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ORIGINAL PAPER

Shifts in fungal biomass and activities of hydrolase and oxidative enzymes explain different responses of litter decomposition to nitrogen addition

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Abstract

Exogenous nitrogen (N) input is a key factor affecting litter decomposition. However, we have limited understanding on how anthropogenic N deposition affects the kinetics and thermodynamics of enzymes involved in litter decomposition. To understand how N enrichment influences litter decomposition, we conducted a field N-addition experiment with *Castanopsis chinensis* (CC) and *Schima superba* (SS) leaf litter. We examined microbial community composition, activities of hydrolases and oxidative enzymes, and hydrolase kinetics and thermodynamics. The litter mass remaining after 18 months of decomposition in N-addition plots was 1.9–2.3 and 1–1.3 times higher than control for CC and SS, respectively. During the early stage of litter decomposition, N addition increased hydrolase activities involved in carbon (C) and N mineralization for both litter types. N addition slowed CC litter mass loss, and reduced the activities of lignolytic enzymes and catalytic efficiency (V_{max}/K_m) of hydrolases in the later stage (9–18 months) of decomposition. N addition had minimal effect on the activation energy (Ea) of enzymes. Our study identifies how enzyme kinetics regulate litter decomposition under N fertilization, and lignin enrichment as decomposition progresses due to microbial N mining effect limits the accessibility of lignin-encrusted structural carbohydrates to hydrolases, which subsequently decreases the energy source of the entire microbial community for enzyme production.

Keywords Enzyme kinetics · Enzyme thermodynamics · Litter decomposition · Microbial community · N deposition · Substrate availability

Introduction

Plant litter decomposition is a key biogeochemical process that helps maintain soil fertility and affects the balance between the terrestrial carbon (C) pool and atmospheric CO_2 concentrations. Litter decomposition rate is significantly influenced by climate, litter chemistry, and microbial community composition (Swift et al. 1979). Atmospheric nitrogen (N) deposition in terrestrial ecosystems is expected to range between 60 and 100 Tg N $year^{-1}$ by the end of this century, with profound consequences for ecosystem C dynamics (Lamarque et al. 2005). Although the effect of elevated N deposition on decomposition and soil C stocks has garnered much attention (Berg and Matzner 1997; Sinsabaugh et al. 2002; Zak et al. 2008; Hobbie et al. 2012; Xia et al. 2017; Carrara et al. 2018), we still do not fully understand the regulatory mechanisms (van Diepen et al. 2017; Purahong et al. 2018; Entwistle et al. 2018b).

Previous studies have shown that N addition can initially enhance decomposition by increasing the activity of polysaccharide-degrading enzymes (van Diepen et al. 2015; Djukic et al. 2018), but inhibit decomposition during the later stage of litter decomposition (Sinsabaugh et al. 2002; Hobbie et al. 2012). Several hypotheses have been proposed to

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explain these transitory dynamics (Fig. 1). Litter decomposition rate is regulated by the stoichiometry of plant litter (C/N ratio) and microbial demands for resources (Berg and Staaf 1980). Alleviation of microbial N limitation can accelerate the breakdown of low quality litter with a high C/N ratio (i.e., the "stoichiometric theory") (Hobbie et al. 2012; Talbot and Treseder 2012). The "microbial N mining" hypothesis posits that if microbial N constraints are alleviated with N addition, the production of lignin-degrading enzymes is suppressed and lignin decomposition reduced (Moorhead and Sinsabaugh 2006; Entwistle et al. 2018b). The "browning effect" postulates that exogenous N stimulates chemical reactions between polyphenols and amino compounds, resulting in the formation of recalcitrant aromatic chemicals with a higher activation energy (Ea) and reduction of lignin decomposition (Fog 1988; Davidson and Janssens 2006). The exact mechanisms responsible for reductions in litter decomposition after N enrichment remain unclear (Rinkes et al. 2016; Zhang et al. 2016).

Microorganisms decompose litter through the production of extracellular enzymes (Moorhead and Sinsabaugh 2006; Nannipieri et al. 2018), and they allocate resources to enzyme production depending on energy and/or nutrient availability and specific growth requirements (Allison and Vitousek 2005). Past studies have examined enzyme activity under N enrichment, but it is difficult to build a direct mechanistic connection between enzyme expression and substrate status because enzyme kinetics and thermodynamics have not been thoroughly investigated (Sinsabaugh et al. 2009, 2014). Enzyme activity is controlled by substrate concentrations, the intrinsic property of an enzyme as described by the Michaelis-Menten function (Dick 2011), and the catalytic

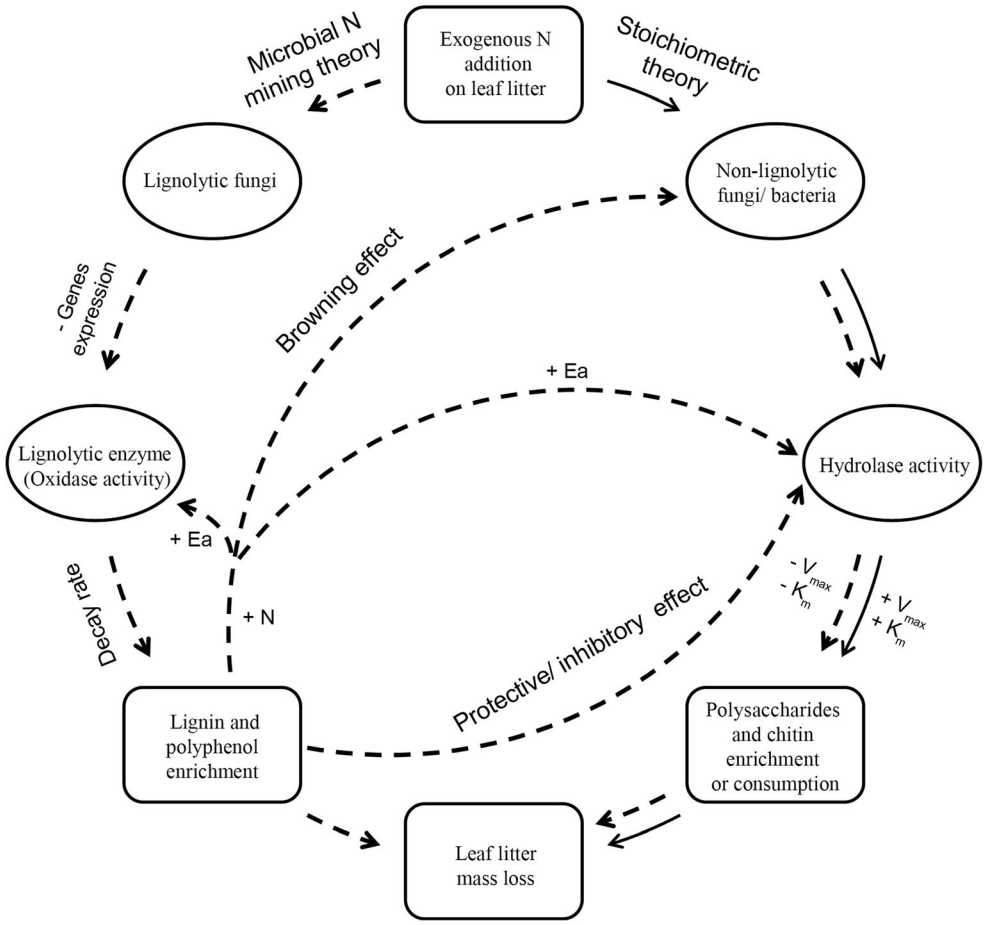

Fig. 1 Conceptual diagram of microbial community and enzyme responses to N addition. Ovals indicate microbial functional guilds and enzyme activity. The polysaccharide (cellulose and hemicellulose) and chitin decomposers are organized into non-lignolytic fungi and bacteria. Squares indicate changes in litter properties during decomposition. Solid and dashed arrows indicate potential positive and negative effects, respectively. We expected exogenous N addition to increase enzyme activity by alleviating the N limitation of microbial communities, which can stimulate the decomposition of available C compounds. As decomposition progresses, N addition decreases lignolytic fungal abundance and activity due to microbial N mining effect. Then, the enrichment of lignin and polyphenol may limit the accessibility of lignin-encrusted celluloses and directly decrease hydrolase activity. Furthermore, exogenous N can cause the "browning effect," resulting in substrates with higher recalcitrance and increased activation energy (Ea). Combined together, these processes determine the extent to which N addition affects litter decomposition

