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Response of decomposing litter and its microbial community to multiple forms of nitrogen enrichment

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Abstract. Despite the importance of litter decomposition for ecosystem fertility and carbon balance, key uncertainties remain about how this fundamental process is affected by nitrogen (N) availability. Resolving such uncertainties is critical for predicting the ecosystem consequences of increased anthropogenic N deposition. Toward that end, we decomposed green leaves and senesced litter of northern pin oak (*Quercus ellipsoidalis*) in three forested stands dominated by northern pin oak or white pine (*Pinus strobus*) to compare effects of substrate N (as it differed between leaves and litter) and externally supplied N (inorganic or organic forms) on decomposition and decomposer community structure and function over four years. Asymptotic decomposition models fit the data equally well as single exponential models and allowed us to compare effects of N on both the initial decomposition rate (k_a) and the level of asymptotic mass remaining (A, proportion of mass remaining at which decomposition approaches zero, i.e., the fraction of slowly decomposing litter). In all sites, both substrate N and externally supplied N (regardless of form) accelerated the initial decomposition rate. Faster initial decomposition rates corresponded to higher activity of polysaccharide-degrading enzymes associated with externally supplied N and greater relative abundances of Gram-negative and Gram-positive bacteria associated with green leaves and externally supplied organic N (assessed using phospholipid fatty acid analysis, PLFA). By contrast, later in decomposition, externally supplied N slowed decomposition, increasing the fraction of slowly decomposing litter (A) and reducing lignin-degrading enzyme activity and relative abundances of Gram-negative and Gram-positive bacteria. Higher-N green leaves, on the other hand, had lower levels of A (a smaller slow fraction) than lower-N litter. Contrasting effects of substrate and externally supplied N during later stages of decomposition likely occurred because higher-N leaves also had considerably lower lignin, causing them to decompose more quickly throughout decomposition. In conclusion, elevated atmospheric N deposition in forest ecosystems may have contrasting effects on the dynamics of different soil carbon pools, decreasing mean residence times of active fractions in fresh litter (which would be further reduced if deposition increased litter N concentrations), while increasing those of more slowly decomposing fractions, including more processed litter.

Key words: *Cedar Creek Ecosystem Science Reserve, Minnesota; extracellular enzyme activity; lignin; litter decomposition; Long-Term Ecological Research site; nitrogen; northern pin oak; phospholipid fatty acids;* Quercus ellipsoidalis.

Introduction

Litter decomposition is a key ecosystem process that influences the recycling of nutrients and thus ecosystem fertility on local scales. It is also the main pathway for return of carbon dioxide fixed during photosynthesis to the atmosphere, and thus influences carbon cycling at larger spatial scales. Despite hundreds, if not thousands, of litter decomposition studies (Adair et al. 2010), there remain uncertainties about how this fundamental process is influenced by the availability of nitrogen (N). This is especially true for externally applied N, which may have substantial, but inconsistent, effects on decomposition in empirical studies. The inconsistencies of these effects have impeded their inclusion in biogeochemical models that, with very few exceptions (Moorehead and Sinsabaugh 2006, Gerber et al. 2010), consider only substrate N effects on decomposition (Parton et al. 1988). Thus, resolving uncertainties about N effects on decomposition is critical for predicting ecosystem consequences of increasing global cycling of anthropogenic reactive N (Galloway et al. 2004).

Numerous studies have demonstrated a positive relationship between litter N concentration and decomposition rate (Adair et al. 2008, Cornwell et al. 2008), presumably because at low substrate N concentrations, N limits the rate of carbon use and thus mass loss. Nevertheless, uncertainties remain about substrate N effects on decomposition. For example, some studies

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have shown that this relationship may switch to a negative one during the later stages of decay, whereby substrates with higher initial N concentrations end up with lower accumulated mass loss in the long term (i.e., with larger, slowly decomposing fractions) (Berg et al. 1982, 2010, Berg 1986, Berg and Meentemeyer 2002). Yet many decomposition studies do not last long enough to reveal whether such negative effects of substrate N occur in the later stages of decomposition, nor are these kinds of effects included in biogeochemical models, which nearly always model the relationship between litter N concentration and decomposition rate as positive.

