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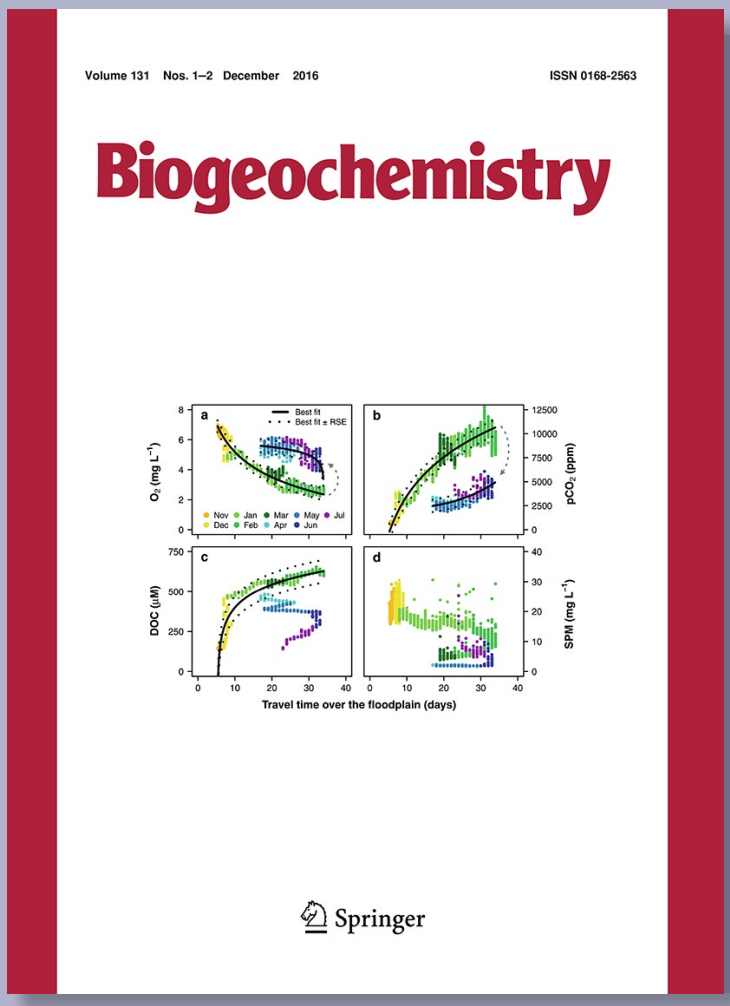

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Aging exo-enzymes can create temporally shifting, temperature-dependent resource landscapes for microbes

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Abstract The rate at which catalytic capacity of microbial exo-enzymes degrades post-exudation will influence the time during which return on microbes' investment in exo-enzyme production can be realized. Further, if exo-enzyme degradation rates vary across exo-enzymes, microbial investment returns may vary by element across time. We quantify how aging of two soil organic matter (SOM)-decaying enzymes (β-D-cellobioside, BGase; and N-acetyl-β-D-glucosaminide, NAGase) influences enzyme-substrate V_{max} at multiple temperatures (5, 15, 25 °C), and compute how enzyme age influences relative availabilities of C and N. Both BGase and NAGase exhibited similar, exponential declines in catalytic rate with age at 25 °C (0.22 ± 0.02 and 0.36 ± 0.14 d^{-1}, respectively). At 15 °C, NAGase exhibited exponential declines in catalytic rates with age (0.79 ± 0.31 d^{-1}), but BGase exhibited no decline. Neither enzyme exhibited a decline in catalytic rate over 72 h at 5 °C. At 15 °C, the amount of C liberated from cellulose and chitin analogues relative to N increased, on average, by more than one order of magnitude. The ratio of C:N liberated from the two substrates remained constant across enzyme age at 25 and 5 °C, but for different reasons: no differences in decay rate across enzymes at 25 °C, and no observed decay at 5 °C. Thus, temperature-dependent decreases of catalytic activity over time may influence microbial resource allocation strategies and rates of SOM decomposition. Because the enzyme decay rates we observed differ considerably from values assumed in most models, such assumptions should be revisited when parameterizing microbial process models.

Keywords Exo-enzymes · Soil organic matter decay · Enzyme kinetics · Microbial process models

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Introduction

Ecosystem scientists often use biogeochemical measurements to infer soil organic matter (SOM) transformations (e.g. Fissore et al. 2008; Craine et al. 2010; Li et al. 2012; Frey et al. 2013; Zhang et al. 2015; Razavi et al. 2016), which are important because they promote ecosystem productivity and can result in feedbacks to climate. However, empirical biogeochemical measurements typically integrate many simultaneous and sometimes competing processes (Subke and Bahn 2010). As such, it is difficult to definitively implicate particular processes as underlying causes for observed responses to environmental change, and their associated rates. For example,

apparent temperature responses of SOM decay in soils assessed via exo-enzymatic activities, one type of biogeochemical measurement (e.g. Wallenstein et al. 2009), may not accurately reflect in situ activities and rates of decomposition because conditions during exo-enzyme assays as typically conducted differ substantially from edaphic conditions (Wallenstein and Weintraub 2008). The approach thus only permits comparisons of relative activity rates across soils or environmental conditions. From such measurements, it is challenging to infer underlying, in situ rates of enzyme activity and SOM decay.

