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A litter-slurry technique elucidates the key role of enzyme production and microbial dynamics in temperature sensitivity of organic matter decomposition

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ABSTRACT

The rate at which organic matter decomposes generally increases with temperature, unless it is physico-chemically protected from enzymatic depolymerization. The temperature sensitivity of decomposition should increase with decreasing reaction rates, corresponding to increasing activation energy of the decomposing compounds. One approach to testing this carbon-quality temperature hypothesis is to study the effect of temperature on leaf litter decomposition, because fresh surface litter is unprotected. However, other factors such as humidity co-vary with temperature, and biological processes such as enzyme production and microbial population growth may also be thermally sensitive. We developed a litter slurry approach to isolate the effect of temperature and litter quality on decomposition. We found that pine litter decomposed faster than oak litter, consistent with a lower C:N and lignin:N ratio. During the first 14 days of decomposition, there was no difference in decomposition rate for litter incubated at 25 °C compared to 35 °C. Lower potential enzyme activity at 35 °C suggested that enzyme production was suppressed at 35 °C compared to 25 °C, resulting in similar *in situ* enzyme activities at the two temperatures. After 14 days, enzyme pools were similar between the two incubation temperatures, which resulted in faster decomposition at the warmer temperature, consistent with enzyme kinetic theory. At Day 14, the decomposition rate of the high quality pine litter was more temperature sensitive than the decomposition rate of the lower quality oak litter, suggesting that the quality of soluble pool rather than bulk chemistry determined the temperature sensitivity during this stage. After 28 days of incubation, oak litter decomposition was more temperature sensitive than pine litter, consistent with the carbon temperature-quality hypothesis. The litter slurry approach revealed that biological responses to temperature can affect the apparent temperature sensitivity of decomposition, and highlight a need for further research into microbial responses to temperature.

1. Introduction

The temperature sensitivity of decomposition should be inversely related to substrate quality based on fundamental theory of enzyme kinetics (Arrhenius, 1889; Davidson and Janssens, 2006). The carbon quality-temperature hypothesis (CQT) posits that enzyme-substrate reactions are the rate-limiting step in decomposition and on a relative basis, reactions with a higher activation energy should increase more rapidly with warming than reactions with lower activation energies (Bosatta and Agren, 1999). This hypothesis implicitly assumes that microbial uptake and catabolism of the soluble products of enzymatic depolymerization is not a rate-limiting step and should therefore not affect decomposition temperature sensitivity.

Soil organic matter (SOM) decomposition often exhibits responses to temperature that appear to be inconsistent with kinetic theory (von Lützow and Kögel-Knabner, 2009). Either the theory is wrong, or other factors constrain the temperature sensitivity of decomposition. Conant et al. (2011) developed a synthetic framework to elucidate temperature controls on decomposition, and suggested that enzyme kinetics do not affect decomposition rates when SOM is physico-chemically protected and thus unavailable for biological decomposition. When substrates are accessible to enzymes, enzyme kinetics are the main determinant of their decomposition rate.

Previous attempts to test the temperature-quality hypothesis have been equivocal due to the myriad of factors influencing the temperature sensitivity of SOM. There is a clear need for

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a technique to isolate the biological component of decomposition by eliminating the effects of physicochemical protection. Fierer et al. (2005) approached this problem by examining the short-term temperature sensitivity of leaf litter that differed in quality (decomposability, lignin:N ratio), since fresh litter on the soil surface is not subject to physical protection or interactions with other SOM constituents. The temperature sensitivity of decomposition of leaf litter should be inversely related to litter quality in the same manner as SOM (Bosatta and Agren, 1999), and the results of the Fierer et al. (2005) study were consistent with this expectation. However, even in controlled laboratory incubations litter decomposition rates are also affected by relative humidity (Nagy and Macauley, 1982), which co-varies with temperature, and other factors such as the composition of the decomposer community (Wall et al., 2008; Ayres et al., 2009). Thus, it can be difficult to isolate the direct effect of temperature on litter decomposition rates using traditional approaches.

We developed a litter slurry incubation technique to isolate the effect of temperature on litter decomposition. The advantages of this approach are that litter decomposition is rapid, all abiotic factors can be controlled and optimized, and it is easy to monitor biological drivers of decomposition such as microbial population size and composition and enzymes throughout the experiment. We applied this approach to test whether the temperature sensitivity of decomposition differed between pine and oak leaf litter, which differ in chemistry and stoichiometry. Based on the CQT hypothesis, we predicted that decomposition of oak litter would be more temperature sensitive than pine litter due to its lower quality. We also examined whether the temperature sensitivity of decomposition changed as its quality changed during the decomposition process. We predicted that temperature sensitivity would increase as litter quality decreased following an initial period of decomposition. Finally, to examine the biological drivers of decomposition, we measured enzyme activities and their temperature sensitivity as well as bacterial and fungal biomass in the slurries.

2. Methods

2.1. Litter preparation

We used two different types of litter that differed in chemistry, Oak and Pine, in a laboratory experiment designed to examine the effect of temperature on microbial dynamics during decomposition. The oak (*Quercus rubra* L.) litter was collected at the Harvard Forest hurricane pulldown experiment near Worcester, MA in 2006. The pine litter (*Pinus ponderosa* Douglas ex Lawson) consisted of dead needles still attached to branches, many of which were beetle-killed, collected along Cache la Poudre River near Fort Collins, CO (40°41′43″N, 105°17′12″W) in 2009. Litter from both sites was dried at 60 °C and then ground separately using a 0.75 mm-mesh screen equipped Wiley mill. In order to obtain a consistent sized litter and maximize litter surface area, we processed the ground litter samples through stacked catchment pans and obtained litter sized between 90 μm and 53 μm. By milling the litter prior to incubation, we minimized the effects of differences in structure, surface area, and physical protection between the two litter types so that we could isolate the influence of substrate chemistry. Individual subsamples were composited for each litter for the incubation experiment. Total C and total N litter concentrations were determined with a LECO CHN-1000 autoanalyzer (LECO Corp., St. Joseph, MI) prior to incubation. Percent cellulose and lignin were determined using a modified Vansoest and Wine (1967) acid detergent digest with the percent cellulose and lignin reported being corrected for ash content.