Those studies that are sufficiently long to discern negative effects of substrate N on decomposition may obscure changes in the effects of substrate N on decomposition over time if data are fit to single rather than multi-pool exponential decay models. For example, in our past research, decomposition rate was positively related to initial substrate N across a wide range of substrates when data were fit to a single exponential decay model (Hobbie 2008). However, fitting the data to an asymptotic decomposition model revealed that higher initial N, while positively correlated with initial rates of decomposition, was associated with larger slowly decomposing litter fractions (Appendix A), consistent with the studies by Berg and colleagues cited in the previous paragraph. Isolating the effect of substrate N is further complicated by the consistent correlation between litter N concentrations and those of other nutrients such as phosphorus and potassium across different species (Garten 1976, Reich and Oleksyn 2004, Cornwell et al. 2008), which may (or may not) drive relationships between litter N concentrations and decomposition.

Another major uncertainty regarding the effects of N on litter decomposition is why the effects of externally supplied N are so often negative, often opposite to those of litter N, and why they are variable among systems. For example, in contrast to the positive relationship between litter N and decomposition mentioned above (Cornwell et al. 2008), a meta-analysis of decomposition studies (Knorr et al. 2005) revealed that externally supplied N had negative effects on litter decomposition on average, but effects varied considerably. Negative effects were particularly evident where ambient N deposition rates were high, litter quality was low, and N addition rates were high.

The magnitudes and mechanisms responsible for the sometimes negative effects of N on decomposition appear to vary among systems. Some studies showed them to be associated with suppression of the activity of oxidative enzymes involved in lignin degradation (Carreiro et al. 2000, DeForest et al. 2004, Zak et al. 2008), whereas other studies demonstrating negative effects of N on decomposition found no evidence for such enzymatic effects (Hobbie 2008) or for reduced lignin decomposition with added N (Sjöberg et al. 2004). Even where N demonstrably suppressed ligninolytic enzyme activity, the effects varied among sites and among litter types (DeForest et al. 2004, Keeler et al. 2009, Weand et al. 2010). Alternatively, inorganic N may react with carbon constituents in litter (e.g., polyphenols, carbohydrates) to form recalcitrant compounds and thereby reduce decomposition rates (Nömmik and Vahtras 1982, Fog 1988, Berg and Matzner 1997), although one study found little evidence for such reactions (Knicker et al. 1997).

Additional negative effects of N on decomposition could result from N-induced changes in the composition and/or physiology of the decomposer community. For example, a modeling study suggested that negative effects of N on decomposition could be explained by an N-induced increase in decomposition carbon-use efficiency (Ågren et al. 2001). An N-induced decrease in microbial C:N stoichiometry could conceivably lead to reduced demand for carbon and thus lower decomposition with added N. However, to our knowledge, empirical evidence for such physiological changes in the microbial community in response to N enrichment is lacking. A meta-analysis of N effects on microbial communities demonstrated reduced microbial biomass with added N on average (Treseder 2008) (the mechanism underlying such effects is unknown), which theoretically could lead to declines in decomposition, but could also be caused by them (Moorehead and Sinsabaugh 2006).

In previous research at eight different forest or grassland sites at the Cedar Creek Ecosystem Science Reserve in central Minnesota, USA, substrate N was consistently positively related to decomposition, while addition of inorganic N had either neutral, positive, or negative effects on decomposition initially (Hobbie 2005) and neutral or negative effects later on (Hobbie 2008, Keeler et al. 2009). Negative effects of N on decomposition were unrelated to initial substrate lignin or N concentrations, and there was no evidence for N inhibition of oxidative (lignin-degrading) enzymes (Keeler et al. 2009). Here we aim to build upon our prior work to address the question: "How do different forms of N (organic vs. inorganic; substrate vs. externally supplied) affect decomposition and the related function and composition of the decomposer community, and do these effects depend on the decomposition stage?"