Baseline measures of key soil processes such as intrinsic SOM decay rates (the maximum reaction rate for a given environmental condition; Lehmeier et al. 2013; Min et al. 2014) are useful because they permit parsing of maximum, fundamental process rates from phenomena like the availability of enzymes and microbial adaptation that also drive apparent process rates. These baseline rates are the most useful starting point for parameterizing detailed process models (Schimel and Weintraub 2003; Allison 2012; Moorhead et al. 2012; Wieder et al. 2014; Manzoni et al. 2014, 2016; Sierra et al. 2015), because such models explicitly formulate dynamics based on intrinsic rates of multiple processes. The aggregate of these processes of interest ultimately determine apparent or realized rates. Baseline, intrinsic rates of SOM decay also can be used to compute how environmental conditions such as temperature and pH regime, *sans* microbes, influence the landscape of resources potentially assimilable by microbes. The C:N flow ratio, a concept that reveals the ratio of assimilable C and N as particular compounds undergo decay (Billings and Ballantyne 2013; Bárta et al. 2014), is particularly useful when invoked for abiotic, biochemical reactions (Lehmeier et al. 2013; Min et al. 2014). Parsing the response of SOM decay to a changing environment in isolation from microbial communities can provide us with baseline measures of how the relative availability of assimilable resources can change solely due to a shift in environmental conditions, even without changes in microbial form or function (Billings et al. 2015). Baseline data is critical for parameterizing models that explicitly include exo-enzyme pools and their dynamics.

Though intriguing, work quantifying intrinsic rates and temperature responses of SOM decay *sans* microbes has not yet resolved how exo-enzyme age influences catalytic rates of distinct enzymes important for the release of C- vs. N-liberation from SOM. This is important because the rate of catalytic decline sets the timescale of turnover for exo-enzyme pools in microbial process models, and influences enzyme pool size (Schimel and Weintraub 2003; Manzoni et al. 2014, 2016; Allison 2006). Furthermore, the rate at which catalytic capacity of exo-enzymes degrades, post-exudation, will influence the time during which potential return on a microbe's investment in exo-enzyme production can be realized. If declines in exo-enzyme catalytic rate vary with temperature, microorganisms may need to modify rates of exo-enzyme production with temperature fluctuations to maintain balanced resource acquisition. Intriguingly, if exo-enzyme degradation rates vary across exo-enzymes, microbial investment returns may vary by element across time. Variation in some exo-enzymes' abilities to attain V_{max} across diverse temperature and pH regimes (Min et al. 2014) suggests that rates of decline over time in exo-enzyme catalysis indeed may vary across enzymes. Given that some exo-enzymes continue to catalyze decay reactions well past exudation (Skujins 1978; Boschker et al. 1995; Steen and Arnosti 2011) and declines in enzyme catalytic rates over time (Asuri et al. 2007; Yan et al. 2010; Kishore et al. 2012; Kedi et al. 2013a; Goyal et al. 2014; Tsai and Meyer 2014, Park et al. 2015; Shirke et al. 2015), we might expect that declines in exo-enzyme catalytic rates as they age may be an important factor influencing resource acquisition of soil microbial communities.

Modelers recognize the importance of formally describing how microbial resource allocation to exo-enzyme production can benefit microbial functioning (Schimel and Weintraub 2003; Allison 2012; Manzoni et al. 2014, 2016; Wieder et al. 2014). However, we lack the knowledge needed to inform models about diverse enzymes' catalytic rate declines over time. Models that consider exo-enzyme catalytic decline prescribe values derived from specific soil types that likely are not applicable to all soils (e.g. Allison 2006), or consider catalytic decline to occur at similar rates regardless of enzyme identity (Schimel and Weintraub 2003; Allison 2012; Manzoni et al. 2014, 2016). However, if the catalytic rates of enzymes important for the release of C- vs. N-rich monomers decline at different rates, resource return

on investment in enzymes may change over time in ways important for microbial functioning and, indeed, may change differently depending on temperature and pH (Lehmeier et al. 2013; Min et al. 2014). We cannot know how to incorporate declines over time in exo-enzyme catalytic rates in diverse environments into models, however, without empirical quantification of decay rates of biogeochemically-relevant enzymes in controlled environments.

Here, we take a first step toward addressing soil enzyme longevity by quantifying how the age of two enzymes important for SOM decay (β-D-cellobioside, BGase; and N-acetyl-β-D-glucosaminide, NAGase) influences V_{max} at multiple temperatures. We quantified the enzyme-catalyzed reaction rates at three ecologically relevant temperatures, using freshly generated enzyme solutions and those aged between 2 and 3 days. We considered the time at which enzyme solutions were generated via mixing of purified enzymes and buffer solution to be analogous to the time at which a soil microbe releases an exo-enzyme into the soil matrix. We then used the resulting V_{max} values over time to compute how enzyme age influences the relative availabilities of C and N.

Given that thermodynamics suggest that biochemical process rates tend to increase with ecologically relevant temperatures, we predicted that increasing temperatures would promote faster degradation of enzymatic catalytic rates for both enzymes assayed. We also predicted that BGase would exhibit greater stability with aging, even at warmer temperatures, than NAGase. This prediction was based on two concepts. First, enzyme kinetics indicate that while chitinases such as NAGase can remain stable for up to 60 min at 60 °C (Parham and Deng 2000; Liang et al. 2014), β-glucosidase activity remains robust at 50 °C after multiple days (Weiss et al. 2013; Tsai and Meyer 2014). Also, some glucosidases exhibit half-lives of >60 days at 37 °C (Goyal et al. 2014), and greater stability in sea water than phosphatase and leucine aminopeptidase (Steen and Arnosti 2011). Second, our previous work suggests that BGase can maintain relatively high catalytic rates across wider temperature and pH ranges than NAGase (Min et al. 2014), suggesting that BGase may exhibit greater stability than NAGase as it ages as well. We observe a complicated dependence of enzyme catalytic rate on enzyme age and temperature, which has implications for how the return on microbial resource acquisition strategies is likely to change with temperature. The results we present here help us understand how time since cellular exo-enzyme exudation can influence microbial realization of any benefits from the selected strategy at different temperature, and represent an important first step towards amassing the empirical evidence needed to integrate exo-enzyme aging as a mechanistic feature in theoretical frameworks describing microbial strategies for life.