properties of the performing isoenzymes (Wallenstein et al. 2011). The maximum catalytic rate (V_{max}) is used as an indicator of overall isoenzyme activities and the amount of enzymes. The half saturation constant (K_m) reflects the apparent affinity of enzymes to substrates, which may vary with microbial community succession (e.g., r- vs. k-strategists) during the process of litter decomposition (Tischer et al. 2015; Loeppmann et al. 2016). The catalytic efficiency (V_{max}/K_m) is an indicator of the potential enzyme activity with respect to substrate quantity and quality (Triebwasser-Freese et al. 2015; Loeppmann et al. 2016). Previous studies found that N addition can increase V_{max} by increasing microbial energy demand, but the effects on K_m depended on soil nutrient status (Stone et al. 2012; Zhang et al. 2017). Substrate reaction rates can also be increased through reductions of enzyme activation energy (Ea), as described in the Arrhenius function (Davidson and Janssens 2006). Investigations of enzyme kinetic (V_{max} and K_m) and thermodynamic (Ea) parameters could elucidate the effects of N enrichment on decomposition dynamics.

The goal of this study was to determine how N addition affects the kinetic and thermodynamic properties of enzymes, microbial community composition, and litter chemistry. We posited that (1) N addition would accelerate litter decomposition via increasing V_{max} and K_m of enzymes during the early stage of decomposition; (2) N addition would retard lignin decomposition during the later stage of decomposition by reducing the V_{max} and K_m of enzymes; and (3) N addition would increase the Ea of enzymatic reactions.

Materials and methods

Experimental site

To test the above hypotheses, we conducted a field N addition experiment at the Dinghushan Biosphere Reserve (DHSBR) in southern China (23° 09′ 21″~23° 11′ 30″ N, 112° 30′ 39″ ~112° 33′ 41″ E). The site has a subtropical monsoon climate. Mean annual precipitation is 1927 mm, with approximately 75% of precipitation occurring between March and August. Mean annual temperature is 21 °C, ranging from 12.6 °C in January to 28 °C in July (Mo et al. 2006). In the study area, the evergreen broadleaf forest is about 110 years (Mao et al. 2017), with average tree height and the average diameter at breast height at 5.7 m and 6.6 cm, respectively (Wang et al. 2019). The ecosystem is dominated by *Castanopsis chinensis*, *Schima superba*, *Cryptocarya chinensis*, *Cryptocarya concinna*, *Machilus chinensis*, and *Syzygium rehderianum* (Wang et al. 1982). The main soil type is mountain red soil (Argic Rhodic Alliti-Udic Ferrosols). In 2008–2009, annual N wet deposition in the region surrounding DHSBR was 29.5 kg N ha^{-1} $year^{-1}$ (Sheng et al. 2013).

Experimental design

The N addition experiment was established in September 2014 in an area of about 100 m × 70 m, with a slope of 20° at 300–355 m elevation, with 50 m perpendicular throw (Fig. A1). We added NH_4NO_3 (AR, Guangzhou Chemical Reagent Factory, China) to represent the main components of N deposition in this area (Fang et al. 2015). We established four N treatments that span the range of N deposition rates expected at our site (Fang et al. 2015; Xu et al. 2015): Control (0 N added), Low-N (35 kg N ha^{-1} $year^{-1}$), Medium-N (70 kg N ha^{-1} $year^{-1}$), and High-N (105 kg N ha^{-1} $year^{-1}$). The experimental treatments were randomized and replicated ($n = 3$). Each experimental plot area was 225 m^2 (15 m × 15 m) with 10-m-wide buffer strips between plots. We applied NH_4NO_3 using a backpack sprayer during the last week of every month. The fertilizer was mixed with 30 L of stream water, while control plots received an equivalent volume of stream water. The total amount of water applied over the course of the experiment was equivalent to 1.6 mm of annual precipitation. The main soil biochemical properties are shown in Table 1. We sampled soils (0–10 cm) from each plot in July 2015. Soil samples were air-dried and sieved (2.0 mm) prior to chemical analyses (details described in Nie et al. (2018)).

Litter decomposition study

We measured litter mass loss using the litterbag method. While we are well aware that the litter bag method may underestimate mass loss due to inhibited fragmentation (Cotrufo et al. 2010, 2015), it is a valid method to retrieve litter at different stages of decomposition. We chose two leaf litter from two plant species (*Castanopsis chinensis*, CC and *Schima superba*, SS) that were abundant at the site and contrasted in chemistry. SS litter had higher lignin, P, Ca, Mg, Mn, and soluble sugars than CC litter, but had lower K concentration than CC litter (Table A1). Freshly fallen leaves were collected from the forest floor immediately after abscission during peak litterfall in March 2015. All litters were air-dried before use. Litter sub-samples were dried at 65 °C for dry weight correction and chemical analyses.

Litter bags were 15 × 25 cm, made of 1 mm mesh polyvinyl screen, filled with 10.00 g of air-dried litter of one litter species and sealed by nylon strings. In order to have enough material for subsequent analyses (see below), we incubated five bags connected together as replicate units per harvest and plot. A total of 420 litter bags of each species were prepared. Thirty-five litterbags of each species were evenly deployed on each plot at the end of April 2015. To prevent the litter bags from turning, we used plastic rods to fix them to the forest floor. Litter bags were collected at 0, 2, 4, 6, 9, 12, and 18 months from the start of incubation.

Table 1 Soil properties under low (LN, 35 kg N ha^{-1} $year^{-1}$), medium (MN, 70 kg N ha^{-1} $year^{-1}$), and high (HN, 105 kg N ha^{-1} $year^{-1}$) N treatments. TOC: total organic C (g kg^{-1}), TN: total N (g kg^{-1}), TP: total P (g kg^{-1}), MBC: microbial biomass C (mg kg^{-1}), MBN: microbial biomass N (mg kg^{-1}). Data are means (SD). Significant differences ($P < 0.05$) among treatments are indicated by different letters

Parameter	Control	LN	MN	HN
pH	3.90 (0.03) a	3.90 (0.01) a	3.84 (0.02) b	3.80 (0.02) c
TOC	25.5 (3.33) a	25.8 (4.69) a	25.7 (1.74) a	27.6 (2.87) a
TN	1.86 (0.13) a	1.70 (0.38) a	1.99 (0.52) a	1.89 (0.32) a
TP	0.23 (0.02) a	0.24 (0.04) a	0.24 (0.01) a	0.23 (0.04) a
MBC	300 (87.8) a	139 (42.8) b	136 (47.3) b	192 (14.3) b
MBN	40.0 (18.7) a	24.2 (11.6) ab	15.7 (2.97) b	18.5 (0.74) ab
C/N	13.6 (0.84) a	15.3 (0.97) a	13.3 (2.42) a	14.7 (1.01) a
C/P	110 (14.9) a	108 (8.38) a	109 (10.1) a	121 (10.6) a
MBC/MBN	8.42 (3.71) a	6.02 (0.98) a	8.66 (2.45) a	10.4 (1.16) a

At every harvest, bags were put into sterile plastic bags and brought to the lab with a cooler. Soil, root, and other objects attached to the bags were removed by hand, then bags were opened, and the remaining litter collected and weighed within 2 days. The litter was then cut into 0.25-cm^2 pieces using scissors after soil particles attached to litter were removed by brush. Litter subsamples were used to assess residual oven-dry mass (65 °C) and chemical properties. Additional subsamples were frozen (− 20 °C) for 2 weeks before analyses of microbial biomass, phospholipid fatty acid (PLFA), enzyme activity, and kinetics assays.

