>-1</sup><sub>Dopa</sub>), followed by NAGase/NAG (41.3 kJ mol<sup>-1</sup><sub>NAG</sub>) and BGase/BG (31.4 kJ mol<sup>-1</sup><sub>BG</sub>). We use a simple model, parameterized with empirical data from these reactions in three different ways, to illustrate how the flow of carbon relative to nitrogen can change with temperature as these resources are liberated from their organic precursors. The results highlight the importance of relative temperature sensitivities among reactions and the substrates' carbon to nitrogen ratio as key determinants of temperature-mediated changes in relative availabilities of carbon and nitrogen to microorganisms.

© 2012 Elsevier Ltd. All rights reserved.

### 1. Introduction

Soil microorganisms produce exoenzymes, which they release into the soil matrix to decompose dead organic material. Through this process, resources such as carbon (C) and nitrogen (N) derived from macromolecular compounds become present in the form of smaller molecules. Such molecules can be taken up by microorganisms, and serve as substrate for their growth and metabolism. Most organic inputs to soils are eventually degraded by microorganisms; their exoenzymes are thus key components in the global

cycling of C and N (Lavelle et al., 1993; Sinsabaugh and Moorhead, 1994; Conrad, 1996; Rillig et al., 2007; Nannipieri and Paul, 2009; McGuire and Treseder, 2010; Henry, 2012; Todd-Brown et al., 2012).

Of particular interest for understanding the cycling of C and N in a changing climate is the relationship between temperature and the rates of soil organic matter (SOM) decomposition (Smith et al., 2008; Von Lützow and Kögel-Knabner, 2009; Kleber, 2010; Singh et al., 2010; Conant et al., 2011; Pritchard, 2011; Sierra, 2012). The rate at which a soil exoenzyme degrades a substrate is determined by a multitude of factors, including pH (for all exoenzymes) and O<sub>2</sub> availability (for oxidative exoenzymes), the physical conditions influencing the coupling of exoenzyme and substrates, the substrate concentration, and the temperature (Von Lützow et al., 2006; Kleber, 2010; Kleber and Johnson, 2010). The Van't Hoff–Arrhenius law of reaction kinetics predicts that the probability of an enzymatic reaction occurring increases with rising temperature (see Laidler, 1984; Bosatta and Ågren, 1999; Davidson and Janssens, 2006; Sierra, 2012), and that the response of the reaction rate to

\* Corresponding author.

E-mail addresses: [Christoph.Lehmeier@gmail.com](mailto:Christoph.Lehmeier@gmail.com) (C.A. Lehmeier), [kjmin.21@gmail.com](mailto:kjmin.21@gmail.com) (K. Min), [niehuesn@gmail.com](mailto:niehuesn@gmail.com) (N.D. Niehues), [fb4@ku.edu](mailto:fb4@ku.edu) (F. Ballantyne), [sharonb@ku.edu](mailto:sharonb@ku.edu) (S.A. Billings).

<sup>1</sup> Present address: Odum School of Ecology, The University of Georgia, 140 E. Green St., Athens, GA 30602, USA.

temperature increases with the activation energy required to initiate substrate decay:

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

$V$  is the reaction rate,  $A$  the pre-exponential factor,  $E_a$  the activation energy,  $R$  the gas constant, and  $T$  the temperature in K. The degree to which a specific enzyme–substrate reaction, not impeded by limiting enzyme or substrate concentrations or any physical constraints (i.e. when  $V$  equals the maximum specific enzyme activity  $V_{\max}$ ), responds to temperature represents the intrinsic temperature sensitivity of the reaction.

In spite of the great theoretical utility of the Van't Hoff–Arrhenius relationship in studies of SOM decomposition (Davidson and Janssens, 2006; Craine et al., 2010; Conant et al., 2011; Davidson et al., 2012; Sierra, 2012), intrinsic temperature sensitivities of enzyme–substrate reactions relevant for biogeochemical processes remain largely unknown. What is observed in most studies of SOM decay is an apparent temperature sensitivity of reactions, where other variables such as low soil water content, low substrate availability or altered microbial exoenzyme production may constrain or obscure the intrinsic temperature sensitivities of the reactions (Davidson and Janssens, 2006; Subke and Bahn, 2010; Billings and Ballantyne, 2012). Thus, apparent temperature sensitivities of SOM decomposition can deviate substantially from intrinsic temperature sensitivities, as has been concluded in numerous studies (e.g. Giardina and Ryan, 2000; Dioumaeva et al., 2002; Biasi et al., 2005; Fang et al., 2005; Leifeld and Fuhrer, 2005; Wetterstedt et al., 2010; German et al., 2012). Recent studies attempt to determine the extent to which factors such as substrate and exoenzyme availabilities (Davidson et al., 2012; German et al., 2012) and microbial adaptation (Bradford et al., 2008; Hartley et al., 2008) drive any discrepancies between intrinsic and apparent temperature sensitivities of SOM decay. However, because the intrinsic temperature sensitivity of decay for key SOM compounds paired with relevant exoenzymes is not known, quantifying the extent to which microbial adaptation or substrate availability influence apparent temperature sensitivities of decay is difficult. Predictions of how temperature responses of biochemical reaction rates will influence the exoenzyme-mediated release of C and N (and other resources) assimilable by microorganisms are therefore not possible at present.

To this end, we quantified reaction rates of purified (1)  $\beta$ -D-cellobioside and  $\beta$ -glucosidase, (2) N-acetyl- $\beta$ -D-glucosaminide and  $\beta$ -N-Acetyl glucosaminidase and (3) 3,4-Dihydroxy-L-phenylalanine and peroxidase at multiple temperatures relevant globally to soil profiles. The three pairings are employed as representatives of fundamental reactions occurring during the natural enzymatic degradation of cellulose (1), chitin (2) and lignin (3), respectively, prevalent organic compounds in most soil profiles. We determined the concentrations at which substrate and enzyme availability was not limiting for the reaction rates, and quantified the intrinsic temperature sensitivities of the reaction rates between 5 °C and 25 °C or 7.5 °C and 27 °C, depending on the reaction. We then used the temperature responses of specific  $\beta$ -glucosidase and  $\beta$ -N-Acetyl glucosaminidase activities to assess the influence of temperature on relative C and N liberation rates from their respective substrates. Our work provides a baseline for quantifying differences between intrinsic and apparent temperature sensitivities for these reactions, highlights the importance of distinguishing between relative and absolute temperature sensitivities, and reveals how temperature-induced variations in the rates of these purely

biochemical reactions may influence fluxes of liberated C and N in soils.

## 2. Materials and methods

We measured the fluorescence of fluorescently tagged substrates as they decay to quantify reaction rates of  $\beta$ -D-cellobioside (abbreviated in the following as “BG”) with  $\beta$ -Glucosidase (BGase; EC 3.2.1.21; Megazyme, Ireland) and N-acetyl- $\beta$ -D-glucosaminide (NAG) with  $\beta$ -N-Acetyl glucosaminidase (NAGase; EC 3.2.1.52; New England BioLabs, USA). The substrates (both from Sigma–Aldrich, USA) contained a methylumbelliferyl (“MUB”) label, which causes a fluorescence signal when cleaved by the enzyme (Mead et al., 1955). The two reactions are considered to simulate the naturally occurring enzymatic cleavage of single molecules from a chain of glucose molecules (representing cellulose) and from a chain of NAG molecules (representing chitin), respectively (see Lynd et al., 2002; Howard et al., 2003; Baldrian and Valášková, 2008).