Materials and methods

Enzyme assays

We quantified fluorescence of 4-Methylumbelliferyl (MUB)-tagged substrates β-D-cellobioside (MUB-BG, Sigma-Aldrich, USA) and N-acetyl-β-D-glucosaminide (MUB-NAG, Sigma-Aldrich, USA) exposed to, respectively, β-glucosidase (BGase; EC 3.2.1.21; Megazyme, Ireland) and β-N-acetyl glucosaminidase (NAGase; EC 3.2.1.52; New England BioLabs, USA). Because MUB tags fluoresce when excited by light energy after enzymatic cleavage from a substrate (Mead et al. 1955), we can use fluorescence to measure specific activity of enzymes and their respective, MUB-labeled substrates (e.g. DeForest 2009). As in Lehmeier et al. (2013) and Min et al. (2014), we assume the cleavage of MUB from these substrates approximates the cleavage of glucose and NAG monomers from cellulose and chitin, respectively, as they are subjected to decay by these same enzymes. We conducted enzyme assays using freshly constituted enzymes as well as enzyme solutions that had aged between 1 h and 66.5 h (BGase) and 1 h and 54 h (NAGase).

For all assays, we dissolved MUB-BG (273 μM), MUB-NAG (400 μM), and a MUB standard (10 μM; Sigma-Aldrich, USA) in deionized water, and reconstituted purified enzymes in 0.2 M sodium acetate buffer at pH 6.5. Previous experiments revealed that the substrate concentrations used here resulted in substrate saturation of these enzymes, and that these saturation concentrations were appropriate for all experimental temperatures (Min et al. 2014). For each assay, we pipetted 50 μl substrate (either MUB-

BG or MUB-NAG) and 200 µl enzyme solution into two, eight-row columns of a black, 96-well plate (Costar, USA). Each well of the 96-well plate containing an enzyme-substrate reaction thus contained 0.024 units of BGase or 0.16 units of NAGase. Into additional columns, we pipetted 250 µl of four categories of controls: quench control (50 µl MUB solution and 200 µl enzyme solution); enzyme control (200 µl enzyme solution and 50 µl buffer); substrate control (50 µl substrate solution and 200 µl buffer); and standard control (50 µl MUB solution and 200 µl buffer). Upon completion of all pipetting, we placed each plate into a Synergy HT microplate reader (BioTek Instruments, In., USA). Fluorescence was assessed using excitation and emission wavelengths of 350 and 450 nm, respectively, for BGase/MUB-BG and NAGase/MUB-NAG reactions. We measured fluorescence over time, every minute for the BGase/MUB-BG reactions and every two minutes for the generally slower NAGase/MUB-NAG reactions. We assessed fluorescence for a sufficiently long period to capture the initial, linear increase in fluorescence; the slope of that line was considered V_{max}. This protocol follows that of DeForest (2009), modified according to the amount of enzyme present in each of our plate wells to permit computation of V_{max} as the specific enzyme activity per unit enzyme mass. Because our buffer-enzyme solution is free of mineral and organic compounds, our experiments reflect enzyme catalytic rates free from the potentially negative influence of enzyme adsorption (Asuri et al. 2007; Bakshi and Varma 2011).

Ambient laboratory temperature was 25 °C. To observe reaction rates at two temperatures lower than ambient (5° and 15 °C), we used protocols detailed in Lehmeier et al. (2013) and modified in Min et al. (2014). Briefly, all solutions and material with which they came into contact were incubated at the desired temperature in separate incubators. For each assay, we generated four, identical 96-well plates. A first plate was placed in the microplate reader, and immediately after measurement it was placed in the incubator. Subsequent plates were incubated at the desired temperature immediately after pipetting. We measured fluorescence over time by alternating plates every minute for the BGase/MUB-BG reactions and every two minutes for the NAGase/MUB-NAG. This process was repeated until all four identical plates were measured 6 to 8 times; thus, each V_{max} estimate was derived from 24 to 32 data points. To vary the age of the purified enzymes from freshly constituted solutions, enzyme-buffer solutions were incubated for the desired duration at the desired temperatures prior to conducting enzyme assays.

Data analyses and calculations

We used V_{max} of multiple ages of BGase and NAGase solutions to address three questions: (1) Do activities of the exo-enzymes BGase and NAGase decline with age; (2) Does temperature influence the rate of that decline; and (3) At a given temperature, does the rate of decline differ between these two exo-enzymes? Using R's nls function (version 3.1.0), we fit exponential decay functions ($V_{max(0)} * e^{(-\lambda * t)}$) to specific activity rates across time, because of both the trends in the data and our expectation of exponential declines in activity over time derived from literature (e.g. Kedi et al. 2013b; Weiss et al. 2013). We considered specific exo-enzyme activity as the response variable, enzyme age a continuous independent variable, and temperature and enzyme identity categorical predictors. This approach permitted us to estimate parameters for decay rates and assess the influence of age on each exo-enzyme at each temperature, and to test if the two exo-enzymes varied in their rate of catalytic decline at a given temperature. After initial visual inspection of the data, we used R's glht function to perform linear contrasts and tested if decay rates for BG and NAG differed at 15 and 25 °C (such contrasts were not relevant at 5 °C). All data satisfied assumptions of normality. We considered results to be statistically significant when $P < 0.05$.