2.2. Litter incubation

The 53–90 μm-sized litter was used in a litter slurry incubation. The incubation consisted of 0.05 g of litter, 19.5 mL of nutrient broth, and 0.5 mL of soil inoculum in 50 mL centrifuge tubes fitted with septa. Hoagland's solution with 50% of the standard N concentration was used as a nutrient broth. The soil inoculum used was produced using methods similar to those described by Plante et al. (2011). Field moist soil from the Boston Area Climate Experiment (BACE) was used to create the soil inoculum. To increase the microbial population in the soil prior to extracting the inoculum, 1 mL of cellobiose solution (2% cellobiose by weight in distilled water) along with either 0.25 g of pine or oak litter was added to two 30 g samples of moist soil. Both soils were mixed to incorporate cellobiose solution and litter and water was added to reach 50% water filled pore space. The soils were incubated for one week to increase the active microbial population at which point 3.0 g was taken from each sample and added to 27 mL of water in a centrifuge tube. The two samples were shaken for 30 min, allowed to settle overnight, and then centrifuged at 2095 *g* for 3 min to spin down the majority of the soil still in suspension. The supernatant was filtered through a 20 μm nylon mesh to catch any litter still in suspension and the extracted liquid was used as the soil inoculum. The inoculum created using the pine litter was added to the pine slurries and the oak inoculum was added to the oak slurries.

Once the inocula were added to the litter slurry, the centrifuge tubes were tightly capped and flushed with CO_2 free air for 10 min. The slurry samples were set into a horizontal shaker at 25 °C and 35 °C and were shaken constantly to keep the slurries aerated. To determine the background activity of the soil inoculum, a set of samples that contained only Hoagland's solution and oak or pine inoculum was also incubated at both temperatures. We incubated three sets of litter to understand the effect of temperature and temperature change on litter decomposition over time. The first set was incubated at 25 °C and samples were sequentially harvested at Day 14, 28, and 42. The second set was incubated at 35 °C and samples were sequentially harvested as for the first set. The last set began the incubation at 25 °C and was then moved to 35 °C (bumped) at Day 14 and 28, as the litter quality declined over time and these samples were used to calculate the Q_{10} of the litter respiration. The samples bumped at Day 14 were harvested at Day 28 and 42 and the samples bumped at Day 28 were subsequently harvested at Day 42. The samples that only contained Hoagland's solution and inoculum were harvested at Day 0 and 42.

CO_2 concentrations were, measured on the subset of samples not harvested until the end of the incubation using an LI-6525 (LI-COR, Lincoln, NE) infrared gas analyzer (IRGA). Measurements were taken daily for the first week of the experiment, the first week after each temperature bump, and then every two-three days for the second week. After each CO_2 measurement was taken on the subset of samples, all samples were flushed with CO_2 free air. Q_{10} was calculated for the bump samples using the cumulative CO_2 respired after the first day of that bump at the 35 °C temperature divided by the cumulative respiration over the same time for the soils incubated at 25 °C.

2.3. Enzyme assays

Each litter sample was filtered through a 20 μm mesh filter and the slurry solution was diluted by 50% for use in the enzyme assay. The diluted slurry solutions were prepared for enzyme analysis using a fluorimetric deep-well microplate technique. The six enzymes measured are involved in the hydrolytic depolymerization of different components of organic matter decomposition:

α-glucosidase (AG), β-glucosidase (BG), cellobiohydrolase (CB), xylosidase (XLY), N-acetyl glucosaminidase (NAG), and leucine-amino peptidase (LAP). A full range (2.5 μM-100 μM) of standards was used to calibrate the enzyme activity of each assay. 4-methylumbelliferone was used as a standard for the AG, BG, CB, NAG, and substrates XYL and 7-amino-4-methylcoumarin was used as the standard for the LAP substrate. The standards were assayed at 35 °C. One hundred microliters of 400 μM fluorometric substrate was added to 400 μL of each litter solution and enzyme assays were conducted at 25 °C and 35 °C and incubated for 3 h and 1.5 h respectively. The fluorescence was measured on a Tecan Infinite M200 (TECAN, Männedorf, Switzerland) microplate reader with an emission wavelength of 450 nm and an excitation wavelength of 365 nm. The enzyme activity Q_{10} was calculated by dividing the total enzyme activity of the sample assayed at 35 °C by the total activity of the sample assayed at 25 °C for a particular enzyme. Since we measured enzymes associated with both the C and N cycle, we were able to calculate the stoichiometry of enzyme acquisition activity following the index developed by Sinsabaugh et al. (2008). The stoichiometry of potential enzyme acquisition activities (C:N) was calculated as (ln(BG)/ln (LAP + NAG) (Sinsabaugh et al., 2008), which provides insight into the relative allocation of resources towards the acquisition of C versus N.