The enzyme peroxidase (EC 1.11.1.7) catalyzed the oxidation and structural transformation of the substrate 3,4-Dihydroxy-L-phenylalanine (L-Dopa; both reagents from Sigma–Aldrich, USA) to 2-carboxy-2,3-dihydroindole-5,6-quinone (Mason, 1948) in the presence of hydrogen peroxide (see Nappi and Vass, 2001; Puiu et al., 2010). The oxidation of the chromogenic substrate causes a color change of the reaction volume, which we measured spectrophotometrically to determine the enzyme activity. This reaction is considered to represent an important step in the peroxidase-mediated degradation of lignin in soils (Skujins, 1978).

### 2.1. Enzyme-substrate assays for fluorescence measurements

Crystalline substrate (BG or NAG) was weighed into deionized water and stirred to dissolution. Aliquots of the enzymes (BGase and NAGase, respectively) were dispensed in 0.2 M sodium acetate buffer solutions at a pH of 6.5. For each assay, 50  $\mu$ l substrate and 200  $\mu$ l enzyme solution were pipetted in two columns (i.e. 16 wells) of a black, 96-well plate (Fisher Scientific, USA). One column of the same plate was loaded with substrate controls (50  $\mu$ l substrate solution and 200  $\mu$ l buffer), another with enzyme controls (200  $\mu$ l enzyme solution and 50  $\mu$ l buffer). The fluorescence of these wells served to correct for any signal detected for the enzyme-substrate wells not caused by the reaction product MUB itself. Ten  $\mu$ M 4-Methylumbelliferone (MUB reference; Sigma–Aldrich, USA) suspensions served to quantify the fluorescence signals of the MUB released in the enzyme-substrate reaction. One plate column was the quench control (50  $\mu$ l MUB reference solution and 200  $\mu$ l enzyme solution), and another was the standard control (50  $\mu$ l MUB reference solution and 200  $\mu$ l buffer).

We measured the evolution of fluorescence across time with a Spectramax Gemini XS fluorescence plate reader (Molecular Devices, USA) at excitation and emission wavelengths of 365 nm and 460 nm, respectively. Preliminary tests ensured that these wavelengths provided for the highest detection sensitivity to MUB. Values for each control are the average fluorescence values of eight single wells of the respective columns. Fluorescence values of one replicate enzyme-substrate assay refer to the average of fluorescence signals from the 16 assay wells of the plate. Over all measurements, the average coefficient of variation of 16 assay wells was 0.042 for BGase/BG (max. 0.105, min. 0.018) and 0.046 for NAGase/NAG (max. 0.118, min. 0.026). The specific enzyme activities were calculated based on DeForest (2009) and German et al. (2011) as follows:

$$\text{Corrected assay fluorescence} = ((\text{Assay fluorescence} - \text{Enzyme control}) / \text{Quench coefficient}) - \text{Substrate control} \quad (2)$$

The quench coefficient accounts for any possible physical or chemical reduction in fluorescence of the released MUB within the reaction wells. It was calculated as

$$\text{Quench coefficient} = (\text{Quench control} - \text{Enzyme control}) / \text{Standard control} \quad (3)$$

We calibrated the quantity of MUB released in the enzyme-substrate reactions (i.e. the product accumulation) by dividing the corrected assay fluorescence by the emission coefficient, which was obtained as

$$\begin{aligned} \text{Emission coefficient (fluorescence nmol}^{-1}\text{)} \\ = \text{Standard control} / 0.5 \text{ nmol} \end{aligned} \quad (4)$$

with 0.5 nmol as the amount of MUB substance in the average standard control well. We plotted the evolution of product accumulation for each replicate assay as a function of time (Fig. 1). The slope of a linear (Model I) regression applied to the linear part of the time course yielded the enzyme activity, which was divided by the mass of enzyme present in a single well to obtain the specific enzyme activity in  $\text{nmol h}^{-1} \text{mg}^{-1} \text{enzyme}$ .

## 2.2. Enzyme-substrate assays for spectrophotometric measurements

Crystalline L-Dopa was weighed into deionized warm water (approximately 85 °C) to facilitate dissolution of the substrate (DeForest, 2009). We dispensed aliquots of peroxidase in 0.2 M sodium acetate buffer solution of pH 6.5. Ten  $\mu\text{l}$  of a 0.3%  $\text{H}_2\text{O}_2$  working solution, 50  $\mu\text{l}$  substrate and 200  $\mu\text{l}$  enzyme suspensions were pipetted into two columns of a clear, 96-well plate (Fisher Scientific, USA). Substrate control wells were filled with 50  $\mu\text{l}$  substrate and 200  $\mu\text{l}$  buffer solutions, enzyme controls with 200  $\mu\text{l}$

enzyme solution and 50  $\mu\text{l}$  buffer. Each control well received 10  $\mu\text{l}$  of the  $\text{H}_2\text{O}_2$  working solution as well. As for the hydrolytic assays, absorbance values of one replicate enzyme-substrate assay refer to the average absorbance measurements from the 16 single wells of one plate. Over all measurements, the average coefficient of variation of 16 assay wells was 0.014 (max. 0.10, min. 0.0002). Enzyme and substrate controls are the averages of 8 wells each.

The color change with time was measured with a Spectramax 340 PC 384 absorbance plate reader (Molecular Devices, USA) set to a predetermined optimal wavelength of 460 nm. Calculations were again carried out based on DeForest (2009) and German et al. (2011):

$$\begin{aligned} \text{Corrected assay absorbance} = \text{Assay absorbance} \\ - \text{Enzyme control} \\ - \text{Substrate control} \end{aligned} \quad (5)$$

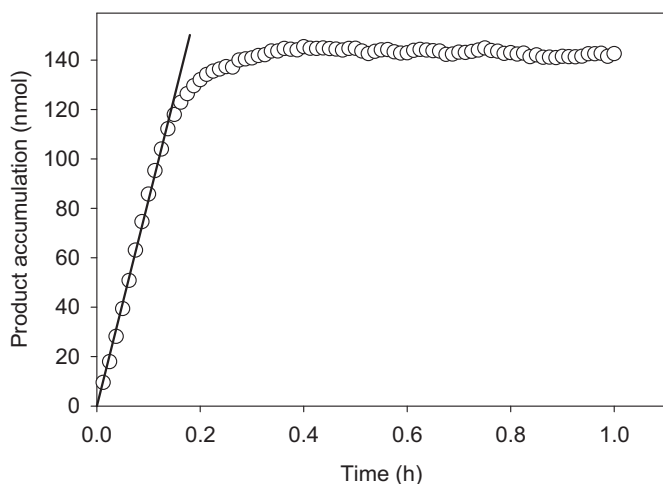
The product accumulation with time was quantified by dividing the corrected assay absorbance by a molar absorbance coefficient of  $10.3 \mu\text{mol}^{-1}$ , which was the slope of the linear relationship between peak absorbance vs. substrate concentration (data not shown). This value is well within the range of coefficients reported in comparable studies (DeForest, 2009; German et al., 2011; Donovan German, personal communication). As described above for fluorescence measurements, the specific enzyme activity in  $\mu\text{mol h}^{-1} \text{mg}^{-1} \text{peroxidase}$  was obtained via the slope of the product accumulation as a function of time (see Fig. 1).