Litter mass loss was best fit (Table A3) by the single negative exponential decomposition function (Olson 1963):

$$X_t = X_0 e^{-kt}$$

where X_t is the amount of litter remaining at time t (month), X_0 is the original amount of litter. This was used to estimate mass loss rates (k).

Litter chemistry

The dry litter was milled to a fine homogenous powder (through a 0.165-mm sieve) before chemical analyses. Total organic C (TOC) content was measured based on the oil bath heating method (Nelson and Sommers 1996). The total N content of the litter was analyzed using the Kjeldahl method (Bremner and Mulvaney 1982). Total phosphorus (P) concentration was measured with molybdenum-antimony anti-spectrophotometric method after samples were digested with mixed acid ($H_2SO_4 + HClO_4$) (Bray and Kurtz 1945). The concentrations of elements (K, Mg, Mn, and Ca) were determined by flame atomic absorption spectrophotometer (contrAA 700, Analytik Jena AG, Germany) after the samples were wet acid oxidized ($HNO_3 + HClO_4$) (Zarcinas et al. 1996). In order to check the validity of the analysis, one national standard litter sample (GBW07603, China) was used as reference sample for chemical element concentration. Soluble sugars were extracted with aqueous ethanol and then determined by anthrone reagent (Chow and Landhausser 2004).

Total acid detergent fiber (ADF) and lignin were determined gravimetrically as the dry weight of solids after sequential hydrolysis with sulfuric acid using FiberteCap TM2021/2023 fiber analysis system (FOSS, Sweden). Subsequently, 0.5 g of dry litter samples was weighed into specimen cups and soaked in boiling acid detergent (dissolve 20 g cetyltrimethylammonium bromide in 1 L 0.5 M H_2SO_4) for 1 h, then washed three times with hot distilled water and acetone. Cups were dried for 5 h at 105 °C. The dry solid residue in the cup was the litter ADF component. The dried cups were soaked in cooled 72% sulfuric acid for 3 h, and then washed several times with hot distilled water until free of acid. Cups were dried at 105 °C for 5 h and weighed. Then, cups were combusted in a muffle furnace at 550–600 °C for 4 h. The acid non-hydrolyzable fraction was determined by subtracting the ash weight, and used as a proxy for lignin (Soong et al. 2015). The cellulose content was determined by subtracting the ash and lignin weight from ADF.

Microbial biomass and available substrate

Litter microbial biomass C (MBC) and N (MBN) were analyzed by the chloroform fumigation-extraction (0.5 M K_2SO_4, ratio of litter to K_2SO_4 solution, 1:5 dry wt/v) procedure (Wu et al. 1990). Litter extraction was measured with a TOC auto-analyzer (Shimadzu, Kyoto, Japan). Microbial biomass P (MBP) was estimated by extracting both fumigated and non-fumigated litter samples with 0.03 M NH_4F-0.025 M HCl (litter/solution ratio of 1:4) (Wu et al. 2000). The inorganic P concentration in the extracts was determined colorimetrically after it was digested with $HClO_4$ (70%, v/v) for 1 h. The MBC, MBN, and MBP concentrations were calculated using an extraction efficiency of 0.45, 0.54, and 0.40, respectively (Wu et al. 1990). Microbial biomass was expressed in milligrams per kilogram of dry litter. The concentration of available C, N, and P was equal to the concentration of extractable C, N, and P in the non-fumigated samples.

Microbial community

The PLFA profiles were analyzed following the method of Frostegård et al. (1993). In total, we obtained PLFAs (40–65 species per gram dried litter) having 12–24 C atoms in all samples. PLFAs were assigned to microbial groups according to Smith et al. (2015) represented species of broad taxonomic groups. Bacterial-specific PLFAs were 16:0 10 methyl, 17:0 10 methyl, 18:0 10 methyl, 19:0 10 methyl, 14:0 iso, 15:0 anteiso, 15:0 iso, 16:0 iso, 16:0 anteiso, 17:0 iso, 17:0 anteiso, 16:1 ω7c, 16:1 ω9c, 17:1 ω8c, and 18:1 ω5c. The amounts 14:0 iso, 15:0 anteiso, 15:0 iso, 16:0 iso, 16:0 anteiso, 17:0 iso, and 17:0 anteiso were considered to represent the relative abundance of gram-positive bacteria (G^+). The amounts, 16:1 ω7c, 16:1 ω9c, 17:1 ω8c, and 18:1 ω5c were considered gram-negative bacteria (G^-). Actinobacteria PLFAs were 16:0 10 methyl, 17:0 10 methyl, 18:0 10 methyl, 19:0 10 methyl, 18:1 ω9c and 18:2 ω6, 9c were considered fungal indicators (Smith et al. 2015). The ratio of fungal to bacterial (F/B) and G^+/G^- PLFAs were also used to describe microbial community composition.

Enzyme activity, kinetics, and thermodynamics

For our study, we chose primary rate-limiting enzymes (Baldrian and Šnajdr 2011). These were: 1,4-β-glucosidase (GLU) and cellobiohydrolase (CB) for cellulose decomposition; 1,4-β-xylosidase (XYL), involved in hemicellulose mineralization; phenol oxidase (PPO) and peroxidase (POD), typical ligninolytic oxidoreductase; 1,4-β-N-acetylglucosaminidase (NAG), involved in chitin decomposition; acid phosphomonoesterase (ACP); and leucine amino peptidase (LAP) which hydrolyzes ester-bonded phosphate and protein, respectively. According to the methods of Bell et al. (2013), hydrolases (GLU, CB, XYL, NAG, and ACP) were assayed fluorometrically using 4-methylumbelliferone substrates, while L-leucine-7-amido-4-methylcoumarin was used for the LAP. Oxidative enzymes, PPO and POD, were measured using L-3,4-dihydroxyphenylalanine (L-DOPA) and hydrogen peroxide as substrates, respectively (Bach et al. 2013). All substrates, 4-methylumbelliferone, and 7-amido-4-methylcoumarin were purchased from Sigma-Aldrich Corporation.

Before enzyme activity was assayed, we measured the litter enzyme activity with different concentrations in order to find the saturated substrate concentration for each hydrolase. GLU, NAG, and LAP were saturated at a fluorigenic substrate concentration of 1000 μM in these samples; CB, ACP, and XYL were saturated at a fluorigenic substrate concentration of 750, 2000, and 1500 μM, respectively. Hydrolase activity was calculated according to standard curves correlating with fluorescence. The activities of oxidative enzymes were calculated according to Bach et al. (2013). Enzyme activity was expressed in nanomoles per gram per hour.

To measure the kinetics and thermodynamics of each enzyme, we used substrate concentrations ranging from 50 to 1000 μM for GLU, NAG, and LAP, 35 to 750 μM for CB, 100 to 2000 μM for ACP, and 150 to 1500 μM for XYL. Litter slurry and substrate were incubated at four temperatures (10, 20, 30, and 40 °C) for 2–6 h. We chose this temperature range (10–40 °C) in order to match the range of topsoil temperature variation (10–42 °C) in the study area. Within this temperature range, the thermodynamic characteristics of soil enzymes can remain stable and the Arrhenius plots are linear for most enzymes (Trasar-Cepeda et al. 2007; Paz-Ferreiro et al. 2015). Similar temperature range has been adopted to study soil enzyme thermodynamics in many previous studies (Yan et al. 2010; Khalili et al. 2011; German et al. 2012; Stone et al. 2012; Triebwasser-Freese et al. 2015). To analyze the effect of N on enzyme properties, the enzyme activity and kinetic parameters measured at 40 °C were used. This temperature is slightly higher than the commonly used temperature of 37 °C for soil enzyme assay (Hui et al. 2013; Dick et al. 2018; Margenot et al. 2018; Nannipieri et al. 2018), but is the closest temperature level with respect to the designed temperature gradient (10–40 °C). We chose to focus on the kinetic characteristics of hydrolases, which can be readily measured due to the binding of a single substrate to a single catalytic site (Triebwasser-Freese et al. 2015). However, as the catalytic reaction of oxidoreductase is affected by the concentration of the reducing substrate and other electron acceptor (H_2O_2), its saturation kinetics rarely follow the Michaelis-Menten function (Triebwasser-Freese et al. 2015).