2.4. Fungal and bacterial biomass

The remaining slurry solution was frozen and subsequently used to estimate bacterial and fungal biomass in the solutions using microscopic methods described by Bloem et al. (1995) as modified by Frey et al. (1999) (fungi) and Chelius et al. (2009) (bacteria). The slurry solution was thawed, vortexed for 30 s, and then 10 μL was pipetted into each 5 wells of a 10-well glass microscope slide with separate slides being prepared for bacteria and fungi. The slides were dried in a laminar flow hood. For the bacteria slide 15 μL of DTAF stain (5-(4, 6 dichlorotriazin-2-yl) aminofluorescein) was then added; for the fungi slide, 15 μL of Calcoflour stain (fluorescent brightener) was added. The slides were then rinsed three times in phosphate buffered saline solution and allowed to dry in a laminar flow hood. The number of bacteria in 3257 microscopic fields at 1250× magnification were counted. Bacteria cell counts were converted to bacterial biomass by using an estimate of 6.65×10^{-13} g bacteria cell^{-1} (Ilic et al., 2001). The length of the fungi was estimated using a line intersect method developed for estimating root length (Newman, 1966) and the length was converted to biomass using the estimate of 2.3×10^{-6} g m^{-1}. Since some individual replicates had no observations of fungi or bacteria, the fungi to bacteria ratio was calculated by dividing the average fungi biomass by the average bacteria biomass for a treatment and the error reported is the ratio standard error (Kendall and Stuart, 1977) calculated from the average fungi and bacteria standard error.

2.5. Statistical analysis

All errors reported are standard errors with four sample replicates for each mean. Duncan's multiple range test in the ANOVA procedure in SAS v9.2 (SAS Institute, CaryNC) was used to determine statistical differences between treatments at each harvest point for potential enzyme activities and fungal and bacterial biomass. For all response variables, we tested for main effects of incubation temperature in separate models for each liter type and harvest date. In all figures, treatment means with different letters indicate that they are significantly different (at $p < 0.05$).

Table 1
Summary of initial litter chemistry prior to incubation.

Litter	%C	%N	%Cellulose	%Lignin	C:N	Lignin:N
Quercus rubra (oak)	54.18	1.198	20.5	39.4	45.2	32.9
Pinus ponderosa (pine)	54.25	2.184	12.5	37.4	24.8	17.1

3. Results

3.1. Litter quality and decomposition

The oak and pine litter used in the experiment differed in quality with the pine having almost twice as much nitrogen as the oak (Table 1). Both litters had similar lignin contents (39.4% for oak and 37.4% for pine), but oak had greater cellulose content (20.5%) than the pine (12.5%). C:N ratio and lignin:N ratio have both been found to be good indicators of litter quality (Melillo et al., 1982) and by both indicators, pine had a lower ratio and would be considered a higher quality litter.

The respiration patterns for the pine and oak litter were similar, with both litters respiring the same amount at 25 °C and 35 °C for the first two weeks of the incubation (Fig. 1a and b). At Day 14 the first set of litters were bumped from 25 °C to 35 °C with

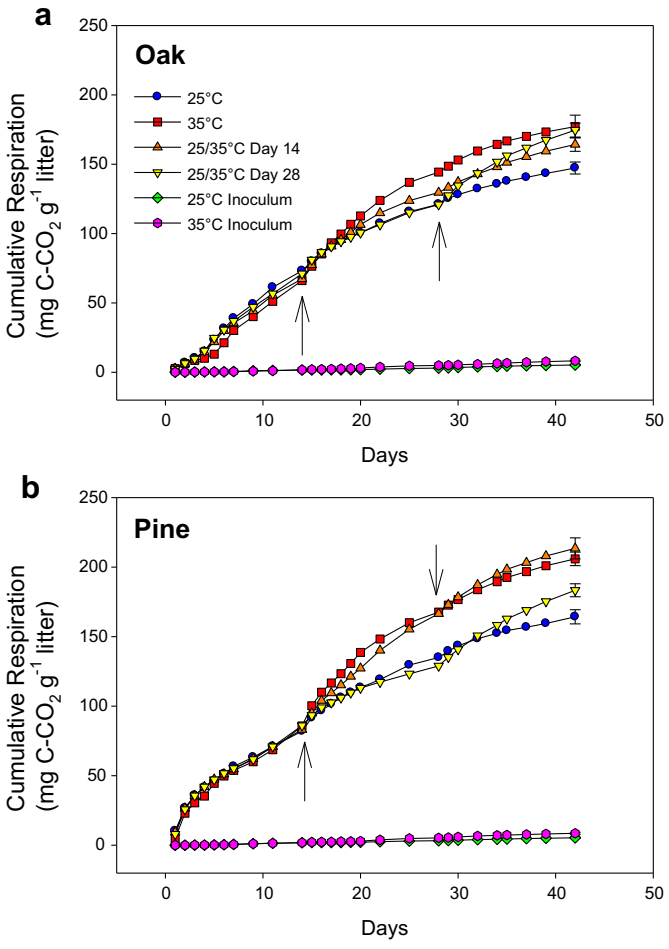

Fig. 1. Cumulative respiration over the 42 day incubation for oak (a) and pine (b) litter at 25 °C, litter increased from 25 °C to 35 °C, litter at 35 °C, and samples with only soil inoculum. Arrows indicate when temperature bumps occurred. Mean ± 1 s.e.m, $n = 4$, s.e.m was calculated at all sampling points, but was removed for visual clarity.

a subsequent increase in the cumulative respiration for both litter types. The Q_{10} of the respiration for the bumped litter calculated after the first day was higher for the pine (1.68 ± 0.65) than the oak (1.49 ± 0.10), indicating a higher temperature sensitivity for the higher quality pine litter. For the pine litter the cumulative respiration of the bumped litter surpassed that of the 35 °C litter two weeks after the temperature bump (Fig. 1b), but the cumulative respiration of the bumped oak litter was lower than the 35 °C litter throughout the remaining incubation (Fig. 1a). At Day 28 the second set of litter slurries were bumped and again there was a subsequent increase in respiration with the Q_{10} for the bumped oak being higher than the pine (1.77 ± 0.31 versus 1.49 ± 0.32) and the cumulative respiration of the bumped litter almost reaching that of the litter that had been at 35 °C the entire incubation after 2 weeks. A large amount of C was respired during the 42 day incubation with the oak respiring 27% of the total C at 25 °C and 33% at