## 2.3. Temperature control and general assay protocol

We conducted assays of BGase/BG at 7.5, 10, 15, 20 and 27 °C, and those of NAGase/NAG and Peroxidase/L-Dopa at 5, 10, 15, 20 and 25 °C. All objects used to set up an enzyme-substrate assay were brought to the desired assay temperatures in a temperature incubator located in the immediate vicinity of the plate readers. This included the substrate and enzyme suspensions, buffer and MUB, as well as the 96-well plates and pipette tips, and aimed to minimize departures from the nominal assay temperatures during handling. Temperature of all reactants was checked with a thermometer immediately before starting the pipetting sequence. Measurements at temperatures of 25 °C and 27 °C were straightforward. Immediately after the pipetting sequence, plates were put in the plate reader cuvettes, which were held at 25 °C or 27 °C, and kept there at least until the linear phase of product accumulation (Fig. 1) was projected. Such readings were taken every minute.

Because the plate readers are not capable of cooling the cuvettes below room temperature, we customized our protocol for measurements at assay temperatures of 20 °C and below. For enzyme-substrate assays with comparably slow reaction rates, i.e. where the linear phase of product accumulation (Fig. 1) lasted about one hour or longer, one individual plate was only measured every 10 min. Each measurement required 25 s in the plate reader, and the plate was kept in the incubator at the appropriate assay temperature for the remaining time. This procedure limited warming of the plate and reaction volumes in the reader cuvette during the assay. The time of measurements relative to the start of the assay was always recorded.

Some enzyme-substrate assays exhibited relatively high reaction rates, with linear phases of product accumulation as short as



**Fig. 1.** Example time course of product accumulation for one replicate enzyme-substrate assay (see Materials and methods section). The transition to the asymptotic phase indicates that all substrate was processed by the enzyme. The slope of a linear regression applied to the initial part of the time course, represented by the solid line, yields the enzyme activity per unit time.

10 min. For these assays at the lower temperatures, we set up several plates at the same time, each of which was subjected to one measurement of 25 s duration. One plate was measured immediately. The others were kept in the incubator and measured successively. These plates were all assayed using the same (fresh) suspensions, and were therefore treated as one replicate plate. In other words, one of those plates yielded one data point of a time course (Fig. 1), and all those plates constituted one replicate time course. The  $R^2$  for the linear regressions applied to the timespan corresponding to the initial product accumulation (Fig. 1) was always higher than 0.90, suggesting that the protocol customization was an effective means of assessing enzyme activity across time at the lower temperatures.

All substrate and enzyme suspensions were generated immediately prior to performing each assay. Reference MUB suspensions were renewed frequently according to recommendations given by DeForest (2009). Each assay followed a strict pipetting protocol: The smallest suspension volumes were pipetted first, and largest volumes (200  $\mu$ l) last. This resulted in repeatedly well-mixed reactants, which was not achieved by the plate shaking function of the plate readers alone. The optimized pipetting protocol was one key criterion to obtain a high reproducibility of the results. We also pre-determined plate reader wavelength optima for the highest sensitivity to fluorescence and spectrophotometric signals (Wariishi et al., 1990) and only used enzyme suspensions immediately after their set up such that observed reaction rates were not biased by age-related decreases in enzyme activity. Because we controlled for these assay features, the observed temperature sensitivities of the reactions appear to be actually related to changes in assay temperature, and not confounded by other factors.

#### 2.4. Calculating the C to N flow ratio of liberated resources

The liberation of MUB molecules from BG and NAG effectively simulates the BGase-mediated cleavage of glucose monomers ( $C_6H_{12}O_6$ ) from cellulose and the NAGase-mediated cleavage of NAG monomers ( $C_8H_{15}NO_6$ ) from chitin, respectively (see Lynd et al., 2002; Howard et al., 2003; Baldrian and Valášková, 2008). Thus, we can employ the effect of temperature on the specific activities of the purified BGase/BG and NAGase/NAG reactions to illustrate how temperature could alter the C to N ratio of the pool of monomers assimilable by soil microbes, using a theoretical concept recently proposed by Billings and Ballantyne (2012).

Assuming that all C and N contained within liberated monomers is assimilable by microbes and that decomposition reactions are neither substrate- nor enzyme-limited, the change in C liberation rates with temperature ( $T$ ) is given by

$$\frac{dC}{dT} = V_{\max\text{BGase}}(T) \left[ \frac{C}{\text{BG}} \right] + V_{\max\text{NAGase}}(T) \left[ \frac{C}{\text{NAG}} \right] \quad (6)$$

where  $V_{\max\text{BGase}}$  and  $V_{\max\text{NAGase}}$  depend only on temperature. The change in N liberation rates follows analogously. Substituting the C and N atomic contents of BG and NAG,  $[C/\text{BG}] = 6$ ,  $[N/\text{BG}] = 0$ ,  $[C/\text{NAG}] = 8$ , and  $[N/\text{NAG}] = 1$  into Equation (6) and the corresponding equation for N, yields a simple equation for the C to N flow ratio from the two substrates in question at specific temperatures (Billings and Ballantyne, 2012):

$$\frac{dC}{dN} = \frac{V_{\max\text{BGase}}(T)}{V_{\max\text{NAGase}}(T)} \cdot 6 + 8. \quad (7)$$

In contrast to the straightforward BGase/BG and NAGase/NAG reactions, the oxidation and structural transformation of L-Dopa decomposition via peroxidase represents only part of a series of reactions required to yield assimilable compounds (Skujins, 1978).

We therefore omitted results from the peroxidase/L-Dopa reactions from the assessment of temperature-induced changes on the C to N flow ratio.

### 3. Results

#### 3.1. Assessment of $V_{\max}$

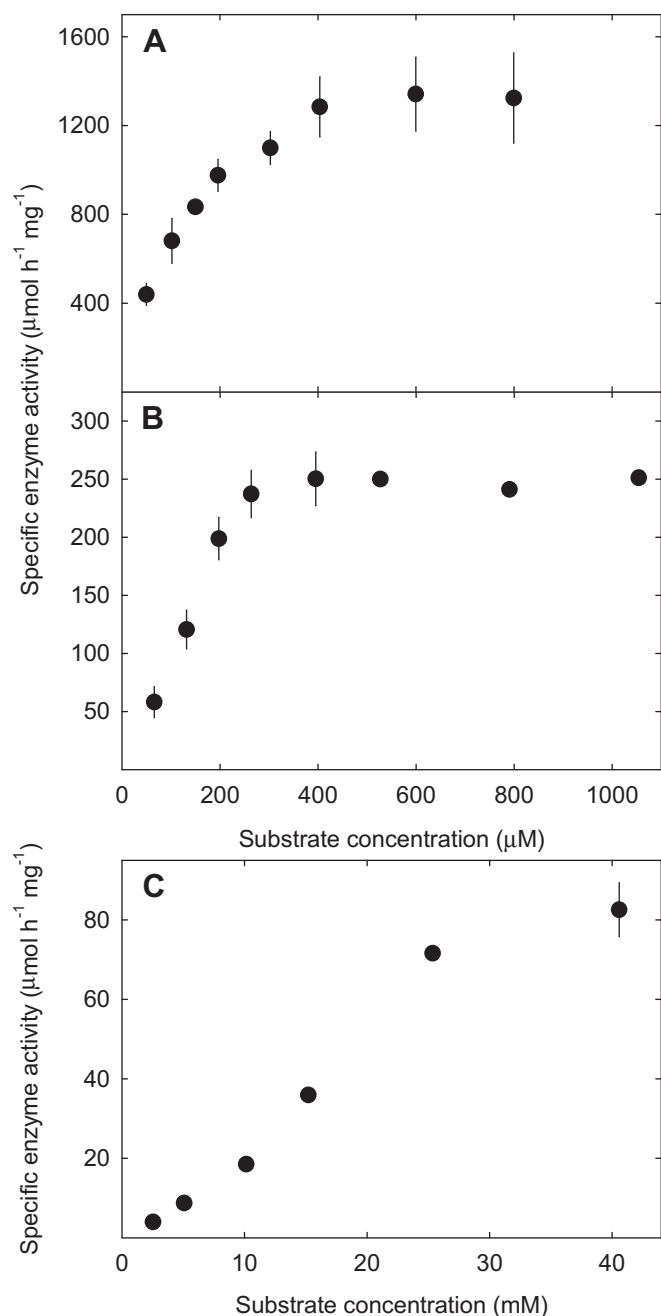
We first needed to ensure that any differences in enzyme-substrate reaction rates observed at different temperatures were not related to limitations in substrate or enzyme availabilities. As such, we determined the concentrations at which an additional increase in enzyme and substrate concentrations did not yield faster reaction rates, i.e. where  $V_{\max}$  of the reaction was reached. This was performed at reference temperatures of 27 °C for BGase/BG and at 25 °C for NAGase/NAG and Peroxidase/L-Dopa. BGase and NAGase activities responded to variations in substrate concentration in a manner similar to enzymes exhibiting Michaelis–Menten kinetics (Fig. 2A, B). The specific activities of both enzymes increased strongly with an increase in substrate concentration up to 300–400  $\mu$ M; higher substrate concentrations did not yield significantly higher specific enzyme activities. An exponential rise to the maximum, expressed as  $f(x) = a \cdot (1 - e^{-b \cdot x})$  exhibited better goodness of fit and distribution of residuals than did a Michaelis–Menten-type function. The fitted parameter  $a$  was therefore considered as the best estimate for the maximum specific activities (1338  $\mu\text{mol h}^{-1} \text{mg}_{\text{BGase}}^{-1}$  and 260  $\mu\text{mol h}^{-1} \text{mg}_{\text{NAGase}}^{-1}$ ). The concentrations of enzyme in one single plate well were 0.024 units BGase and 0.16 units NAGase, respectively. An increase in the concentrations did not yield higher  $V_{\max}$  (data not shown), indicating that neither substrate nor enzyme were limiting.