This work differs from past work in its consideration of different forms of externally applied N on decomposition, including organic N, and in its simultaneous characterization of the effects of added N on decomposition and on the composition and enzymatic activity of the decomposer community.

We tested the following two hypotheses:

Hypothesis 1.—In the initial stages of decomposition, addition of N will increase decomposition rates, but because of lower N assimilation costs, addition of

organic forms of N, either as higher substrate N or organic N fertilizer, will promote microbial abundance, investment in carbon-degrading enzymes, and decomposition rate more than addition of inorganic N fertilizer.

Hypothesis 2.—In the later stages of decomposition, when lignin decomposition dominates, all forms of N will have negative effects on decomposition, because of suppression of oxidative enzyme activity and reduced microbial biomass.

Methods

Decomposition experiment

We established a decomposition experiment at three forested sites at the Cedar Creek Ecosystem Science Reserve, a Long Term Ecological Research (LTER) site in central Minnesota, USA (latitude 45.40 N, longitude 93.20 W, elevation 270 m), located on sandy, poorly developed soils. Average ambient wet N deposition rates are 5.6 kg $N{\cdot}ha^{-1}{\cdot}yr^{-1}$ (Hobbie 2008). We chose these sites because in a prior study of eight sites (forests and grasslands) including these, they encompassed the among-site range of variability in N effects on decomposition, especially in the initial stages (Hobbie 2005, 2008). The temperate climate is characterized by mean annual temperature of 6.7°C and mean annual precipitation of 801 mm. Sites included two forest stands dominated by *Quercus ellipsoidalis* and one stand dominated by *Pinus strobus*. (For more detail see Hobbie [2008].)

We initiated a nutrient enrichment experiment at each site in fall 2004. Plots (3 m^2, 1.5×2 m) received one of the following treatments (five replicate plots per treatment): control (CONT, no nutrient addition), inorganic N addition (IN, 10 g $N{\cdot}m^{-2}{\cdot}yr^{-1}$ as NH_4NO_3), organic N addition (ON, 10 g $N{\cdot}m^{-2}{\cdot}yr^{-1}$ as 3.33 g $N{\cdot}m^{-2}{\cdot}yr^{-1}$ each of alanine, glycine, and glutamate), carbon addition (CARB, 28.5 g $C{\cdot}m^{-2}{\cdot}yr^{-1}$ as D-glucose, the same rates of C addition as in the ON treatment), inorganic N + carbon addition (INC, 10 g $N{\cdot}m^{-2}{\cdot}yr^{-1}$ as NH_4NO_3 + 28.5 g $C{\cdot}m^{-2}{\cdot}yr^{-1}$ as D-glucose), long-term N addition (LTN, 10 g $N{\cdot}m^{-2}{\cdot}yr^{-1}$ as NH_4NO_3, initiated in 1999 as part of another experiment, Hobbie 2008), and non-N nutrient addition (NON, 10 g $P{\cdot}m^{-2}{\cdot}yr^{-1}$, 12.6 g $K{\cdot}m^{-2}{\cdot}yr^{-1}$ as KH_2PO_4, 10 g $Ca{\cdot}m^{-2}{\cdot}yr^{-1}$ as $CaCl_2$, 5 g $Mg{\cdot}m^{-2}{\cdot}yr^{-1}$ and 6.6 g $S{\cdot}m^{-2}{\cdot}yr^{-1}$ as $MgSO_4$, 0.5 g $Fe{\cdot}m^{-2}{\cdot}yr^{-1}$ as FeNaEDTA). All nutrients were added in aqueous solution divided into three applications over the season (May, July, October, 1 L solution per plot per application; control plots received water only). Except in the LTN treatment, treatments were initiated in August 2004 with one-third of the annual application rate.