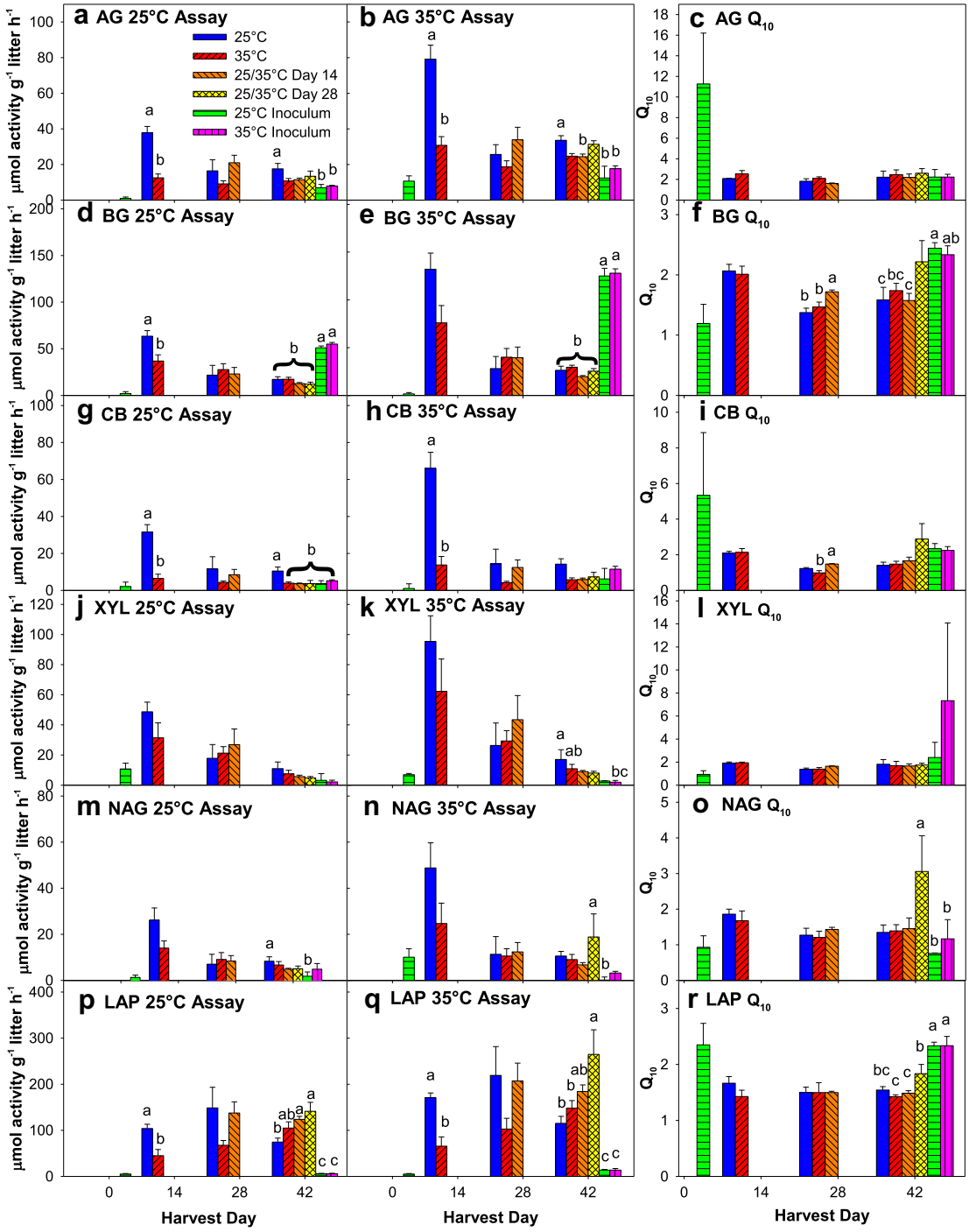

Fig. 2. Potential enzyme activities in oak litter slurries and Q_{10} of enzyme activity over time for litter at 25 °C, litter increased from 25 °C to 35 °C, litter at 35 °C, and samples with only soil inoculum. Enzymes are α-glucosidase (AG) (a, b, c), β-glucosidase (BG) (d, e, f), cellobiohydrolase (CB) (g, h, i), xylosidase (XLY) (j, k, l), N-acetyl glucosaminidase (NAG) (m, n, o), and leucine-amino peptidase (LAP) (p, q, r). Left panel are the assays performed at 25 °C (a, d, g, j, m, p), center panel are the assays performed at 35 °C (b, e, h, k, n, q), and right panel are the Q_{10} values calculated from the two assay temperatures (c, f, i, l, o, r). Means with different letters at each harvest point are significantly different.

35 °C. The pine litter respired slightly more total C than the oak over the course of the incubation with 30% being respired at 25 °C and 38% at 35 °C. Very little respiration came from the samples containing only soil inoculum with the cumulative respiration from the litter samples at the end of the experiment being 20–30 times greater than the samples with only soil inoculum (Fig. 1a and b) and the samples containing only the soil inoculum having a constant respiration rate over time (data not shown).