The evolution of the specific activity of peroxidase with increasing L-Dopa concentration (Fig. 2C) did not show such a pronounced asymptote as was observed for BGase and NAGase (Fig. 2A, B). Full solubility of L-Dopa during preparation of the substrate suspension, however, was only provided up to a concentration of 15 mM. At higher nominal concentrations, L-Dopa precipitated in the suspension, hindering an accurate enzyme activity assessment at substrate concentrations higher than 15 mM. As a result, we considered the specific activity of peroxidase at the substrate concentration of 15 mM as the most meaningful, achievable proxy of  $V_{\max}$ . The peroxidase concentration in one single plate well was four units; an increase of this concentration did not yield higher  $V_{\max}$ . Further, increasing the  $H_2O_2$  concentrations in the assays did not yield faster reaction rates, indicating that the oxidant was not limiting for the peroxidase/L-Dopa reactions observed.

#### 3.2. Effect of temperature on specific enzyme activities

First, we determined the substrate concentrations high enough to saturate enzymes and optimized other assay parameters for measuring  $V_{\max}$  at 25 °C (NAG and L-Dopa) and 27 °C (BG). We assumed that these concentrations also yielded  $V_{\max}$  at cooler temperatures and performed all subsequent assays at substrate concentrations of 600  $\mu$ M (BG), 1000  $\mu$ M (NAG) and 15 mM (L-Dopa). As expected from theory, the specific enzyme activities of all three pairings increased with higher temperature (Fig. 3). At each temperature at which the assays were performed, the specific activity of BGase was highest, followed by NAGase and Peroxidase. The two hydrolytic enzymes exhibited a different pattern of response to increasing temperature than did peroxidase. Peroxidase activity demonstrated a relatively sharp increase in activity between 15 °C and 20 °C, whereas increases in hydrolytic enzyme activities were more pronounced at lower temperatures. In the range from 7.5 °C to 25 °C, the specific activities of BGase, NAGase and

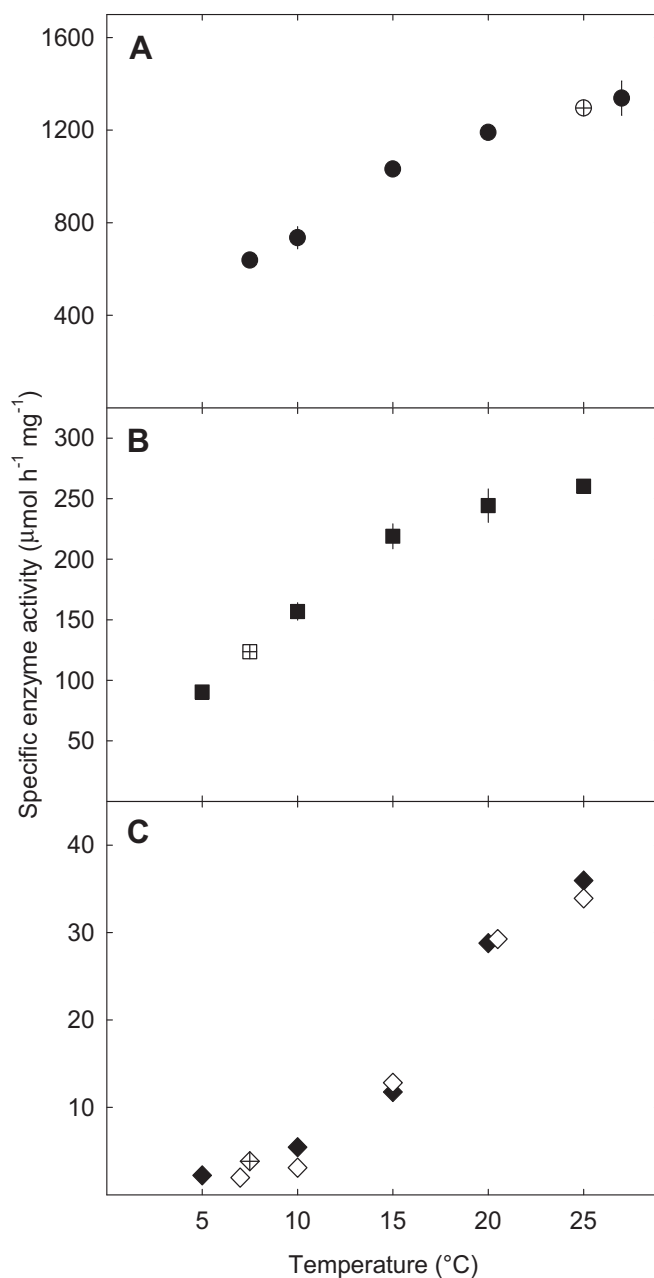




**Fig. 2.** Specific enzyme activity in  $\mu\text{mol}$  product accumulated per hour per mg of enzyme, as a function of substrate concentration for three enzyme-substrate pairings:  $\beta$ -Glucosidase and  $\beta$ -D-cellobioside (A);  $\beta$ -N-acetyl glucosaminidase and N-acetyl- $\beta$ -D-glucosaminide (B); and peroxidase and L-Dopa (C). Note the different unit on the X-axis for (C). Each data point is the mean of three to six replicate measurements. Error bars represent one standard error of the mean. See Fig. 1 legend and the Materials and methods section for the definition of a replicate.

Peroxidase increased by 103%, 111% and 835%, respectively, demonstrating the highest relative temperature sensitivity of the Peroxidase/L-Dopa reaction, and little difference in relative temperature sensitivities of the BGase/BG and NAGase/NAG reactions.