Data analyses

The values of K_m and V_{max} are estimated by the slop and intercept of the Lineweaver-Burk function, respectively (Nannipieri and Gianfreda 1998; Dick 2011):

$$\frac{1}{v} = -\frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}}$$

where V_{max} (nmol L^{-1} h^{-1}) is the apparent maximum velocity, K_m (nmol L^{-1}) is the apparent Michealis-Menten constant, V is the initial velocity (nmol L^{-1} h^{-1}), and $[S]$ is the initial substrate concentration (μM). In this study, we observed that the amount of released products increased linearly with the incubation time (< 9 h). Therefore, we used the velocity at the regular incubation time as the initial velocity for each temperature.

The activation energy (Ea, kJ mol^{-1}) was calculated from the Arrhenius equation (Davidson and Janssens 2006):

$$\ln(k) = \ln A - \frac{Ea}{RT}$$

where k is the catalytic turnover rate, A is a pre-exponential factor, R is the ideal gas constant, and T is the assay temperature (K). In this study, k is substituted as V_{max} because V_{max} is proportional to k when the concentration of enzyme keeps constant (Allison et al. 2018). In this study, we estimated the Ea of oxidative enzyme by their activities at 25 mM dopamine that may approximate to V_{max} (Rodríguez-López et al. 2000).

We found that there were lower amounts of DOC, DON, and DOP after 6 months of decomposition in this experiment, which we used to denote the early versus later stages of decomposition (Soong et al. 2015). Effects of N deposition were assessed on cumulative enzyme activities (early vs. later stage) that can be tallied as activity-days, which is analogous to the accumulated temperature used to quantify the cumulative influence of temperature (Sinsabaugh et al. 2002). These were calculated for each enzyme by integrating the area under the curve of the enzyme activity plotted against time using the trapezoidal rule (Carreiro et al. 2000). All the data were explored for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene's test). Microbial properties and litter chemistry were log-transformed prior to analysis where necessary to approximate normal distribution. For each litter type, differences in N treatments in cumulative enzyme activity and litter mass remaining were examined using one-way ANOVA. Effect of litter type, N treatment, decomposition time (all the months of sample collection), and their interactions on element concentration, microbial biomass, enzyme kinetics, and thermodynamics were examined using three-way ANOVA. In this model, block was selected as random effect. The Tukey honest significant difference test was use for pairwise comparisons. The Pearson correlation analysis on elements concentration, microbial biomass, microbial abundance, enzyme activity, and kinetic parameters was performed to reveal how litter chemistry may correlate with microbial community and activity during decomposition. To investigate how substrate availability drove microbial enzyme allocation, we used linear regressions between the concentration of available substrate (available C and N) and kinetic parameters (OriginPro 2016, OriginLab, MA, USA). The F test was used to estimate the goodness of fit for all models. Statistical significant differences were set at $P < 0.05$ unless otherwise stated. All statistical analyses and calculations were conducted with the SPSS 22.0 software (SPSS Inc., Chicago, USA) and Excel 2013 (Microsoft Corporation, Redmond, USA).

Results

Litter mass remaining and chemical dynamics during decomposition

During the 18 months of study, mass remaining for the CC and SS litter in all treatments ranged from 17.8 to 40.1%, and from 24.8 to 31.5%, respectively (Fig. 2a and e). Overall, litter type and N treatment affected litter mass loss (Table A2). CC litter had more remaining mass than SS litter except for the control treatment ($P < 0.001$). When we separated by litter type to examine the effects of N on mass loss, we found N addition significantly increased the remaining mass of CC litter ($P < 0.001$), but had no effects on SS litter ($P > 0.05$). The post hoc pairwise comparisons showed that the LN and MN, MN and HN treatments significantly increased the mass remaining of CC litter at 9 and 18 months, respectively (Fig. 2a, $P < 0.05$). After 18 months of decomposition, the amounts of mass remaining for CC and SS litter in the N treatments were 1.9–2.3 and 1–1.3 times more than the control, respectively (Fig. 2a and e). However, mass loss rates (k) were not significantly affected by either the litter type or N treatment ($P > 0.05$, Table A3).

During decomposition, N, P, ADF, and lignin concentrations increased (fraction of initial concentration > 100%) in both litter types, while there was substantial loss of other nutrients ($P < 0.001$, Tables A2, A4, and A5). In comparison with control, overall N treatments increased the loss of DOC, DON, Ca, Mg, and Mn from CC litter, but decreased C/N ratio and lignin decomposition ($P < 0.001$, Fig. 2d and Tables A2 and A4), with the post hoc pairwise comparisons showing that the MN and HN treatments significantly increased lignin mass remaining at 6 and 12 months (Fig. 2d, $P < 0.05$). After 18 months of decomposition, the amounts of soluble sugars and cellulose mass remaining for CC litter in the N treatments were 0.4–0.9 times lower and 1.4–1.6 times higher than control, respectively (Fig. 2b and c). By contrast, N addition decreased Mg concentration and had varying effects on lignin decomposition in SS litter ($P < 0.05$, Fig. 2h and Tables A2 and A5). The post hoc pairwise comparisons showed that the amount of cellulose mass remaining in the MN treatment was significantly lower than the LN and control treatment at 9 months (Fig. 2g, $P < 0.05$), and the HN treatment significantly decreased lignin mass remaining in comparison with the LN and control treatment at 9 months (Fig. 2h, $P < 0.05$). However, the amount of lignin mass remaining in the HN treatment was only significantly lower than the LN treatment at 18 months.

Microbial dynamics during decomposition

N amendment decreased total PLFAs in CC litter ($P < 0.05$) due to the decrease in fungal PLFAs (Fig. 3a, $P < 0.05$). By contrast, the total PLFAs in SS litter were not affected by N addition (Fig. A2). N addition also significantly decreased MBC, MBN, and MBP associated with CC litter, but only significantly decreased MBP in the SS litter ($P < 0.05$). The composition of main