3.2. Enzyme activities

At the Day 14 harvest point, in almost all instances potential enzyme activities were higher for the litters incubated at 25 °C than the litters incubated at 35 °C at both assay temperatures for oak (Fig. 2) and for pine (Fig. 3). On average, potential enzyme activities were twice as high in the 25 °C slurry incubations compared to the 35 °C incubations. By the Day 28 harvest, potential enzyme

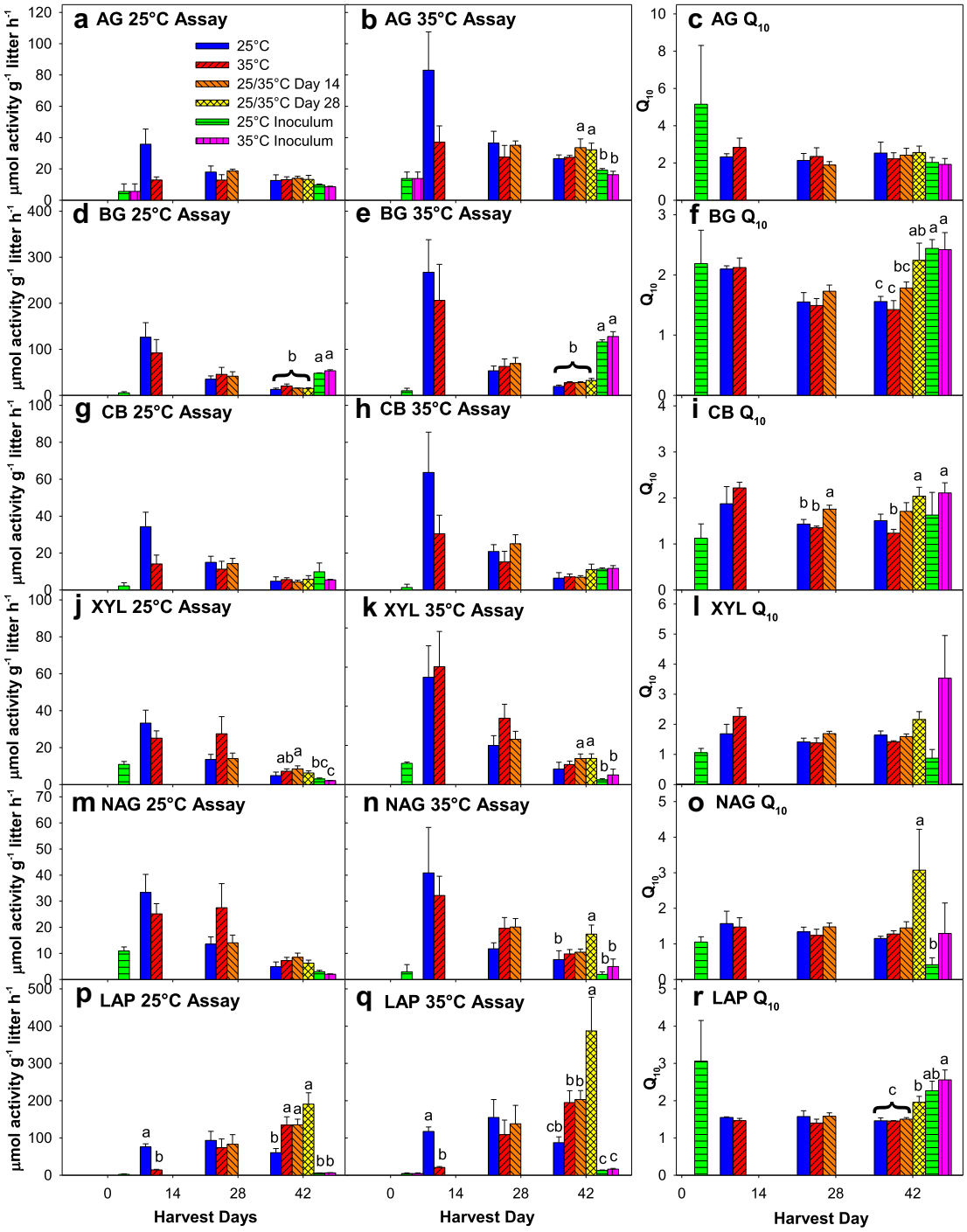

Fig. 3. Potential enzyme activities in pine litter slurries and Q_{10} of enzyme activity over time for litter at 25 °C, litter increased from 25 °C to 35 °C, litter at 35 °C, and samples with only soil inoculum. Enzymes are α-glucosidase (AG) (a, b, c), β-glucosidase (BG) (d, e, f), cellobiohydrolase (CB) (g, h, i), xylosidase (XLY) (j, k, l), N-acetyl glucosaminidase (NAG) (m, n, o), and leucine-amino peptidase (LAP) (p, q, r). Left panel are the assays performed at 25 °C (a, d, g, j, m, p), center panel are the assays performed at 35 °C (b, e, h, k, n, q), and right panel are the Q_{10} values calculated from the two assay temperatures (c, f, i, l, o, r). Means with different letters at each harvest point are significantly different.

activities had decreased for the litters incubated continuously at 25 °C with the exception of LAP for both pine and oak (Fig. 2p and q, Fig. 3p and q). At this time point, potential enzyme activities for the 25 °C, 35 °C, and bumped slurry incubations were similar. With the exception of the LAP enzyme, there tended to be a decline in the enzyme activity of the incubated litters over time with similar trends for both the oak and pine litter (Figs. 2 and 3). The LAP activity either increased or stayed constant over time with the samples bumped at Day 28 having the highest enzyme activity at the last harvest point (Fig. 2p and q, Fig. 3p and q). The pine litter had higher BG activity than the oak (Fig. 3e versus Fig. 2e), but otherwise both litters had similar amounts of enzyme activity. The enzyme activity of the soil inoculum added to the litter was relatively low at the time 0 harvest point and at the Day 42 harvest point was still relatively low. The one exception to this was the BG enzyme where the enzyme activity of the soil inoculum was twice as high as the enzyme activity of the litters (Fig. 2d and e, Fig. 3d and e). The Q_{10} of the enzyme activity for the litters, calculated from the 25 °C and 35 °C assay temperatures, was similiar for the different temperature treatments in most instances and over time and was approximately 2 (Figs. 2 and 3, right panel). The one exception to this Q_{10} trend occurred on Day 42, when the NAG Q_{10} for both the oak and pine litter bumped at Day 28 for NAG was approximately 3 (Figs. 2o and 3o). The soil inoculum also had high Q_{10} values in some instances, but there was high variability in these values.