We also explored how exo-enzyme aging could influence the C:N flow ratio, or the relative liberation of C compared to that of N, as catalytic rates declined during exo-enzyme aging. As described in full in Billings and Ballantyne (2013) and employed in Lehmeier et al. (2013) and Min et al. (2014), the C:N flow ratio resulting from decline in MUB-BG and MUB-NAG catalytic rates can be computed if we consider the rate of these substrates' decay reactions and the number of C and N atoms liberated upon monomer liberation. The consistent use of the MUB fluorophore liberated from both substrates upon their decay permits direct conversion of fluorescence into numbers of atoms liberated. Specifically, the C:N

flow ratio resulting from decay of MUB-BG and MUB-NAG can be computed using

$$\frac{dC}{dN} = \frac{V_{maxBGase}(T)}{V_{maxNAGase}(T)} \cdot 6 + 8$$

as described in Lehmeier et al. (2013). The constants derive from the six assimilable C atoms liberated upon the release of a glucose monomer from cellulose, and the eight C atoms and one N atom liberated upon release of a NAG monomer from chitin. We used estimates of $V_{max(0)}$ and λ derived from the model fits to estimate the average change in C:N flow ratio as the two exo-enzymes age at each temperature. To create confidence envelopes for the C:N flow ratio over time at each temperature, we sampled from the distributions of $V_{max(0)}$ and λ associated with fitting exponential decay functions to generate 100,000 decay trajectories for each enzyme at each temperature. We then used estimated $V_{(t)}$ values and quantiles (0.025 and 0.975) to bound the confidence regions for each temperature over time. This exercise provides an opportunity to observe how the landscape of assimilable resources that becomes available for microbial uptake may vary over time, well after cellular exudation of these exo-enzymes has ceased.

Results

Initial BGase activities were significantly different across the three temperatures assessed, rising with temperature (Table 1). Initial NAGase activities were greater at 15 and 25 °C than at 5 °C (Table 1). BGase activities were generally higher than NAGase activities (Fig. 1). Both BGase and NAGase exhibited exponential declines in catalytic rate with age at 25 °C ($P < 0.0001$, $P = 0.006$, respectively; Fig. 1, Table 1). BGase decline in catalytic rate with age at 25 °C was greater than at either of the cooler temperatures ($P < 0.0001$ for 25 vs. 15 °C, $P = 0.0001$ for 25 vs. 5 °C, Table 1). NAGase declines in catalytic rate with age did not differ across temperatures (Table 1). There was not a significant difference between rates of catalytic decline for BGase and NAGase at 25 °C (Table 1). At 15 °C, NAGase exhibited an exponential decline in catalytic rate with age (H_0: $\lambda = 0$; $P = 0.0100$), but BGase exhibited no such decline, driving a significant

Table 1 Parameter estimates describing influence of age on catalytic rate of two enzymes, β-glucosidase (BGase) and β-N-acetyl glucosaminidase (NAGase) at the specified temperatures

Enzyme	Term	5 °C			15 °C			25 °C		
		Estimate, s.e.	P_0	P_{temp}	Estimate, s.e.	P_0	P_{temp}	Estimate, s.e.	P_0	P_{temp}
BGase	*Intercept*	477.3 (45.7)♯	**<0.0001**	**<0.0001**	647.9 (35.3)♯	**<0.0001**	**<0.0001**	1458.0 (40.9)□	**<0.0001**	**<0.0001**
	Rate constant	−0.003 (0.003)◇	0.36	0.353	0.000 (0.002)◇	0.79	**<0.0001**	0.009 (0.001)◇	**<0.0001**	**0.0001**
NAGase	*Intercept*	226.6 (52.7)♯	**<0.0001**	**0.037**	429.9 (81.9)♯	**<0.0001**	0.428	359.3 (34.9)♯	**<0.0001**	**0.036**
	Rate constant	0.009 (0.010)◇	0.35	0.145	0.033 (0.013)□	**0.01**	0.219	0.015 (0.006)◇	**0.006**	0.576

Units for intercept estimates are $\mu mol_{activity}\ h^{-1}\ mg^{-1}_{enzyme}$; rate constant values represent estimated rates of catalytic decline (h^{-1}). Values in parentheses are standard errors. *P* values indicating whether intercepts and rate constants are significantly different from zero are designated P_0. *P* values indicating whether intercepts or rate constants differ across temperatures for a given enzyme are designated P_{temp} and reflect contrasts with the temperature positioned to the right in the table, except for those associated with 25 °C, which reflect contrasts with 5 °C data. P values considered significant at $\alpha = 0.05$ are emboldened. Different cross symbols (□, ♯) represent statistical differences between exo-enzymes' intercepts at a given temperature; different shapes (◇, □) represent statistical differences between exo-enzymes' rate constants at a given temperature

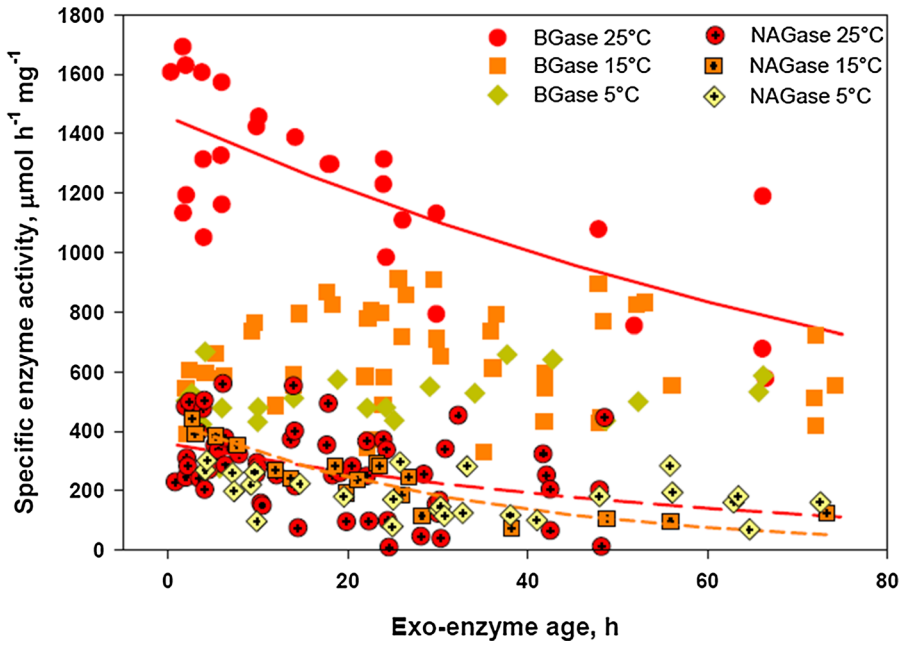

Fig. 1 Specific enzyme activities (V_{max}) for β-glucosidase (BGase) and β-N-acetyl glucosaminidase NAGase) at the specified temperatures as enzyme-buffer solution aged. Decline in catalytic rate is statistically significant for BGase at 25 °C, and for NAGase at 25 and 15 °C, represented by plotted functions. See Table 1 for parameter estimates and significance

difference between the two enzymes in their catalytic decline (H_0: $\lambda_{NAG} = \lambda_{BG}$; $P = 0.0263$). Neither enzyme exhibited a significant decline in catalytic rate with age over the timescales assessed here at 5 °C.