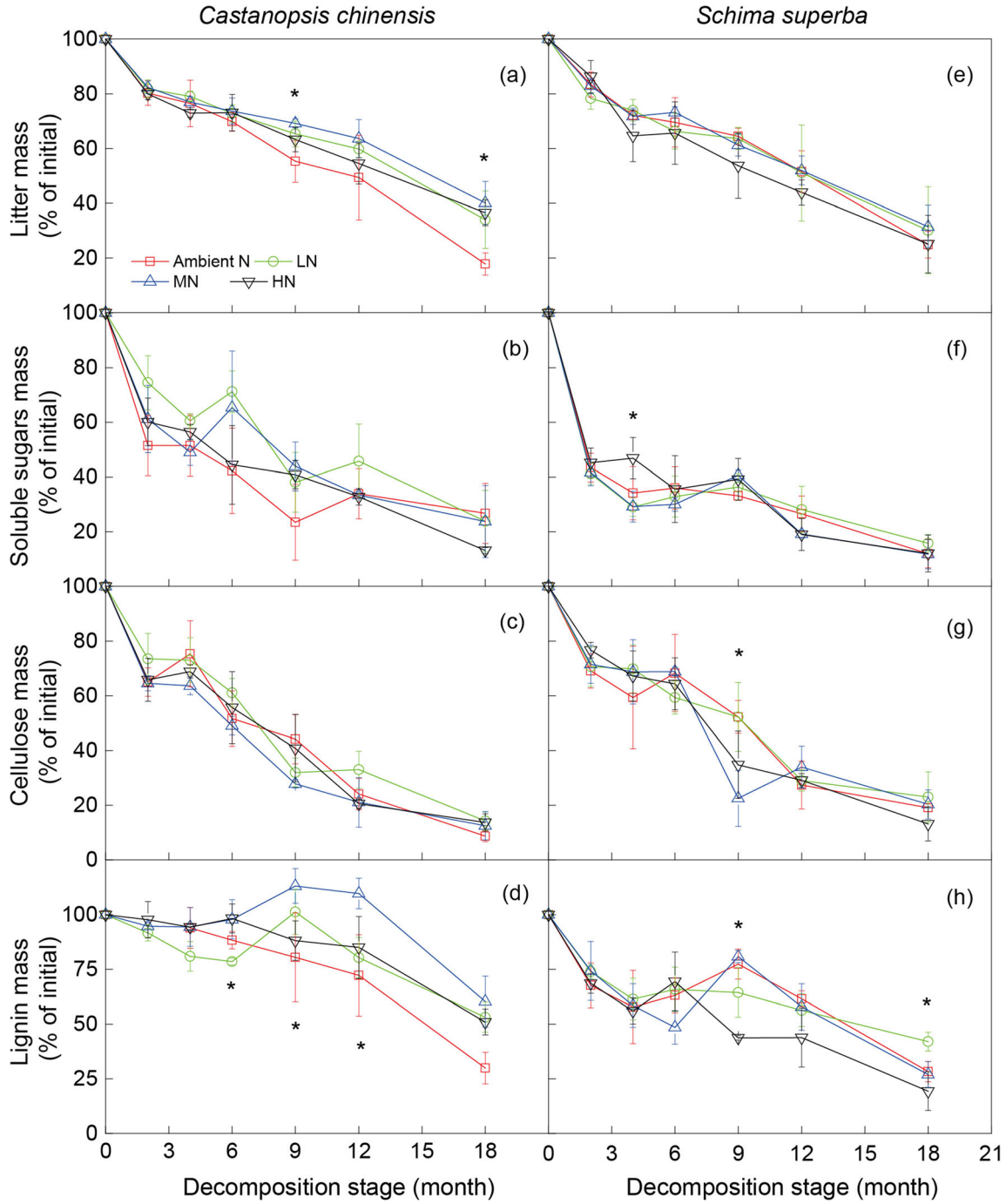

Fig. 2 Litter, soluble sugars, cellulose, and lignin mass remaining of two litter species exposed to four levels of N addition. Control: ambient N deposition, LN: low N addition, MN: medium N addition, HN: high N addition. Asterisks denote a significant difference ($P < 0.05$, ANOVA) among N treatments per time. Error bars mean standard deviation ($n = 3$)

microbial groups (F/B and G^+/G^-) was not significantly affected by N addition in either of the two litter types (Fig. A2). Overall, total PLFAs and F/B associated with the two litters decreased steadily through time, while G^+/G^- increased (Fig. A2). N and lignin concentration exhibited significantly negative correlations with fungal PLFAs, while significantly positive correlations existed with bacterial PLFAs (Table A6).

Enzyme activities

Enzyme activities varied with decomposition time and N treatment in both litters (Table A7). The effects of N addition on relative cumulative enzyme activity varied with litter type and decomposing time (Table A7 and Fig. 4). At the early decomposition stage (0 to 6 months), HN treatment generally increased the activities of hydrolases involved in C and N

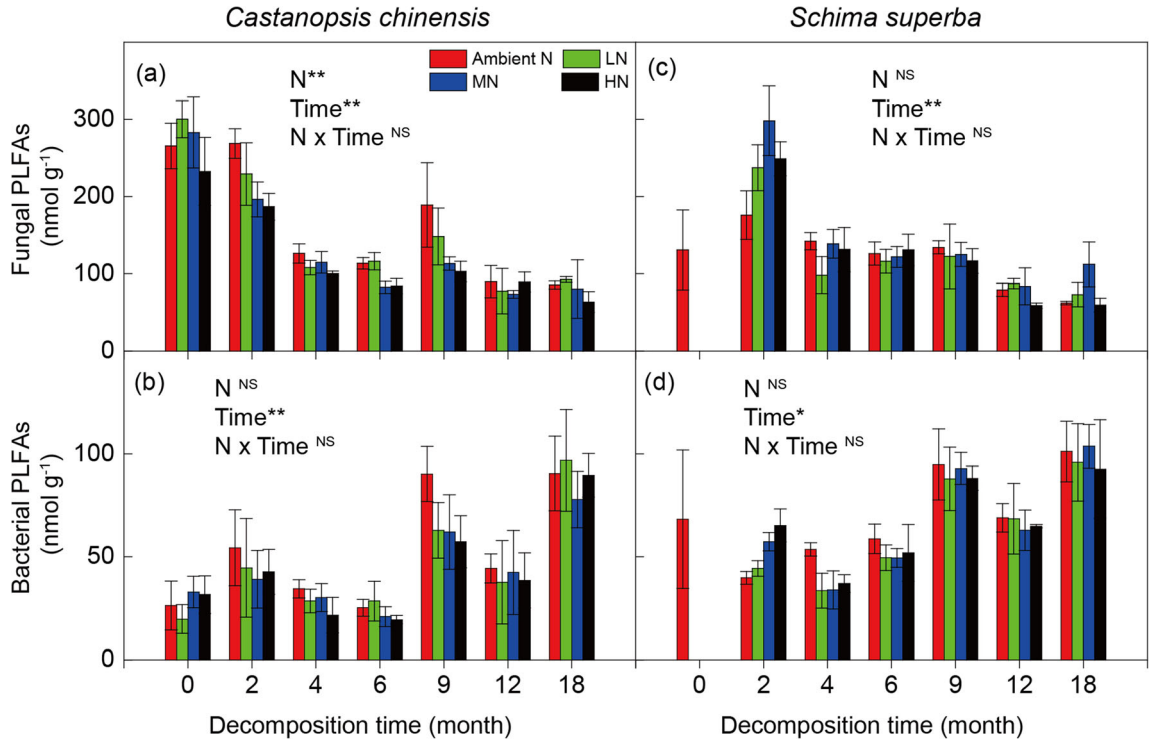

Fig. 3 Abundance of fungi and bacteria during the decomposition of two litter species exposed to four levels of N addition. Asterisks indicate a significant effect of N or decomposition time (*$P < 0.05$ and **$P < 0.01$, ANOVA). Error bars mean standard deviation ($n = 3$). NS represents no significant difference among N treatment and/or decomposition time

mineralization for both litter types (CC: GLU, $P < 0.05$; NAG, $P < 0.001$; SS: GLU, CB, XYL, $P < 0.05$), but decreased XYL ($P < 0.05$) activity for CC litter (Fig. 4a and b). Additionally, N treatments (except SS litter in LN treatment) did not significantly decrease the activities of oxidative enzymes (POD and PPO) (Fig. 4a and b). At the later decomposition stage (> 6 months), all N treatments significantly decreased PPO activity regardless of litter type (except LN treatment in SS litter); however, POD activity was only inhibited by HN treatment in SS litter (Fig. 4c and d, $P < 0.05$). N enrichment significantly decreased hydrolase activities in CC litter (Fig. 4c, $P < 0.05$). However, hydrolase activities in SS litter did not significantly decrease with N addition (Fig. 4d).

Kinetic and thermodynamic characteristics

Kinetic parameters varied with N treatments, litter type, and decomposition stage (Table A7). In general, N addition increased the V_{max} (average lnRR = 1.03–1.12, Fig. 5a) and K_m (average lnRR = 1.06–1.24, Fig. 5b) of most hydrolases in CC litter during the early stage of decomposition, but this effect diminished over time and even reversed during later stage (> 6 months) of decomposition except for XYL (average V_{max} lnRR = 0.60–0.77, average K_m lnRR = 0.60–0.94, Fig. 5a and b). Significant effects of N addition on V_{max}/K_m were found for GLU, CB, XYL, NAG, and LAP ($P < 0.05$). Later in decomposition, the average lnRR of V_{max}/K_m ranged from 0.60 to 0.99 (Fig. 5c). In SS litter, N addition decreased the V_{max} of GLU, CB, and LAP but increased that of XYL, NAG, and ACP at the early stage of decomposition. N addition decreased K_m of hydrolases except that of ACP. However, at the later stage of decomposition, N addition decreased V_{max} and K_m of hydrolases except V_{max} of NAG (Fig. 5d and e). The average lnRR of V_{max} and K_m ranged from 0.87 to 1.02 and 0.75 to 1.08, respectively, at both decomposition stages (Fig. 5d and e). In contrast to CC, N increased the V_{max}/K_m at the later decomposition stage in SS with the average lnRR ranging from 1.02 to 1.26. However, the only significant effect of N addition was found for CB V_{max}/K_m ($P < 0.05$).