At Day 14, the C:N acquisition ratio was wider in the litters incubated at 35 °C and the pine had wider C:N acquisition ratios that the oak (Table 2). This was also the trend at Day 28 with the bump litters having C:N acquisition ratios in between the two incubation temperatures for the oak and the values closer to the 35 °C incubation temperature for the pine. By Day 42 the differences between incubation treatments and the litter types was much smaller. There was a general decline in enzyme activity C:N over time for both litters. The 35 °C enzyme assay temperature always had a slightly wider C:N acquisition ratio than the 25 °C assay temperature (Table 2) indicating that C-degrading enzymes are more temperature sensitive than N degrading enzymes, consistent with previous studies (Koch et al., 2007; Wallenstein et al., 2009).

3.3. Fungal and bacterial biomass

Direct counts of fungi and bacteria of our slurry solution showed that our system was dominated by fungi on a biomass basis (Fig. 4e and f). In both the oak and pine litter, fungal and bacterial biomass was similar between the 25 °C and 35 °C incubation temperatures at Day 14. The oak had a wider fungi:bacteria ratio at this point which tended to narrow over time at the subsequent sampling points (Fig. 4e). At Day 28 there was a greater difference in fungal and bacterial biomass between the temperature treatments with the oak slurry incubated at 35 °C having significantly more fungal biomass than the oak slurry incubated at 25 °C (Fig. 4a). The pine litter slurry had a fairly constant fungi:bacteria ratio over time (Fig. 4f). As can be seen by the time 0 sampling point of the soil inoculum (Fig. 4), the inoculum provided an ample population of both fungi and bacteria for the system with the fungi:bacteria ratio initially widening from what was provided in the inoculum for both litters from 8 to 58 for the oak (Fig. 4e) and 1 to 8 for the pine after 14 days (Fig. 4f).

4. Discussion

The litter slurry approach enabled us to assess the temperature sensitivity of decomposition and the biological mechanisms driving decomposition rates in the absence of physical protection such as mineral interactions or soil aggregation. Temperature had little effect on respiration rates during the first 14 days of the incubation. There was also no effect of incubation temperature on fungal or bacterial biomass, indicating that microbial population growth was not thermally stimulated. Rather, microbial growth rates may have been limited by the supply of assimilable substrates that result from enzymatic depolymerization, even though enzyme activities should follow Arrhenius kinetics and thus be sensitive to temperature. The production of soluble C by enzymatic depolymerization has been proposed as the rate-limiting step in SOM decomposition (Bengtson and Bengtsson, 2007). In this experiment, when C-degrading enzyme activities were assayed at the respective temperature of the two slurry incubation temperatures, potential enzyme activity rates did not differ, indicating that *in situ* enzyme activities did not differ between the 25 °C and 35 °C incubations during this period. These data suggest that enzyme production decreased at 35 °C compared to 25 °C, consistent with a soil decomposition model that predicted that decreased carbon use efficiency (CUE) with increasing temperature (Steinweg et al., 2008), would result in decreased allocation of assimilated C towards enzyme production in response to warming (Allison et al., 2010). Although enzyme activities were sensitive to temperature in

Table 2
C:N stoichiometry of potential enzyme activities assayed at 25 °C and 35 °C on three harvest dates for the oak and pine litter incubated at 25 °C, 35 °C, and 25 °C bumped to 35 °C. Mean ± 1 s.e.m, $n = 4$. Shading corresponds to the magnitude of each mean enzyme C:N with darker shading indicating higher values.

	Harvest Day 14		Harvest Day 28		Harvest Day 42	
	25°C Assay	35°C Assay	25°C Assay	35°C Assay	25°C Assay	35°C Assay
Oak 25°C	0.85±0.03	0.90±0.02	0.55±0.07	0.57±0.06	0.64±0.03	0.67±0.03
Oak 35°C	0.90±0.02	0.98±0.04	0.75±0.06	0.77±0.07	0.60±0.01	0.67±0.01
Oak 25/35°C Day 14			0.62±0.08	0.67±0.07	0.52±0.01	0.57±0.01
Oak 25/35°C Day 28					0.50±0.04	0.58±0.02
Pine 25°C	1.03±0.08	1.09±0.06	0.79±0.07	0.81±0.06	0.60±0.04	0.65±0.04
Pine 35°C	1.23±0.08	1.30±0.06	0.83±0.11	0.87±0.11	0.60±0.03	0.62±0.02
Pine 25/35°C Day 14			0.83±0.10	0.87±0.09	0.56±0.02	0.62±0.03
Pine 25/35°C Day 28					0.51±0.03	0.58±0.02