At 15 °C, C:N flow ratio estimates derived from exo-enzyme activities increased from significantly below that at 25 °C to equal or greater than that at 25 °C after 70 h, due to the decline across time in NAGase activity and invariant BGase activity (Fig. 2). At 25 °C, though catalytic rates declined with enzyme age for both BGase and NAGase, the lack of a significant difference in decline rates at this temperature resulted in a constant C:N flow ratio as enzymes aged. At 5 °C, C:N flow ratio estimates also remained constant, but for a different reason than at 25 °C; at 5 °C, the constant C:N flow ratio reflects the lack of decline in activity with aging for either enzyme across the investigated timescale.

Discussion

Quantification of specific enzyme activities (V_{max}) driving decay of biogeochemically relevant substrates in ideal conditions (Lehmeier et al. 2013, Min et al. 2014) provides a valuable baseline for comparison with apparent decay rates (Billings et al. 2015). This is true particularly if purified enzymes adequately reflect activities of the distinct isozymes that microbes may generate in different temperature regimes, and enzyme-enzyme interactions (Billings and Ballantyne 2013; Bradford 2013). Apparent SOM decay rates in natural environments (e.g., Sinsabaugh 2010; Tiemann and Billings 2011; German et al. 2012) and apparent temperature sensitivities of exo-enzymes responsible for SOM decay (e.g. Wallenstein et al. 2009; Li et al. 2012; Razavi et al. 2016) all reflect potential rates modified by restrictions on substrate availability, varying enzyme production rates and any diffusion limitations of substrate and/or enzyme (Sinsabaugh and Follstad Shah 2012). In the current work, we demonstrate yet another mechanism by which apparent rates of SOM decay, and apparent temperature sensitivities of SOM decay, may also deviate from their potential in the natural environment: age-dependent decreases in exo-enzyme catalytic rates. Quantifying this decrease provides a fundamental starting point for parameterizing coupled microbial-enzyme process models.

Enzyme aging and specific activities

The V_{max} values of freshly reconstituted enzyme solutions (i.e. near-zero values on X-axis in Fig. 1) were obtained under similar conditions as those

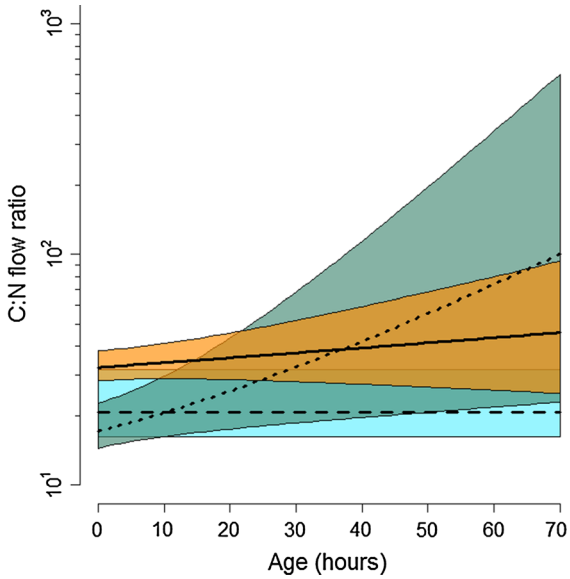

Fig. 2 The C:N flow ratio (ratio of microbially assimilable C and N resources) resulting from the release of glucose and N-acetylglucosamine monomers from cellobiose and chitin, respectively, as these substrates decompose via the catalysis induced by aging enzymes β-glucosidase (BGase) and β-N-acetyl glucosaminidase (NAGase), at 5, 15, and 25 °C (*dashed*, *dotted*, and *solid lines*, respectively). *Shaded regions* (*light blue*, 5 °C; *green*, 15 °C; *orange*, 25 °C) represent confidence envelopes generated from sampled distributions of parameters describing fitted exponential decay functions. Y-axis is log scaled to enhance separation between polygons for clarity. *Note* that C:N flow ratio is constant at 5 °C due to a lack of catalytic decline rate with enzyme aging at that temperature across 70 h; because of the lack of an effect of either enzymes' age at 5 °C, we have overlaid this *light blue rectangle* by the other temperatures' polygons. At 25 °C, rates of catalytic decline with enzyme aging were not significantly different across enzymes, but the C:N flow ratio at 25 °C appears to increase as an arithmetic consequence of small, non-significant differences in catalytic declines with time between BGase and NAGase. At 15 °C, C:N flow increases significantly across enzyme age. See text for complete description of confidence envelope generation and C:N flow ratio calculations. (Color figure online)

observed in related work (Lehmeier et al. 2013; Min et al. 2014), and yet were more variable than the values reported in those investigations. We cannot explain this enhanced variability. However, the detectable instability of both enzymes as they aged at 25 °C, and of NAGase at 15 °C, points to several phenomena with potentially profound implications for SOM decay and microbial abilities to acquire resources. First, the generally greater catalytic rate of BGase compared to NAGase means that even with similar rates of catalytic decline over time at 25 °C (see similar parameter estimates across enzymes at 25 °C, Table 1), the absolute decrease in the rate of monomer liberation from cellulose per unit time is greater than the absolute decrease in monomer cleavage from chitin (Fig. 1). Thus, the absolute amount of glucose-C potentially provided to a microbe by BGase experiences a much larger decline over time than the absolute amount of NAG-borne C and N, per unit time. Second, though the decline in BGase at 25 °C is impressive in absolute terms, its stability at 15 °C is consistent with the idea that BGase can remain stable in a wider range of environmental conditions (Min et al. 2014); this may reflect the ubiquity of glucose-based metabolism across diverse taxa. Assays using yet-older enzyme-buffer solutions are needed to quantify the (presumed eventual) catalytic decline of BGase at 15 °C and of both enzymes at 5 °C.