Ea varied substantially across enzyme species, litter type, and decomposition time (Fig. A3 and Table A8). N addition only affected the Ea of XYL and NAG (Fig. A3 and Table A8). On average, Ea of the enzymes involved in C and N mineralization (except GLU and XYL in SS litter) positively correlated with increasing decomposition time. In contrast, Ea of POD and PPO in CC litter negatively correlated with decomposition time (Fig. A3).

Relationships among enzymatic parameters and biotic and abiotic factors

Enzymatic activities and kinetic parameters were generally significantly correlated with most litter chemical and microbial properties ($P < 0.05$, Tables A9 and A10), with DOC and

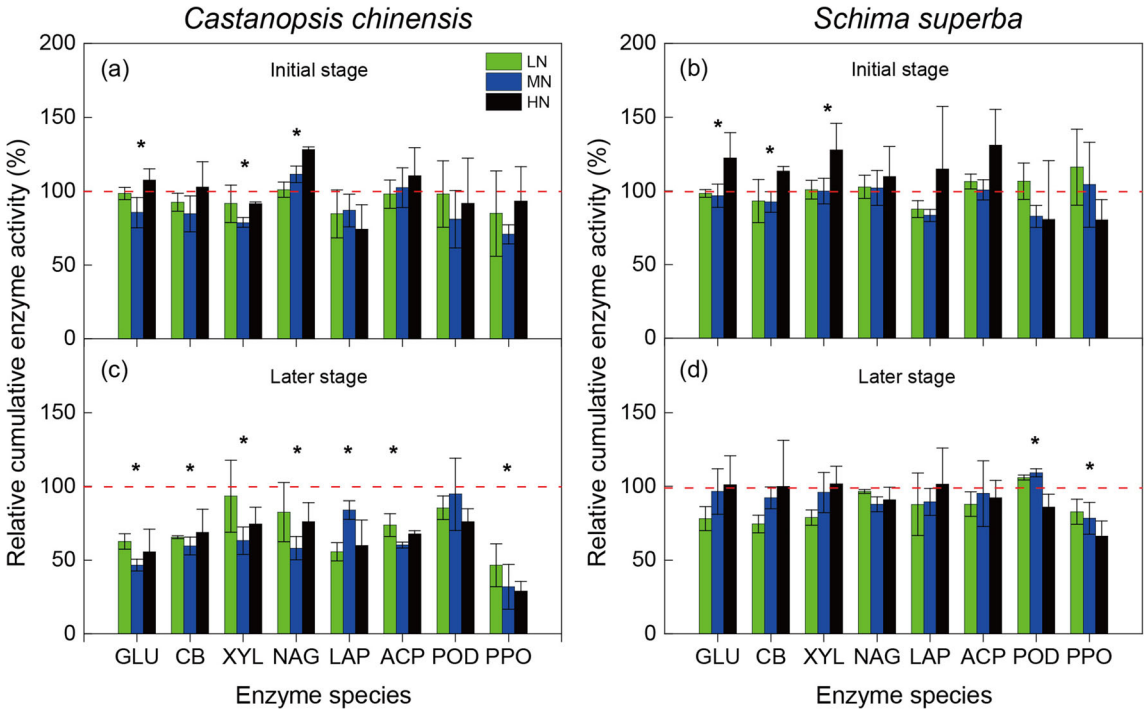

Fig. 4 Relative cumulative enzyme activity (REA) during the early and later stages of decomposition following N addition. The effect of N addition on REA was quantified as the relative change to that of control. Red dashed lines represent the REA% under the ambient N level. Bars higher than the 100% REA line indicate a stimulation effect; those lower than the 100% REA line indicate suppressive effects. An asterisk indicates a significant overall effect of N ($P < 0.05$, ANOVA). Error bars mean standard deviation ($n = 3$)

DON concentrations having the highest correlation coefficients with V_{max} and K_m. There were significant correlations between kinetic parameters (V_{max} and K_m) and fungal PLFAs, MBC, and MBN (Table A9). Especially, fungal PLFAs exhibited stronger statistical correlations with enzyme kinetics than bacterial PLFAs. The V_{max} of LAP significantly decreased as DOC concentration increased, with a scaling exponent of −0.17 (adj. $R^2 = 0.057$, $P < 0.05$, Fig. 6e). Conversely, the V_{max} of other hydrolases increased as DOC concentration increased, with scaling exponents ranging from 0.18 to 0.66 (adj. R^2 from 0.125 to 0.612, $P < 0.001$, Fig. 6a–f). The K_m of all hydrolases significantly increased with DON concentration, with scaling exponents ranging from 0.2 to 1.09 (adj. R^2 from 0.039 to 0.494, $P < 0.01$, Fig. 6g–l).

Discussion

In this study, the effect of N addition on litter decomposition varied depending on litter chemistry, N addition level, and decomposition stage. Previous studies also observed that N addition had varying effects on litter decomposition and the inhibition effect was usually observed at the later decomposition stage (Berg and Matzner 1997; Mo et al. 2006; Hobbie et al. 2012). These inconsistent patterns can be explained by the interactions between litter chemistry and microbial community composition and function (García-Palacios et al. 2016). Such microbial regulations have been examined at the physiological (van Diepen et al. 2017) and metagenomics levels (Entwistle et al. 2018b). These studies found that although changes in fungal abundance and growth rates varied among fungal species, litter decomposition was generally lower with N addition compared with control. However, it remains unclear how N enrichment regulates the resource acquisition strategy of the microbial community (e.g., enzyme production), and the catalytic property of enzymes, which is an essential step in predicting how litter decomposition rate may be affected by future atmospheric N deposition. We found that the effect of N addition on enzyme kinetics varied with decomposition stage, consistent with the dynamics of litter chemistry and microbial community composition and biomass. The observed thermodynamic parameters of enzymes did not follow the expected "browning effect."

Response mechanisms of enzyme activity to N addition

During the early decomposition stage (0 to 6 months), we found that N addition increased the hydrolase activities involved in C and N mineralization, regardless of litter type. These results are consistent with previous studies in boreal forests (Carreiro et al. 2000; Hobbie et al. 2012). Consistent