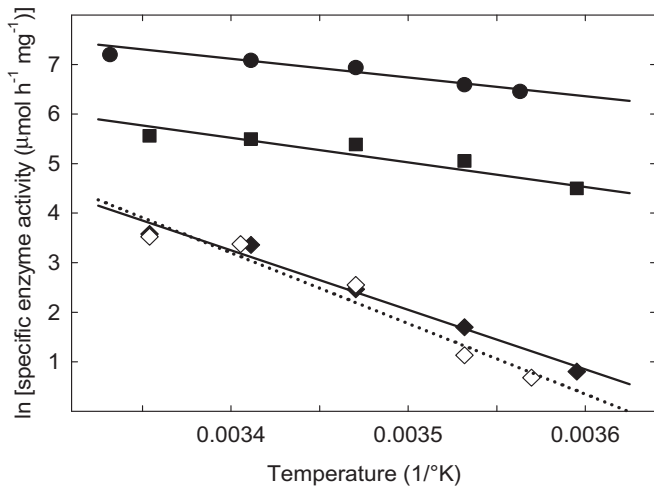
The variation in specific enzyme activity with temperature (Fig. 3) yields information about the activation energy required for a reaction to proceed. We estimated the activation energies for each reaction by transforming the data into Arrhenius plots by plotting the natural log of specific enzyme activities as a function of  $1/T$  (in



**Fig. 3.** Specific enzyme activity in  $\mu\text{mol}$  product accumulated per hour and mg of enzyme as a function of temperature of the enzyme-substrate pairings  $\beta$ -Glucosidase and  $\beta$ -D-cellobioside (A),  $\beta$ -N-acetyl glucosaminidase and N-acetyl- $\beta$ -D-glucosaminide (B) and peroxidase and L-Dopa (C). Data points are the means of at least three replicate measurements, error bars denote standard errors. The data points at 27 °C in (A) and 25 °C in (B) represent  $V_{\text{max}}$  at these temperature, that is, the parameter 'a' of the function  $y = a \cdot (1 - e^{-b \cdot x})$  fitted to the data of Fig. 2A, B, respectively. The open crossed symbols at 25 °C in (A) and 7.5 °C in (B) and (C) are obtained by linear interpolation between the two adjacent data points each (referring to the closed diamonds in (C)). Open diamonds in (C) denote results from peroxidase/L-Dopa assays where the enzyme concentration in the assays was reduced to one fourth of the enzyme concentration underlying the closed symbols, that is, to one unit per single plate well.

K; Fig. 4). The slopes of linear regressions served to obtain activation energy estimates of  $31.4 \text{ kJ mol}^{-1}$  BG for BGase ( $\pm 6.8$ ,  $Cl_{0.95}$ ;  $R^2 = 0.86$ ),  $41.3 \text{ kJ mol}^{-1}$  NAG for NAGase ( $\pm 9.5$ ,  $Cl_{0.95}$ ;  $R^2 = 0.86$ ) and  $99.8 \text{ kJ mol}^{-1}$  L-Dopa for Peroxidase ( $\pm 9.4$ ,  $Cl_{0.95}$ ;  $R^2 = 0.98$ ).

Due to the ambiguity in  $V_{\text{max}}$  assessment of peroxidase/L-Dopa, we conducted additional temperature assays with the same L-Dopa



**Fig. 4.** Arrhenius plot (natural logarithm of the specific enzyme activity vs.  $1/T$  (Temperature in K)) for three enzyme-substrate pairings:  $\beta$ -Glucosidase and  $\beta$ -D-cellobioside (circles),  $\beta$ -N-acetyl glucosaminidase and N-acetyl- $\beta$ -D-glucosaminide (squares) and Peroxidase and L-Dopa (diamonds). Data points are the means of at least three replicate measurements. The data points at 0.00333 for  $\beta$ -Glucosidase and  $\beta$ -D-cellobioside and 0.00335 for  $\beta$ -N-acetyl glucosaminidase and N-acetyl- $\beta$ -D-glucosaminide represent  $V_{\max}$  at these temperatures (at 27 °C and 25 °C, respectively), that is, the parameter 'a' of the function  $y = a \cdot (1 - e^{-b \cdot x})$  fitted to the data of Fig. 2A, B, respectively. The slopes of linear regression to the data sets (solid lines) served to calculate the activation energies of the reactions. The open diamonds denote results from peroxidase/L-Dopa assays where the enzyme concentration in the assays was reduced to one fourth, that is, to one unit per single plate well; the dotted line is the corresponding linear regression.

concentration (15 mM), but with only one fourth the peroxidase concentration in the plate wells (i.e. one unit peroxidase per well) as compared to the assays described above. The temperature response of these assays exhibited a pattern very similar to those with a peroxidase concentration of four units per well (Fig. 3);  $E_a$  assessed for this reaction was  $118.5 \text{ kJ mol}^{-1}$  ( $\pm 16.8$ ,  $CI_{0.95}$ ), close to  $E_a$  assessed for the reaction at high peroxidase concentration (Fig. 4). These findings suggest that the differences in temperature sensitivity of the peroxidase/L-Dopa reaction with the two hydrolytic reactions described above are meaningful, in spite of the difficulties with generating a higher concentration of L-Dopa suspension.

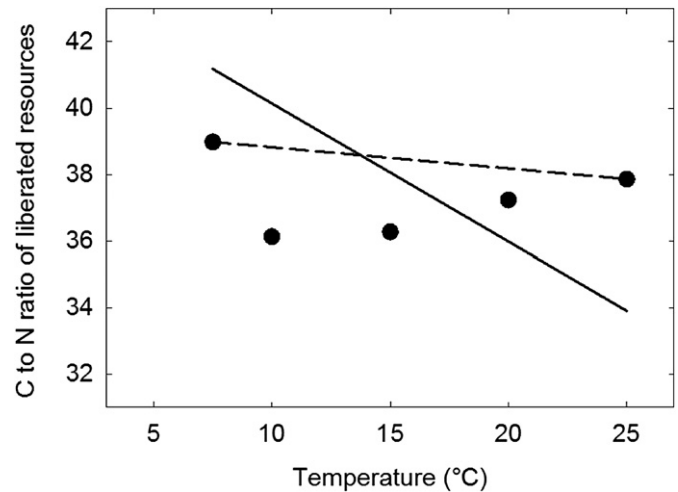
### 3.3. Effect of temperature on the C to N flow ratio of liberated resources

The observed responses of specific BGase and NAGase activities to temperature allow estimating the C to N flow ratios (Eq. (7)) in three different ways.

In the first approach, we calculate  $V_{\max \text{BGase}}$  and  $V_{\max \text{NAGase}}$  via Eq. (1) which employs  $E_a$  of  $31,400 \text{ J mol}^{-1}$  and  $41,300 \text{ J mol}^{-1}$ . The pre-exponential factors (A) are  $e^{19.98}$  and  $e^{22.48} \mu\text{mol mg}^{-1} \text{ h}^{-1}$  for BGase/BG and NAGase/NAG, respectively, derived from the Y-intercepts of the Arrhenius plots (Fig. 4). We obtain  $V_{\max \text{BGase}}(T) = e^{19.98} e^{-31,400/RT}$  and  $V_{\max \text{NAGase}}(T) = e^{22.48} e^{-41,300/RT}$ . Substituting these expressions and  $R = 8.315 \text{ J mol}^{-1} \text{ K}^{-1}$ , Equation (7) reduces to  $dC/dN = (0.08e^{1191/T}) \cdot 6 + 8$ , which is a decreasing function of temperature. At 7.5 °C, the C to N flow ratio is 41, and at 25 °C it is 34 (Fig. 5).