A third emergent feature illuminated by this work is reflected in the rate constants' values describing these two enzymes' catalytic decline as they aged. These constants, and their values in different temperature regimes, provide constraints for trait-based theories of microbial functioning. For example, Schimel and Weintraub (2003) employ a decay constant for exo-enzymes (K_l) of 0.05 d^{-1} in their model linking exo-enzyme activity to soil microbial C and N limitation. This value is also used by Manzoni et al. (2014) for their k_E term, the enzyme deactivation rate. The data presented here suggest that, in the absence of any other confounding factors, 0.22 d^{-1} (0.009 h^{-1}*24) and 0.36 d^{-1} (0.015 h^{-1}*24) are reasonable baseline estimates of rate constants describing the decline in exo-enzyme catalytic rates over time for BGase and NAGase, respectively, at 25 °C (Table 1). At 15 °C, the decay constant for NAGase in an environment with negligible enzyme adsorption appears to be 0.79 d^{-1} (0.033 h^{-1}*24). Although adsorption can protect exo-enzymes from microbially-induced degradation in soils (Nannipier et al. 1982; Monsan and Combes 1988; George et al. 2005; Kedi et al. 2013b; Rosas et al. 2011; Park et al. 2015) and may mitigate catalytic decay rates in natural environments, these parameter estimates suggest that current models of microbial resource acquisition and growth employ enzyme deactivation rates far slower than what potentially can occur. Though current model estimates may represent reasonable phenomenological rate constants for some

soils, discrepancies between baseline rates presented in the current study and those realized in soils remain unclear. These data also highlight that temperature can play an important role not just in accelerating SOM decay, but in differentially augmenting the rate at which enzymes' deactivation can occur. Thus, not only do current models of microbial growth likely employ underestimates of exo-enzyme decay constants, but they do not reflect the meaningful variation with temperature across exo-enzymes in those constants.

Enzyme aging and C:N flow ratios

Temperature governed the processes driving C:N flow ratio behavior as these exo-enzymes aged, and thus changes in resource availability of potential ecological significance. The constant C:N flow ratio at 25 °C, even as BGase and NAGase catalytic rates both decline with exo-enzyme age, reflects the lack of a meaningful difference in the rate at which these enzymes degraded over time. These data suggest that investigators have reasonable justification for simplifying at least one feature of microbial resource acquisition: for these two exo-enzymes at 25 °C, the degradation of catalytic rates across time can be considered the same. In contrast, the constant C:N flow ratio at 5 °C reflects the lack of decline in catalytic rate with enzyme age at that temperature. Contrasting with both of these scenarios, the increase in C:N flow ratio at 15 °C reflects unchanging BGase activity with exo-enzyme age and declining NAGase activity, and the resulting continued availability of glucose-C with a simultaneous decline in NAG-N and NAG-C.

These changes in C:N flow ratio across time at 15 °C represent a change in the landscape of microbially assimilable resources. To the extent that changes in C:N flow ratio occur in a natural environment, microorganisms must adapt to such shifts in resource landscape, either by exhibiting stoichiometric plasticity (Billings and Ballantyne 2013) or by modifying their decomposition strategy to maintain a set supply rate of different elements (Moorhead et al. 2012). For example, in an environment where C and N are derived from BGase and NAGase activities, microbes at 15 °C must cope with a large increase in the C:N flow ratio ~70 h post-exudation of these enzymes. One strategy for maintaining a supply of assimilable N may be to augment their NAGase production rate. Importantly, variation in a C:N flow ratio over time evidently can result solely from the distinct ways in which these two enzyme-substrate reactions respond to enzyme age at different temperatures, and the C and N available after liberation of monomers, in isolation of any changes in the identity of active microorganisms or their decomposition strategies.

In previous work, we have outlined some of the ecological ramifications of altered C:N flow ratios resulting from substrate decay in a changing temperature regime (Billings and Ballantyne 2013; Lehmeier et al. 2013) and with changing pH and temperature (Min et al. 2014). For example, a declining C:N flow ratio with warming, as computed from observed decay rates of cellulose and chitin analogs (Lehmeier et al. 2013), may help explain unexpected declines in response of microbial CO_2 efflux after prolonged exposure to experimental warming (Peterjohn et al. 1994; Oechel et al. 2000; Luo et al. 2001; Rustad et al. 2001; Melillo et al. 2002; Eliasson et al. 2005), particularly at pH 7.5 (Min et al. 2014). Data in the current study highlight another potential mechanism that could drive apparent temperature sensitivities of soil CO_2 efflux in cooler (e.g. 15 °C) vs. warmer (e.g. 25 °C) temperatures: given that C:N flow ratio is modified by aging BGase and NAGase in a temperature-dependent manner, microbial communities may experience relative C limitation at the warmer temperature (Fig. 2) and not exhibit a predicted CO_2 temperature response.

Conclusions

We provide fundamental, temperature-influenced rates of catalytic decline for two exo-enzymes of biogeochemical interest to those empiricists and modelers investigating SOM decay. The data highlight a currently underappreciated mechanism—exo-enzyme aging—likely contributing to variation in apparent exo-enzyme activities. Like potentially changing identity of active microbial groups, stoichiometric plasticity, and altered substrate availability and microbial decomposition strategies, exo-enzyme aging can influence some of the biogeochemical fluxes critical to understand for accurate projection of the resource landscape of soil microbial communities,

microbial CO_2 production throughout soil profiles, and Earth's SOM reservoirs. These data provide baseline data to which we can compare variation in catalytic rates over time as derived from environmental samples. Though exo-enzyme adsorption may retard declines in catalytic rates over time in natural settings, these data provide a starting point from which we can base parameter estimates in models of microbial resource acquisition and growth; these data suggest that rate constants in microbial process models currently may represent underestimates of declining exo-enzyme catalytic rates over time.