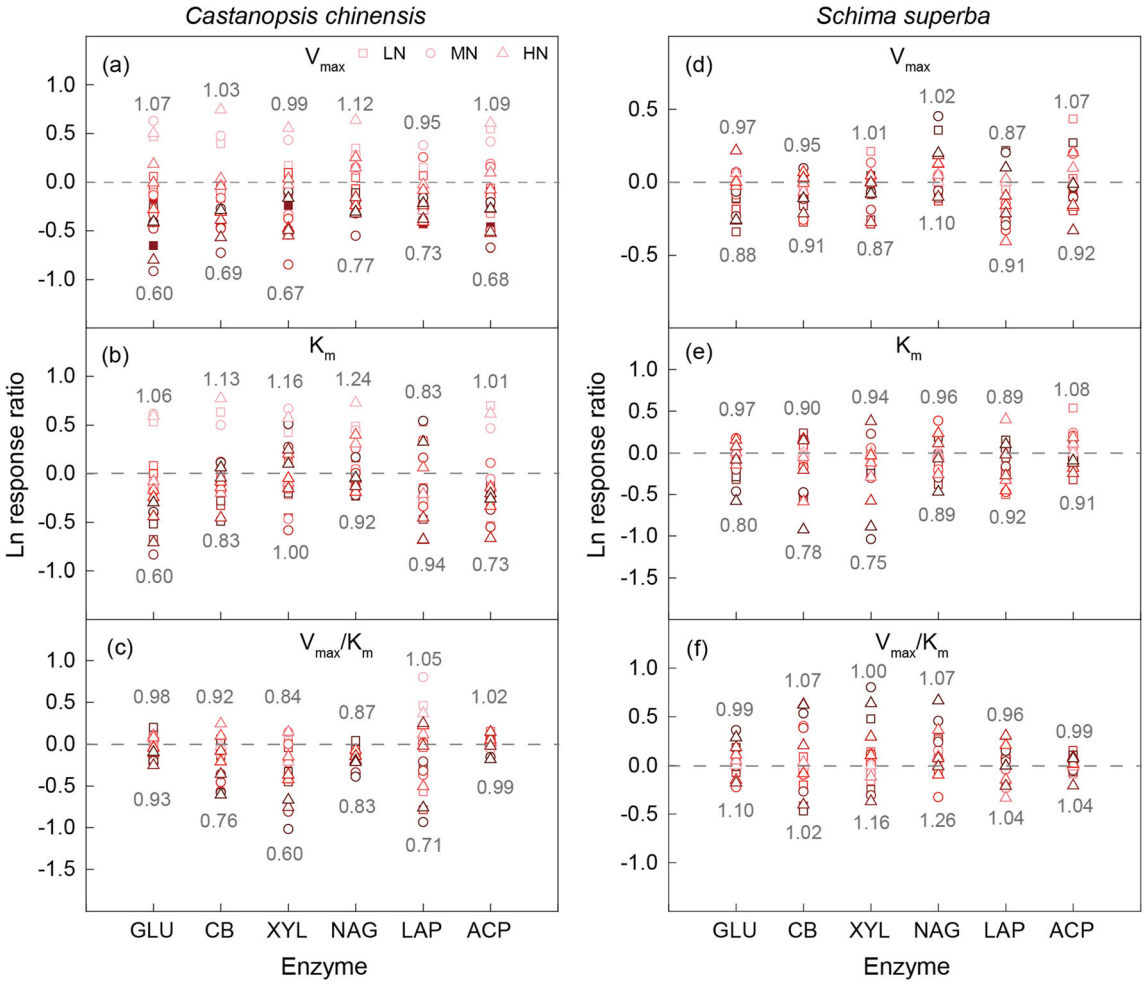

Fig. 5 Hydrolase kinetic parameters (K_m, V_{max}, and V_{max}/K_m) for two decomposing litter responses to N treatments. The x-axis is the enzyme species, and the y-axis is the natural logarithm response ratio of enzyme kinetic parameters (the ratio of kinetic parameters in N treatment to control) under different N treatments, and among the decomposition times. Each point represents the ln of response ratio (lnRR) of a given litter type and N level at each decomposition time. Positive or negative lnRR indicates positive or negative responses to the N treatments, respectively; that is, enzyme kinetic parameters were higher/lower in the N-treated plots than in the control plots. Open square, circle, and triangle indicate each enzyme kinetic parameter measured on LN, MN, and HN treatments, respectively. Seven shades of red from light tint to dark represent the decomposition time, and seven shades of red open square indicate the ln response ratio at 0, 2, 4, 6, 9, 12, and 18 months in LN treatment. The numbers in the top and bottom of each panel indicate the average lnRR (N treatment/control treatment) of kinetic parameters in the early and later decomposition stages, respectively

with our first hypothesis, N addition slightly increased the V_{max} and K_m of hydrolases in CC litter, but decreased V_{max} (except XYL and NAG) and K_m of hydrolases in SS litter during the early decomposition stage. This suggests that N addition increases hydrolase activity involved in structural carbohydrate and protein decomposition by stimulating microbes to produce more hydrolases or enzymes with higher affinity, which is considered an effective strategy for soil microbes to relieve N limitation (Stone et al. 2012). Previous research also found that although N addition stimulates hydrolase activity, enzyme kinetics can be affected differently (Stone et al. 2012).

Consistent with our second hypothesis, we found that the activities of lignolytic enzymes consistently decreased in both litters (Sinsabaugh et al. 2002). Based on the N mining theory, there is widespread acceptance that low quality litter (lignin content > 20%) has lower decomposition rates with high N deposition (Carreiro et al. 2000; Knorr et al. 2005). However, we found that N enrichment only significantly decreased the CC litter decomposition, even though CC liter had lower initial ADF and lignin content than SS litter. Previous studies demonstrated that effects of N addition on litter decomposition were independent of initial lignin content and N enrichment did not affect lignin decomposition (Hobbie 2008; Zhang et al. 2016). They deduced that abiotic interactions between N enrichment and products of microbial degradation or N effects on the decomposer community could result in the above phenomenon (Hobbie 2008; Zhang et al. 2016). Thus, the buildup of chemically recalcitrant compounds

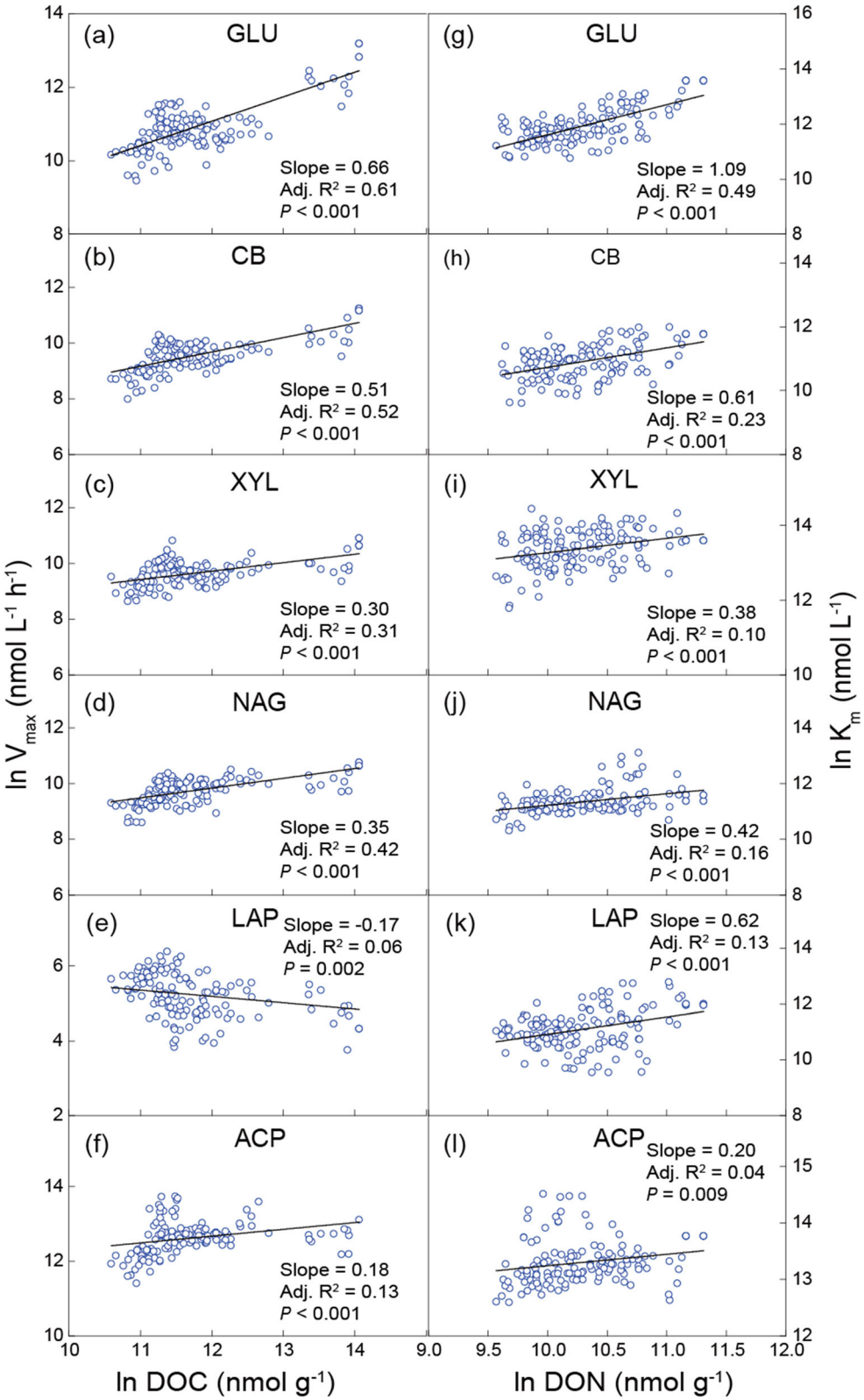

Fig. 6 Linear regression statistics for V_{max} versus dissolved organic C (DOC) and K_m versus dissolved organic N (DON). All data were transformed with natural logarithm

following N addition can result in a high Ea for enzymes, which can subsequently decrease enzyme activities (Fog 1988; Davidson and Janssens 2006). In contrast to our third hypothesis, N did not increase the Ea of enzymes, except for NAG and XYL, suggesting weak or no "browning effect."