The second approach only invokes the specific enzyme activities from the extremes of the experimental temperature range (Fig. 3A, B) to compute  $V_{\max \text{BGase}}/V_{\max \text{NAGase}}$  at 7.5 °C and 25 °C. This approach yields a smaller decline in the C to N flow ratio, from 39 at 7.5 °C to 38 at 25 °C (Fig. 5).

Similar to the second approach, the third approach also uses specific enzyme activities, but those at all individual temperatures



**Fig. 5.** Atomic C to N flow ratio of the pool of monomers (glucose and N-acetyl- $\beta$ -D-glucosaminide) liberated during the enzymatic breakdown of MUB-labeled  $\beta$ -D-cellobioside by  $\beta$ -Glucosidase and MUB-labeled N-acetyl- $\beta$ -D-glucosaminide by  $\beta$ -N-acetyl glucosaminidase, derived from the specific enzyme activities at the respective temperatures (Fig. 3A, B). The atomic flow ratio was estimated via (1) the  $E_a$  of the reactions (solid line), (2) the specific enzyme activities at 7.5 °C and 25 °C (dashed line), or (3) using specific enzyme activities at 7.5, 10, 15, 20 and 25 °C (black dots).

to compute changes in the C to N flow ratio over each, smaller temperature range. Using this approach, the C to N flow ratios at the temperature extremes are identical as for the second approach, but changes in the C to N flow ratio between intermediate temperature points are not consistent with the monotonic predictions of the first two approaches: after an initial decline between 7.5 °C and 10 °C, the C to N flow ratio increases within each interval, to varying degrees (Fig. 5).

## 4. Discussion

This work provides quantitative estimates for the intrinsic temperature sensitivities of three exoenzyme-substrate reactions of global relevance for SOM decay, soil feedbacks to climate, and microbial ecology (Kögel-Knabner, 2002; Lynd et al., 2002; Howard et al., 2003; Sinsabaugh, 2010). Because the reactions proceeded in well-mixed media with purified reactants and were free from the influence of microbial activities, our estimates of temperature sensitivities of substrate decay are as close to intrinsic values as is feasible to compute. As such, they provide novel insights into the influence and the consequences of climate warming on the rates of SOM decomposition in two key ways. First, they contribute empirical data to the enduring discussion about projected temperature sensitivities of SOM pools with different turnover rates and functional attributes (Davidson and Janssens, 2006; Trumbore, 2009; Sierra, 2012). Second, the estimates of intrinsic temperature sensitivities also permit us to assess biochemically-induced changes in flows of C and N liberated from SOM with soil warming in the absence of potential temperature-induced changes in microbial resource demands, acquisition, or use.

### 4.1. Temperature response of single enzyme-substrate reactions

The relative increases in substrate decay with temperature were consistent with the Van't Hoff–Arrhenius law: all three reaction rates increased across the entire temperature range, and they showed varying degrees of response. Higher  $V_{\max}$  values were associated with lower relative temperature sensitivity of the reaction and lower  $E_a$  (Figs. 2–4). The activation energy required for the

NAGase/NAG reaction to proceed was only slightly higher than for BGase/BG (+32%); this agrees with expectations because both enzymes cleave a  $\beta$ -glycosidic bond where the MUB labels are attached to the substrates. In contrast, the peroxidase-mediated oxidation of L-Dopa required approximately three times the activation energy of the hydrolysis of BG and NAG, and the specific activity of peroxidase increased by a factor of nine between 7.5 °C and 25 °C. Thus, the relative increase of the peroxidase/L-Dopa reaction rate with warming was much higher than the relative rate increases of BGase/BG and NAGase/NAG, for which specific activities approximately doubled in the same temperature range (Fig. 3). These findings confirm at a fundamental, biochemical level what some phenomenological studies suggest – that a slower-turnover substrate, which requires a higher  $E_a$  for its decay, experiences a greater percentage increase in decomposition rate with warming than do substrates with a comparably faster turnover in soils (Craine et al., 2010; Wetterstedt et al., 2010; see Conant et al., 2011 for a recent review).

Increasing specific enzyme activities with warming imply a higher efficiency of microbial resource investment in exoenzyme production. One mg of enzyme can process more substrate per unit time in a warmer environment. In other words, soil microorganisms may need to produce fewer enzymes to process the same amount of substrate in the same time. That the relative temperature response for the L-Dopa-peroxidase pairing was highest suggests that microorganisms adept at decomposing lignin (such as *Phanerochaete chrysosporium* or *Coriolus versicolor*; Killham, 1994) would benefit comparably more from a warmer environment than microbes relying on cellulose as their C source. Species-specific changes in resource use efficiency resulting from different responses of decay reactions to temperature might be a factor influencing observed changes in microbial community composition with soil warming (Waldrop and Firestone, 2004; Biasi et al., 2005).

However, we must consider the changes in absolute flux rates per mg of enzyme to assess how temperature sensitivities may influence cellulose and lignin pool sizes with warming. The 103% increase in the decay of BG from 7.5 °C to 25 °C corresponds to an absolute increase of 660  $\mu\text{mol h}^{-1} \text{mg}_{\text{BGase}}^{-1}$  (Fig. 3). This is twenty times higher than the temperature-induced changes for the peroxidase-mediated reaction: the 835% relative increase amounts to an absolute increase of only 32  $\mu\text{mol h}^{-1} \text{mg}_{\text{peroxidase}}^{-1}$  observed across the same temperature range. For a microbial community with unlimited access to cellulose and lignin, these findings suggest that more than twenty times the amount of peroxidase would be required to outweigh the temperature-enhanced decomposition rate of cellulose by BGase. These empirical observations highlight the need to consider absolute temperature sensitivities of SOM decomposition (Sierra, 2012) and the absolute quantities of resources available (Billings and Ballantyne, 2012) when predicting the feedback potential of a soil microbial community's respiration flux to climate change.

#### 4.2. Temperature-induced changes in the C to N flow ratio of liberated resources

All three approaches used to assess the effects of temperature on the flow ratio of C and N liberated from BG and NAG, and thus on the relative availabilities of C and N for microbial assimilation, are meritorious. However, each approach yields a slightly different prediction (Fig. 5). The first approach, akin to that frequently invoked by biogeochemists, assumes that one constant  $E_a$  value is applicable across the entire experimental temperature range. The second approach, perhaps an obvious step to take in SOM incubation studies at two different temperatures, employs the unadulterated, empirical observations at 7.5 °C and 25 °C. Because both of

these approaches assume monotonic changes in the  $V_{\text{maxBGase}}$  to  $V_{\text{maxNAGase}}$  ratio with temperature, they both result in monotonic declines in the C to N flow ratio with warming. The third approach is essentially the same as the second, but uses specific enzyme activities at all individual temperatures assayed to compute changes in the C to N flow ratio over smaller temperature ranges. It therefore requires no assumption about how the ratio of  $V_{\text{maxBGase}}$  to  $V_{\text{maxNAGase}}$  changes over the entire temperature range.

Theoretically, all three approaches should generate declining C to N flow ratios with warming, given the higher  $E_a$  of the NAG-NAGase pairing and its C and N content relative to BG (Billings and Ballantyne, 2012). However, this exercise highlights that C to N flow ratio predictions are sensitive to the type of data collected and the data variability over the selected temperature range. The third approach, yielding non-monotonic changes in the C to N flow ratio, deviates from Van't Hoff–Arrhenius expectations because the differences in intrinsic temperature sensitivities between the BGase/BG and the NAGase/NAG reactions, and thus the differences in  $E_a$ , are small relative to unavoidable measurement variability associated with extracellular enzyme assays (Fig. 3). This feature can challenge attempts to detect meaningful differences in the C to N flow ratios within shorter temperature intervals, for enzymatic reactions with similar kinetics.