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References

Allison SD (2006) Soil minerals and humic acids alter enzyme stability: implications for ecosystem processes. Biogeochem 81:361–373

Allison SD (2012) A trait-based approach for modelling microbial litter decomposition. Ecol Lett 15:1058–1070

Asuri P, Karajanagi SS, Vertegel AA, Dordick JS, Kane RS (2007) Enhanced stability of enzymes adsorbed onto nanoparticles. J Nanosci Nanotechnol 7:1675–1678

Bakshi M, Varma A (2011) Soil enzyme: The state-of-art. In: Sukla G, Varma A (eds) Soil Enzymology. Springer, Berlin, pp 1–24

Bárta J, Šlajsová P, Tahovská K, Picek T, Šantrucková H (2014) Different temperature sensitivity and kinetics of soil enzymes indicate seasonal shifts in C, N and P nutrient stoichiometry in acid forest soil. Biogeochem 117:525–537

Billings SA, Ballantyne F IV (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. Glob Chang Biol 19:90–102

Billings SA, Tiemann LK, Ballantyne F IV, Lehmeier CA, Min K (2015) Investigating microbial transformations of soil organic matter: synthesizing knowledge from disparate fields to guide new experimentation. Soil 1:313–330

Boschker HTS, Bertilsson SA, Dekkers EMJ, Cappenberg TE (1995) An inhibitor-based method to measure initial decomposition of naturally occurring polysaccharides in sediments. Appl Environ Microbiol 61:2186–2192

Bradford MA (2013) Thermal adaptation of decomposer communities in warming soils. Front Microbiol. doi:10.3389/fmicb.2013.00333

Craine JM, Fierer N, McLauchlan KK (2010) Widespread coupling between the rate and temperature sensitivity of organic matter decay. Nat Geosci 3:854–857

DeForest JL (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 Biol Biochem 41:1180–1186

Eliasson PE, McMurtrie RE, Pepper DA, Stromgren M, Linder S, Agren GI (2005) The response of heterotrophic CO_2 flux to soil warming. Glob Chang Biol 11:167–181

Fissore C, Giardina CP, Kolka RK, Trettin CC, King GM, Jurgensen MF, Barton CD, McDowell SD (2008) Temperature and vegetation effects on soil organic carbon quality along a forested mean annual temperature gradient in North America. Glob Chang Biol 14:193–205

Frey SD, Lee J, Melillo JM, Six J (2013) The temperature response of soil microbial efficiency and its feedback to climate. Nat Clim Chang. doi:10.1038/NCLIMATE1796

George TS, Richardson AE, Simpson RJ (2005) Behaviour of plant-derived extracellular phytase upon addition to soil. Soil Biol Biochem 37:977–988

German DP, Marcelo KR, Stone MM, Allison SD (2012) The Michaelis-Menten kinetics of soil extracellular enzymes in response to temperature: a cross-latitudinal study. Glob Chang Biol 18:1468–1479

Goyal M, Chaudhuri TK, Kuwajima K (2014) Irreversible denaturation of maltodextrin glucosidase studied by differential scanning calorimetry, circular dichroism, and turbidity measurements. PLoS ONE 9(12):e115877. doi:10.1371/journal.pone.0115877

Kedi B, Sei J, Quiquampoix H, Staunton S (2013a) Persistence of catalytic activity of fungal phosphatases incubated in tropical soils. Soil Biol Biochem 56:69–74

Kedi B, Abadie J, Sei J, Quiquampoix H, Staunton S (2013b) Diversity of adsorption affinity and catalytic activity of fungal phosphatases adsorbed on some tropical soils. Soil Biol Biochem 56:13–20

Kishore D, Kundu S, Kayastha AM (2012) Thermal, chemical and pH induced denaturation of a mutimeric β-galactosidase reveals multiple unfolding pathways. PLoS ONE 7 (11):50380. doi:10.1371/journal.pone.0050380

Lehmeier CA, Min K, Niehues ND, Ballantyne F IV, Billings SA (2013) Temperature-mediated changes of exoenzyme-substrate reaction rates and their consequences for the carbon to nitrogen flow ratio of liberated resources. Soil Biol Biochem 57:374–382

Li J, Ziegler S, Lane CS, Billings SA (2012) Warming-enhanced preferential microbial mineralization of humified boreal forest soil organic matter: interpretation of soil profiles along a climate transect using laboratory incubations. J Geophys Res Biogeosci 117(G02008):1–13. doi:10.1029/2011JG001769

Liang T-W, Hsieh T-Y, Wang S-L (2014) Purification of a thermostable chitinase from Bacillus cereus by chitin affinity and its application in microbial community changes in soil. Bioprocess Biosyst Eng 37:1201–1209

Luo Y, Wan SQ, Hui DF, Wallace LL (2001) Acclimatization of soil respiration to warming in a tall grass prairie. Nature 413:622–625

Manzoni S, Schaeffer SM, Katul G, Porporato A, Schimel JP (2014) A theoretical analysis of microbial eco-physiological and diffusion limitations to carbon cycling in drying soils. Soil Biol Biochem 73:69–83

Manzoni S, Moyano F, Katterer T, Schimel J (2016) Modeling coupled enzymatic and solute transport controls on decomposition in drying soils. Soil Biol Biochem 95:275–287

Mead JAR, Smith JN, Williams RT (1955) Studies in detoxication. 67. Biosynthesis of the glucuronides of umbelliferone and 4-methylumbelliferone and their use in fluorimetric determination of beta-glucuronidase. Biochem J 61:569–574