Lower oxidase activity decreases the lignin decomposition and elevates polyphenol concentrations in litter

(DeForest et al. 2004), which might reduce enzyme activity and increase cellulose contained in lignified cell walls (Freeman et al. 2001; Adamczyk et al. 2015). We speculated that the enrichment of undecomposed lignin and lignin-derived polyphenol may slow down the rate of formation of enzyme-substrate complexes (decreased V_{max}) by inhibiting binding of the polysaccharides with the enzyme's active center, and/or by binding to the non-active sites of hydrolases leading to an increase in enzyme affinity (decreased K_m).

Interestingly, though hydrolase activities were initially increased by N addition, the overall decreased hydrolase activities in the later stages of decomposition likely resulted in the non-significant effect of N addition on cellulose decomposition. After 18 months of decomposition, the average content of cellulose and soluble sugar was only 9.45% and 7.09% in CC litter, respectively, while lignin accounted for 31.1% of CC litter. This suggests that the accumulated lignin may block the decomposition of other carbohydrates (e.g., hemicellulose, starch, and pectin), proteins, and fat. A recent study documented that N enrichment can slow the decomposition of proteins and condensed tannins (Xia et al. 2017), which supports directly our result that the decreased hydrolase activities may slow down the decomposition of the non-lignolytic components of litter under N treatment. We infer that hydrolase also is one of the major drivers to govern the effects of N addition on litter decomposition at the later decomposition stage.

We also found a significant reduction in hydrolase catalytic efficiency (V_{max}/K_m) of CC litter under N addition at a later stage of decomposition. Mechanically, enzymes operate under non-saturating conditions, where K_m and V_{max}/K_m become an important parameter to determine the rate of catalytic reaction (Tan et al. 2017). To our knowledge, no other litter decomposition studies have measured the effect of N addition on K_m and V_{max}/K_m during litter decomposition, despite its importance. We found that V_{max}/K_m was sensitivity to N enrichment, suggesting that the effect of N addition on the hydrolase activity depends on the change in catalytic efficiency other than its affinity to substrate.

In this study, the neutral N effect on the SS litter mass loss may be due to the short-term nature of N addition (less than 2 years). Fang et al. (2007) found that long term N addition can aggravate the inhibitive effect of N on litter decomposition, because long-term N addition can further change the relative abundance and physiology of lignolytic fungi or accelerate C-limitation for microbial degradation (Entwistle et al. 2018a, b). SS litter is characterized by higher initial dissolved nutrient and soluble sugar concentration than CC litter, which can moderate the C limitation for microbial degradation. Alternatively, Manganese (Mn) has been viewed as one of the main drivers in the later stages of litter decomposition because it can stimulate lignin and ADF decomposition (Berg 2014). A high level of Mn in SS litter appears to moderate the inhibitive effect of N on lignin decomposition. Therefore, the contrasting effects of N on these two litters may have resulted from their different initial chemistries (e.g., availability substrate and Mn content) rather than ADF and lignin content.

Feedback and regulation of enzymes

The response of enzymes to N has the potential to directly decrease available resources for heterotrophic metabolism, thereby limiting microbial growth and subsequent enzyme expression (Schimel and Weintraub 2003; Sinsabaugh et al. 2016). Here, we provide evidence that enzyme kinetic parameters (V_{max} and K_m) are a function of available substrate and fungal biomass during litter decomposition. Indeed, we observed reductions in microbial biomass (fungal PLFAs, MBC) and enzyme activity, as lignin decomposition slowed in CC litter. In contrast, N addition to SS litter did not influence V_{max} and K_m, fungal PLFAs, MBC, and lignin decomposition compared with control. These results suggest that reductions in enzyme production (decrease in V_{max}) may decrease the enzyme catalytic efficiency, which may be a preferred microbial strategy in high N environments.

We also found that the activity, microbial abundance (total PLFA) and composition of main microbial groups (F/B and G^+/G^-) varied with the decomposition process, but N addition only decreased total microbial abundance due to a significant decrease in fungal abundance. Remarkably, we found that kinetic parameters and substrate availability in CC litter decreased at the later stage of decomposition under N treatments. We infer that the adaptation of microbial function to the altered substrate supply, not only resulted in fungal biomass change, but also in a change in the catalytic characteristics and expression of enzymes. This suggests that the physiological metabolic regulation of microorganisms may be one of the important microbial mechanisms controlling litter decomposition in the context of N deposition.

Previous studies have observed that the V_{max} of enzymes in soils was preferentially controlled by specific soil properties such as pH and C/N and by a high abundance of bacteria (Triebwasser-Freese et al. 2015; Tischer et al. 2015). We speculate that the different regulated patterns between litter and soil environment could be attributed to microbial community properties. Throughout litter decomposition, microbial community succession, and the subsequent shift in microbial strategy (r- or k-strategist) usually match with substrate availability (García-Palacios

et al. 2016). Fungal communities are recognized as the primary mediators of litter decomposition, particularly for chemically recalcitrant compounds (Van der Wal et al. 2013). Soil bacteria are also able to decompose cellulose and lignin; however, the direct contribution of bacterial enzymes to degrading these complexes seems to be minor than fungi (Schneider et al. 2012). Bacteria can only play a leading role in the degradation of lignocellulose-rich organic materials in specific soil environments (e.g., periodic anoxic conditions), where fungal growth and activity are restricted (DeAngelis et al. 2011). In general, environmental drivers (biotic and abiotic factors) of enzyme production and catalytic characteristics are temporally and spatially specific, posing a significant challenge for emerging enzyme-explicit decomposition models which nevertheless require parsimony.

Conclusions

We conclude that the decrease in enzyme catalytic efficiency in the later decomposition stage is an alternative mechanism for interpreting the decline in non-lignolytic enzyme activity. This suggests that the protective/inhibitory effect of incomplete lignin decomposition might be equally important to the N mining theory, and the combination of these two mechanisms can holistically explain the effects of N enrichment during the later-stage decomposition. Our study identifies the integrated pathways that contribute to the inhibitive effect of exogenous N addition on litter decomposition. Specifically, as decomposition progresses, lignin enrichment due to microbial N mining effect limits the accessibility of lignin-encrusted structural carbohydrates to hydrolytic enzymes, and subsequently decreases the energy source of the entire microbial community for enzyme production. Our study also provides a novel experimental contribution to the understanding of enzyme kinetics in relation to microbial communities and substrate availability. During litter decomposition, C and N availability were strong predictors of V_{max} and K_m, respectively. Furthermore, the results show that V_{max}/K_m should be incorporated into an enzyme-based model because of decoupling of V_{max} and K_m, and its sensitivity to variation in controlling factors.

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Author contributions S.W. and T.X. designed the study; T.X. analyzed samples and collected the data; T.X., M.M., S.W., and F.C. conceived the ideas; T.X. wrote the manuscript. All authors contributed critically to manuscript editing.

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Compliance with ethical standards

Competing interests The authors declare that they have no competing interests.

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