The decline in the C to N flow ratio observed between 7.5 °C and 25 °C suggests that warming can lower the C to N ratio of the resources made available for microbial assimilation from exoenzymatic cellulose and chitin degradation. These results, obtained from purified reactions, may help illuminate what might be driving observed reductions in soil respiratory responses to warming over time (Peterjohn et al., 1994; Oechel et al., 2000; Luo et al., 2001; Rustad et al., 2001; Melillo et al., 2002; Eliasson et al., 2005), or changes in relative soil C and N mineralization rates with temperature (Koch et al., 2007; Wallenstein et al., 2009). Such changes may induce shifts in stoichiometry of the microbial biomass or may prompt microbes to change their exoenzyme production to target resources better able to match its extant stoichiometry (Billings and Ballantyne, 2012). This latter trajectory seems particularly feasible when assuming a homeostatic regulation of microbial C to N ratios (Goldman et al., 1987). However, we do not know enough about how either response will influence microbial C acquisition and allocation to predict microbial growth and respiratory responses to changing C to N flow ratios.

## 5. Conclusions

The reductionist approach employed here, in strictly controlled experimental conditions, allows estimation of the intrinsic temperature sensitivity of decay and associated  $E_a$  for enzyme-substrate pairings relevant for global decomposition of SOM. The reactions of substrates representative of cellulose, chitin and lignin with exoenzymes known to be produced by soil microbes exhibited contrasting rates and distinct responses to temperature, consistent with knowledge of these substrates' molecular complexity. Estimates of intrinsic temperature sensitivities in the absence of microorganisms provide an important and until now missing baseline for comparison with the apparent temperature sensitivities of extracellular enzymes in studies of real soils.

The estimates of intrinsic temperature sensitivity further permitted quantification of how pure biochemical responses to temperature, and any relationship between substrate  $E_a$  and C to N, can influence the relative flows of C and N liberated during decay. We have demonstrated how the magnitude of the changes in the C to N flow ratio is sensitive to the type of data used for these calculations – specific enzyme activities or  $E_a$ . Further, the direction of change in the C to N flow ratio can be sensitive to the

temperature range over which these data are obtained. Temperature-mediated changes in C to N flow ratio provides one of multiple, potential mechanisms that could influence microbial adaptation to warming (Bradford et al., 2010; Treseder et al., 2012; Billings and Ballantyne, 2012). Calculating changes in the C to N flow ratio with temperature will permit the inclusion of more realistic microbial responses into models of soil system feedbacks to climate.

## Acknowledgments

We are indebted to Ashleigh Price and Anuradha Roy for providing access to the plate readers at the High Throughput Screening Lab at the University of Kansas. Ryan Felton, Chao Song, Christopher Asmus and Donovan German are thanked for valuable discussions. Two anonymous reviewers are thanked for helpful comments.