Melillo JM, Steudler PA, Aber JD, Newkirk K, Lux H, Bowles FP, Catricala C, Magill A, Ahrens T, Morrisseau S (2002) Soil warming and carbon-cycle feedbacks to the climate system. Science 298:2173–2176

Min K, Lehmeier CA, Ballantyne F IV, Tatarko A, Billings SA (2014) Differential effects of pH on temperature sensitivity of organic carbon and nitrogen decay. Soil Biol Biochem 76:193–200

Monsan P, Combes D (1988) Enzyme stabilization by immobilization. Methods Enzymol 137:584–599

Moorhead DL, Lashermes G, Sinsabaugh RL (2012) A theoretical model of C- and N-acquiring exoenzyme activities, which balances microbial demands during decomposition. Soil Biol Biochem 53:133–141. doi: 10.1016/j.soilbio.2012.05.011

Nannipier P, Ceccanti B, Conti C, Bianchi D (1982) Hydrolases extracted from soil: their properties and activities. Soil Biol Biochem 14:257–263

Oechel WC, Vourlitis GL, Hastings SJ, Zulueta RC, Hinzman L, Kane D (2000) Acclimation of ecosystem CO_2 exchange in the Alaskan Arctic in response to decadal climate warming. Nature 406:978–981

Parham JA, Deng SP (2000) Detection, quantification and characterization of β-glucosaminidase activity in soil. Soil Biol Biochem 32:1183–1190

Park S, Kim SH, Won K, Choi JW, Kim YH, Kim HJ, Yang Y-H, Lee SH (2015) Wood mimetic hydrogel beads for enzyme immobilization. Carbohydr Polym 115:223–229

Peterjohn WT, Melillo JM, Steudler PA, Newkirk KM, Bowles FP, Aber JD (1994) Responses of trace gas fluxes and N availability to experimentally elevated soil temperatures. Ecol Appl 4:617–625

Razavi BS, Blagodatskaya E, Kuzyakov Y (2016) Temperature selects for statis soil enzyme systems to maintain high catalytic efficiency. Soil Biol Biochem 97:15–22

Rosas A, Lopez A, Lopez R (2011) Behavior of enzymatic activity in Chilean volcanic soil and their interactions with clay fraction. In: Sukla G, Varma A (eds) Soil enzymology. Springer, Berlin, pp 313–328

Rustad LE, Campbell JL, Marion GM, Norby RJ, Mitchell MJ, Hartley AE, Cornelissen JHC, Gurevitch J, GCTE-News (2001) A meta-analysis of the response of soil respiration, net nitrogen mineralization, and aboveground plant growth to experimental ecosystem warming. Oecologia 126:543–562

Schimel JP, Weintraub MN (2003) The implication of exoenzyme activity on microbial carbon and nitrogen limitation in soil: a theoretical model. Soil Biol Biochem 35:549–563

Shirke AN, Basore D, Butterfoss GL, Bonneau R, Bystroff C, Gross RA (2015) Toward rational thermostabilization of *Aspergillus oryzae* cutinase: insights into catalytic and structural stability. Proteins 84:60–72

Sierra CA, Trumbore SE, Davidson EA, Vicca S, Janssens I (2015) Sensitivity of decomposition rates of soil organic matter with respect to simultaneous changes in temperature and moisture. J Adv Model Earth Syst 7:335–356

Sinsabaugh R (2010) Phenol oxidase, peroxidase and organic matter dynamics of soil. Soil Biol Biochem 42:391–404

Sinsabaugh R, Follstad Shah JJ (2012) Ecoenzymatic stoichiometry and ecological theory. Ann Rev Ecol Evol Syst 43:313–343

Skujins J (1978) History of abiotic soil enzyme research. In: Burns RG (ed) Soil enzymes. Academic Press, New York, pp 1–49

Steen AD, Arnosti C (2011) Long lifetimes of β-glucosidase, leucine aminopeptidase, and phosphatase in Arctic seawater. Mar Chem 123:127–132

Subke JA, Bahn M (2010) On the 'temperature sensitivity' of soil respiration: can we use the immeasurable to predict the unknown? Soil Biol Biochem 42:1653–1656

Tiemann LK, Billings SA (2011) Indirect effects of nitrogen amendments on organic substrate quality increase enzymatic activity driving decomposition in a mesic grassland. Ecosystems 14:234–247

Tsai C-T, Meyer AS (2014) Enzymatic cellulose hydrolysis: enzyme reusability and visualization of β-glucosidase immobilized in calcium alginate. Molecules 19:19390–19406

Wallenstein MD, Weintraub MN (2008) Emerging tools for measuring and modeling the in situ activity of soil extracellular enzymes. Soil Biol Biochem 40:2098–2106

Wallenstein MD, McMahon SK, Schimel JP (2009) Seasonal variation in enzyme activities and temperature sensitivities in Arctic tundra soils. Glob Chang Biol 15:1631–1639

Weiss N, Borjesson J, Pedersen LS, Meyer AS (2013) Enzymatic lignocellulose hydrolysis: improved cellulase productivity by insoluble solids recycling. Biotechnol Biofuels 6:5

Wieder WR, Grandy AS, Kallenbach CM, Bonan GB (2014) Integrating microbial physiology and physio-chemical principles in soils with the MIcrobial-MIneral Carbon Stabilization (MIMICS) model. Biogeosci 11:3899–3917

Yan J, Pan G, Li L, Quan G, Ding C, Luo A (2010) Adsorption, immobilization, and activity of β-glucosidase on different soil colloids. J Colloid Interface Sci 348:565–570

Zhang Q, Katul GG, Oren R, Daly E, Manzoni S, Yang D (2015) The hysteresis response of soil CO_2 concentration and soil respiration to soil temperature. J Geophys Res 120:1605–1618. doi:10.1002/2015JG003047