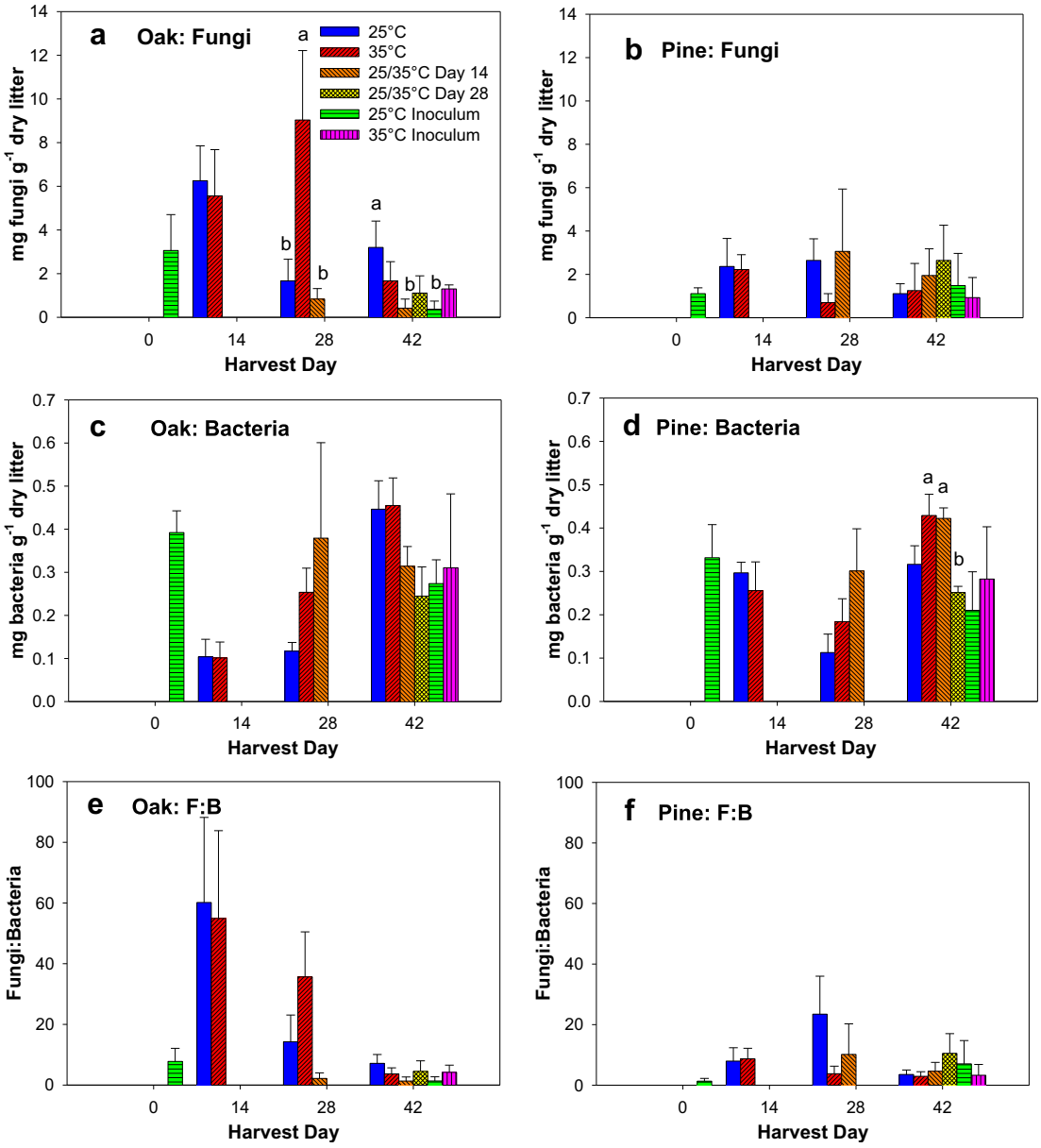

Fig. 4. Direct count microscopy for: fungal biomass for oak (a) and pine (b) litter slurries, bacterial biomass for oak (c) and pine (d) litter slurries, and fungal to bacterial ratios for oak (e) and pine (f) for litter at 25 °C, litter increased from 25 °C to 35 °C, litter at 35 °C, and samples with only soil inoculum. Means with different letters at each harvest point are significantly different, and the absence of letters indicates there are no significant differences.

the assays, biological regulation of enzyme production mitigated fundamental kinetic responses in the litter slurry incubations during this initial period. Since there was no temperature effect on fungal or bacterial biomass up to Day 14, it is unlikely that microbial uptake rates or catabolic rates were the rate-limiting factor in respiration.

After Day 14, decomposition rates were higher in slurries incubated at a constant 35 °C compared to those at 25 °C, consistent with our *a priori* prediction based on kinetic theory. It appears that C-degrading enzyme pools were similar during this period, and thus enzyme activities were higher at the warmer temperature. In oak litter, this coincided with much higher bacterial and fungal biomass in the 35 °C incubation compared to 25 °C at Day 28. We speculate that accelerated enzymatic decomposition in the 35 °C incubations after Day 14 supported increased microbial growth rates in the oak litter slurries. Thus, microbes gained a higher return (in the form of assimilable substrate) for their investment in enzymes at the higher temperature, allowing them to allocate more resources towards growth. When enzyme pool sizes are not affected by temperature, enzyme kinetics appear to drive the temperature sensitivity of decomposition.

For both litter types, the potential activity of C-degrading enzymes peaked by Day 14, indicating either decreased production or increased turnover after that date. Although little is known about the factors controlling enzyme turnover (Wallenstein and Weintraub, 2008), since abiotic conditions were constant, it is more likely that enzyme pool dynamics were the result of changes in enzyme production. The production of enzymes that degrade C-rich substrates may be regulated by substrate concentration (German et al., 2011). Thus, the decrease in C-degrading enzyme

potential activity after Day 14 may have been due to decreasing substrate availability as target substrates were decomposed. The LAP activity was less dynamic, but generally increased throughout the incubation. This may indicate that N became more limiting to microbial growth throughout the incubation, and the microbes were allocating more resources towards the acquisition of N contained in peptides.

Fungi dominated the biomass of the decomposing community, consistent with other field and lab studies, suggesting that the slurry technique did not strongly favor one group over the other. The fungi:bacteria biomass ratio increased in the initial stages of decomposition (from Days 0–14) for both litter types, consistent with other studies suggesting that fungi are the primary decomposers during early decomposition (Šnajdr et al., 2011). After the initial period, the fungi:bacteria ratio decreased suggesting an increasing contribution of bacteria to decomposition and possibly enzyme production. It is also possible that the increase in bacteria through time is due to bacterial utilization of fungal metabolites, which could be viewed as 'cheating' from an enzyme economics viewpoint (Allison, 2005). The litter slurry technique would be ideally suited to the application of proteomics to identify the taxa responsible for producing extracellular enzymes (Schneider et al., 2010).