## References

- Baldrian, P., Valášková, V., 2008. Degradation of cellulose by basidiomycetous fungi. *FEMS Microbiology Reviews* 32, 501–521.
- Biasi, C., Rusalimova, O., Meyer, H., Kaiser, C., Wanek, W., Barsukov, P., Junger, H., Richter, A., 2005. Temperature-dependent shift from labile to recalcitrant carbon sources from arctic heterotrophs. *Rapid Communications in Mass Spectrometry* 19, 1401–1408.
- Billings, S.A., Ballantyne IV, F., 2012. 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*. <http://dx.doi.org/10.1111/gcb.12029>.
- Bosatta, E., Ågren, G.I., 1999. Soil organic matter quality interpreted thermodynamically. *Soil Biology & Biochemistry* 31, 1889–1891.
- Bradford, M.A., Davies, C.A., Frey, S.D., Maddox, T.R., Melillo, J.M., Mohan, J.E., Reynolds, J.F., Treseder, K.K., Wallenstein, M.D., 2008. Thermal adaptation of soil microbial respiration to elevated temperature. *Ecology Letters* 11, 1316–1327.
- Bradford, M.A., Watts, B.W., Davies, C.A., 2010. Thermal adaptation of heterotrophic soil respiration in laboratory microcosms. *Global Change Biology* 16, 1576–1588.
- 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.A.M., Bradford, M.A., 2011. Temperature and soil organic matter decomposition rates – synthesis of current knowledge and a way forward. *Global Change Biology* 17, 3392–3404.
- Conrad, R., 1996. Soil microorganisms as controllers of atmospheric trace gases (H<sub>2</sub>, CO, CH<sub>4</sub>, OCS, N<sub>2</sub>O and NO). *Microbiological Reviews* 60, 609–640.
- Craine, J.M., Fierer, N., McLaughlan, K.K., 2010. Widespread coupling between the rate and temperature sensitivity of organic matter decay. *Nature Geoscience* 3, 854–857.
- 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., Samanta, S., Caramori, S.S., Savage, K., 2012. The dual Arrhenius and Michaelis–Menten kinetics model for decomposition of soil organic matter at hourly to seasonal time scales. *Global Change Biology* 18, 371–384.
- 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.
- Dioumaeva, I., Trumbore, S., Schuur, E.A.G., Goulden, M.L., Litvak, M., Hirsch, A.I., 2002. Decomposition of peat from upland boreal forest: temperature dependence and sources of respired carbon. *Journal of Geophysical Research* 108, 8222. <http://dx.doi.org/10.1029/2001JD000848>.
- Eliasson, P.E., McMurtrie, R.E., Pepper, D.A., Strömberg, M., Linder, S., Ågren, G.I., 2005. The response of heterotrophic CO<sub>2</sub> flux to warming. *Global Change Biology* 11, 167–181.
- Fang, C., Smith, P., Moncrieff, J.B., Smith, J.U., 2005. Similar response of labile and resistant soil organic matter pools to changes in temperature. *Nature* 433, 57–59.
- German, D.P., Weintraub, M.N., Grandy, A.S., Lauber, C.L., Rinkes, Z.L., Allison, S.D., 2011. Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. *Soil Biology & Biochemistry* 43, 1387–1397.
- German, D.P., Marcelo, K.R.B., 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.
- Giardina, C.P., Ryan, M.G., 2000. Evidence that decomposition rates of organic carbon in mineral soil do not vary with temperature. *Nature* 404, 858–861.
- Goldman, J.C., Caron, D.A., Dennett, M.R., 1987. Regulation of gross growth efficiency and ammonium regeneration in bacteria by substrate C:N ratio. *Limnology and Oceanography* 32, 1239–1252.
- Hartley, I.P., Hopkins, D.W., Garnett, M.H., Sommerkorn, M., Wookey, P.A., 2008. Soil microbial respiration in arctic soil does not acclimate to temperature. *Ecology Letters* 11, 1092–1100.
- Henry, H.A.L., 2012. Soil extracellular enzyme dynamics in a changing climate. *Soil Biology & Biochemistry* 47, 53–59.
- Howard, M.B., Ekborg, N.A., Weiner, R.M., Hutcheson, S.W., 2003. Detection and characterization of chitinases and other chitin-modifying enzymes. *Journal of Industrial Microbiology & Biotechnology* 30, 627–635.
- Killham, K., 1994. *Soil Ecology*. University Press, Cambridge.
- Kleber, M., 2010. What is recalcitrant soil organic matter? *Environmental Chemistry* 7, 320–332.
- Kleber, M., Johnson, M.G., 2010. Advances in understanding the molecular structure of soil organic matter: implications for interactions in the environment. *Advances in Agronomy* 106, 77–142.
- Koch, O., Tschirko, 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/10.1029/2007GB002983>.
- Kögel-Knabner, I., 2002. The macromolecular organic composition of plant and microbial residues as inputs to soil organic matter. *Soil Biology & Biochemistry* 34, 139–162.
- Laidler, K.J., 1984. The development of the Arrhenius equation. *Journal of Chemical Education* 61, 494–498.
- Lavelle, P., Blanchart, E., Martin, A., Martin, S., Spain, A., Toutain, F., Barois, I., Schaefer, R., 1993. A hierarchical model for decomposition in terrestrial ecosystems: application to soils of the humid tropics. *Biotropica* 25, 130–150.
- Leifeld, J., Fuhrer, J., 2005. The temperature response of CO<sub>2</sub> production from bulk soils and soil fractions is related to soil organic matter quality. *Biogeochemistry* 75, 433–453.
- Luo, Y., Wan, S., Hui, D., Wallace, L.L., 2001. Acclimatization of soil respiration to warming in a tall grass prairie. *Nature* 413, 622–625.
- Lynd, L.R., Weimer, P.J., van Zyl, W.H., Pretorius, I.S., 2002. Microbial cellulose utilization: fundamentals and biotechnology. *Microbiology and Molecular Biology Reviews* 66, 506–577.
- Mason, H.S., 1948. The chemistry of melanin. III. Mechanism of the oxidation of dihydroxyphenylalanine by tyrosinase. *The Journal of Biological Chemistry* 172, 83–99.
- McGuire, K.L., Treseder, K.K., 2010. Microbial communities and their relevance for ecosystem models: decomposition as a case study. *Soil Biology & Biochemistry* 42, 529–535.
- Mead, J.A.R., Smith, J.N., Williams, R.T., 1955. Studies in detoxication. The biosynthesis of the glucuronides of umbelliferone and 4-methylumbelliferone and their use in fluorometric determination of  $\beta$ -glucuronidase. *Biochemical Journal* 61, 569–574.
- Melillo, J.M., Steudler, P.A., Aber, J.D., Newkirk, K., Lux, H., Bowles, F.P., Catricala, C., Magill, A., Ahrens, T., Morrisseau, S., 2002. Soil warming and carbon-cycle feedbacks to the climate system. *Science* 298, 2173–2176.
- Nappi, A.J., Vass, E., 2001. The effects of nitric oxide on the oxidations of L-Dopa and dopamine mediated by tyrosinase and peroxidase. *The Journal of Biological Chemistry* 276, 11214–11222.
- Nannipieri, P., Paul, E., 2009. The chemical and functional characterization of soil N and its biotic components. *Soil Biology & Biochemistry* 41, 2357–2369.
- Oechel, W.C., Vourlitis, G.L., Hastings, S.J., Zulueta, R.C., Hinzman, L., Kane, D., 2000. Acclimation of ecosystem CO<sub>2</sub> exchange in the Alaskan Arctic in response to decadal climate warming. *Nature* 406, 978–981.
- Peterjohn, W.T., Melillo, J.M., Steudler, P.A., Newkirk, K.M., Bowles, F.P., Aber, J.D., 1994. Responses of trace gas fluxes and N availability to experimentally elevated soil temperatures. *Ecological Applications* 4, 617–625.
- Pritchard, S.G., 2011. Soil organisms and global climate change. *Plant Pathology* 60, 82–99.
- Puiu, M., Babaligea, I., Olmazu, C., Răducan, A., Oancea, D., 2010. Peroxidase-mediated oxidation of L-Dopa: a kinetic approach. *Biochemical Engineering Journal* 52, 248–254.
- Rillig, M.C., Caldwell, B.A., Wösten, H.A.B., Sollins, P., 2007. Role of proteins in soil carbon and nitrogen storage: controls on persistence. *Biogeochemistry* 85, 25–44.
- Rustad, L.D., Campbell, J.L., Marion, G.M., Norby, R.J., Mitchell, M.J., Hartley, A.E., Cornelissen, J.H.C., Gurevitch, J., 2001. A meta-analysis of the response of soil respiration, net nitrogen mineralization, and above ground plant growth to experimental ecosystem warming. *Oecologia* 126, 543–562.
- Sierra, C.A., 2012. Temperature sensitivity of organic matter decomposition in the Arrhenius equation: some theoretical considerations. *Biogeochemistry* 108, 1–15.
- Singh, B.K., Bardgett, R.D., Smith, P., Reay, D.S., 2010. Microorganisms and climate change: terrestrial feedbacks and mitigation options. *Nature Reviews Microbiology* 8, 779–790.
- Sinsabaugh, R.L., Moorhead, D.L., 1994. Resource allocation to extracellular enzyme production: a model for nitrogen and phosphorus control of litter decomposition. *Soil Biology & Biochemistry* 26, 1305–1311.
- Sinsabaugh, R.L., 2010. Phenol oxidase, peroxidase and organic matter dynamics of soil. *Soil Biology & Biochemistry* 42, 391–404.
- Skujins, J., 1978. History of abiotic soil enzyme research. In: Burns, R.G. (Ed.), *Soil Enzymes*. Academic Press, New York, pp. 1–49.
- Smith, P., Fang, C., Dawson, J.J.C., Moncrieff, J.B., 2008. Impact of global warming on soil organic carbon. *Advances in Agronomy* 97, 1–43.
- Subke, J.A., Bahn, M., 2010. On the 'temperature sensitivity' of soil respiration: can we use the immeasurable to predict the unknown? *Soil Biology & Biochemistry* 42, 1653–1656.
- Todd-Brown, K.E.O., Hopkins, F.M., Kivlin, S.N., Talbot, J.M., Allison, S.D., 2012. A framework for representing microbial decomposition in coupled climate models. *Biogeochemistry* 109, 19–33.



- Treseder, K.K., Balser, T.C., Bradford, M.A., Brodie, E.L., Dubinsky, E.A., Eviner, V.T., Hofmockel, K.S., Lennon, J.T., Levine, U.Y., MacGregor, B.J., Pett-Ridge, J., Waldrop, M.P., 2012. Integrating microbial ecology into ecosystem models: challenges and priorities. *Biogeochemistry* 109, 7–18.
- Trumbore, S., 2009. Radiocarbon and soil carbon dynamics. *Annual Review of Earth and Planetary Sciences* 37, 47–66.
- Von Lützw, M., Kögel-Knabner, I., Ekschmitt, K., Matzner, E., Guggenberger, G., Marschner, B., Flessa, H., 2006. Stabilization of organic matter in temperate soils: mechanisms and their relevance under different soil conditions – a review. *European Journal of Soil Science* 57, 426–445.
- Von Lützw, M., Kögel-Knabner, I., 2009. Temperature sensitivity of soil organic matter decomposition – what do we know? *Biology and Fertility of Soils* 46, 1–15.
- Waldrop, M.P., Firestone, M.K., 2004. Altered utilization patterns of young and old soil C by microorganisms caused by temperature shifts and N additions. *Biogeochemistry* 67, 235–248.
- 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, 1632–1639.
- Wariishi, H., Marquez, L., Dunford, H.B., Gold, M.H., 1990. Lignin peroxidase compounds II and III. Spectral and kinetic characterization of reactions with peroxides. *The Journal of Biological Chemistry* 265, 11137–11142.
- Wetterstedt, J.Å.M., Persson, T., Ågren, G.I., 2010. Temperature sensitivity and substrate quality in soil organic matter decomposition: results of an incubation study with three substrates. *Global Change Biology* 16, 1806–1819.