4.1. Was the CQT hypothesis supported?

Pine litter decomposed more quickly than oak litter under equivalent conditions, consistent with its lower C:N and lignin:N ratios. We predicted that temperature would have a greater effect on the relative rate of decomposition for the more resistant oak litter than for the pine litter, based on litter quality. On the contrary, at Day 14 the decomposition of pine litter was more temperature sensitive than the decomposition of oak litter. Specifically, the Q_{10} for litter bumped from 25 °C to 35 °C at Day 14 was greater for pine than for oak. However, at Day 28, the temperature bump induced opposite Q_{10} responses for the two litters (i.e. Q_{10} was higher for oak than pine). Since the Q_{10} was measured immediately following the temperature bump, there should not have been time for significant enzyme production or microbial growth relative to the constant 25 °C incubation.

While chemical indices of litter quality such as C:N or lignin:N are useful in predicting decomposition rates over long periods, the temperature sensitivity of decomposition at any particular time point should be related to the quality of substrates that are being enzymatically depolymerized and subsequently consumed by microbes. At any time point, a diverse population of compounds is subject to enzymatic depolymerization, and the temperature sensitivity should be related to the aggregate activation energy of each enzyme–substrate reaction during the assay period. We speculate that before Day 14 the median activation energy of substrates being degraded in pine litter was higher than for oak litter. At this stage of decomposition, much of the mass loss can be attributed to soluble substrate (Berg and McClaugherty, 2008), which may differ substantially from the quality of bulk litter. By Day 28, the soluble substrate pool was likely depleted, and thus the compounds being decomposed were more representative of the whole-leaf stoichiometry and chemistry. Thus, we hypothesize that the temperature sensitivity of decomposition observed in this experiment was driven by the chemical complexity of the substrate pools that are accessible to enzymes rather than by the bulk chemistry of organic matter.

The stoichiometry of enzyme acquisition activity also changed throughout the incubation and was affected by temperature, suggesting that microbes were changing their relative allocation of resources towards the production of different enzymes. The ratio of C-degrading enzymes (represented by BG) to N degrading enzymes (LAP and NAG) (Sinsabaugh et al., 2008) decreased through time for both litters under all conditions, suggesting that microbes were responding to increased N limitation through time. In both litter types, the C:N enzyme acquisition ratio (when assayed at a common temperature) was generally higher for litter incubated at 35 °C than at 25 °C, indicating that microbes allocated more resources into C degradation than N degradation at the warmer temperature, although these differences declined through time. In addition, the Q_{10} of C-degrading enzymes was higher than that for N degrading enzymes on all harvest dates, suggesting that the production of assimilable C was higher than assimilable N with increasing temperature. Despite the increased efficacy of C-degrading enzymes at warmer temperatures, it appears that microbes still increased production of C-degrading enzymes at the higher temperature. This may indicate that CUE was lower at the higher temperature, requiring greater amounts of C to support microbial activity and growth since a higher proportion of assimilated C was lost through respiration.

4.2. Methodological considerations

The predominant approach to measuring leaf litter decomposition rates is to place litter encased in mesh bags on top of the soil surface. However, the litterbag technique has been recently criticized as a poor predictor of decomposition rates under *in situ* conditions (Cotrufo et al., 2010). Numerous varieties of laboratory incubations have been utilized to study fundamental controls on litter decomposition. Many of these studies do not explicitly inoculate litter with microbes, but rather rely on the extant community of microbes already present on litter at the collection time. Given the fundamental importance of microbial communities in litter decomposition (Strickland et al., 2009), the lack of microbial inoculation may be an important oversight and may affect decomposition dynamics.

The litter slurry approach provides a rapid, highly controlled method to study decomposition. In 42 days, 27–38% of litter mass was lost, based on cumulative respiration rates. While this technique allowed us to isolate the effect of temperature and to monitor biological dynamics, it does not mimic field conditions. For example, moisture is not a factor in this approach. The biological community likely differs from natural conditions and the slurry does not include soil fauna, which play an important role under natural conditions. This method would not be appropriate for estimating field decomposition rates.

4.3. Synthesis

The results of this study add a new dimension to our understanding of temperature effects on decomposition. Microbial responses to temperature, litter quality, and their interactions can affect decomposition in complex ways. Although the slurry incubation developed in this study did not approximate natural conditions for litter decomposition, it revealed that microbial dynamics strongly influence rates of decomposition and the apparent temperature sensitivity of decomposition. While it has previously been recognized that abiotic factors including physicochemical protection can affect the apparent temperature sensitivity for decomposition (Davidson et al., 2006; Gillabel et al., 2010; Conant et al., 2011), this study suggests that biotic factors may also be very important. Specifically, temperature can affect enzyme production, which in turn affects potential enzyme activities. Thus, while enzyme kinetics determine the temperature sensitivity of litter decomposition at any instant, microbial allocation to enzyme production can strongly affect the effect of warming on decomposition rates over longer time periods. This study provides only one

example of temperature effects on enzyme production. Additional studies are needed to understand whether the specific patterns observed here are generalizable.

Although this experiment focused on leaf litter, the mechanisms observed here are also pertinent to SOM decomposition. When SOM is available for enzymatic depolymerization, rates are determined by the frequency of substrate–enzyme interactions, and by enzyme kinetics, which are thermally sensitive. Respiration rates can also be influenced by microbial uptake, which may be rate-limiting in dry soils, and by microbial CUE, which may also be affected by temperature (Steinweg et al., 2008). The combined responses of enzyme production, enzyme kinetics, microbial uptake, and microbial metabolism to temperature are a critical control on ecosystem scale C balance (Allison et al., 2010). The litter slurry technique offers great promise for isolating biological controls on decomposition.

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