The ON and IN treatments were designed to allow comparisons of effects of organic vs. inorganic forms of N, respectively, on decomposition, to elucidate whether differences between externally supplied and substrate N observed in other studies arise because externally supplied N is often added in inorganic forms, while substrate N is organic. The INC and CARB treatments were then included to control for (and isolate) effects of added carbon in the ON treatment. NON and LTN treatments were included to test two alternative explanations for our prior results. NON was included to determine whether the correlation between litter N and decomposition arose because N was correlated with some other factor that limits decay such as phosphorus (P) or potassium (K), both of which were tightly correlated with litter N in previous work (Hobbie 2005). LTN was included to determine whether any potential lack of initial effects of IN treatment arose because inorganic N did not immediately elevate inorganic N supply to litter or change microbial communities in the litter layer. (Past work demonstrated that 3.5 years of inorganic N addition increased inorganic N in the litter layer >10-fold [Hobbie 2008].)

In each plot, we decomposed four substrates chosen to give a wide range in initial N concentration: green leaves and leaf litter of *Quercus ellipsoidalis* from each of two sites that are part of a long-term fire frequency experiment in oak savanna and woodland. As foliar and litter N concentrations are higher in unburned than in frequently burned sites (Reich et al. 2001, Hernández and Hobbie 2008), we collected green leaves (by handpicking fully expanded leaves from branches) and leaf litter (by handpicking freshly fallen litter from the ground) from one unburned stand (Burn Unit 209, never burned) and one frequently burned stand (Burn Unit 103, burned at the rate of 0.7 fire/year). Leaves and litter were dried at 50°C upon collection and subsamples were analyzed for: (1) carbon fractions using an ANKOM Fiber Analyzer (Ankom Technology, Macedon, New York, USA) (cell solubles, hemicelluloses + bound protein, cellulose, and lignin + other recalcitrants, determined on an ash-free dry mass basis); (2) initial N concentrations on a Costech ECS4010 element analyzer (Costech Analytical, Valencia, California, USA) at the University of Nebraska, Lincoln; and (3) initial concentrations of magnesium (Mg), calcium (Ca), manganese (Mn), and potassium (K) by Inductively Coupled Argon Plasma Emissions Spectrometry (ICP, Applied Research Laboratory 3560) following digestion in 10% HCl (Munter and Grande 1981) at the University of Minnesota's Research Analytical Laboratory.

We constructed 20 × 20 cm litter bags from 1-mm fiberglass window screen. Past work has shown that this mesh size is permeable to a variety of soil fauna, including earthworms. Bags were filled with 10 g of litter or leaves from frequently burned and unburned sites, and one bag containing each substrate was attached to a 1-m length of nylon string (four bags per string). Five strings were deployed in each plot in November 2004, and one string was harvested in May 2005 and in October of each year from 2005 to 2008 (after 0.5, 1, 2, 3, and 4 years). At each harvest, replicates 1 and 2 (from all sites) were harvested together, replicates 3 and 4 were

harvested together one week later, and replicate 5 was harvested one week after that, to allow time for processing litter.

At all five harvests, we determined substrate mass loss and N concentration. At the first four harvests (after 0.5, 1, 2, and 3 years), we also determined the activities of five extracellular enzymes. After 1 and 4 years, we determined microbial community composition using phospholipid fatty acid analysis (PLFA).

At each harvest, we removed substrates from mesh bags, cleaned them by hand of insects, roots, and other matter, cut them into ~0.25-cm^2 pieces (using scissors), and subsampled them for fresh/dry mass (65°C) ratio (also used for C and N determination), PLFA, and enzyme activities. Subsamples for PLFA and enzyme assays were frozen (−20°C) until analysis. Substrate C and N concentrations were determined as described for initial samples. We multiplied substrate N concentration by mass at each time point to determine total substrate N content, which was divided by the initial N content to determine the proportion of initial N remaining over time.

To determine whether treatments were affecting decomposition by altering forest floor pH, we measured the pH of the O horizon in July 2009. Random samples of O horizon were collected from four 400-cm^2 areas. Composite samples (by plot) were coarsely chopped and equilibrated with deionized water (1:10) before measuring pH.

Phospholipid fatty acid analysis

Lipids were extracted from 1 g litter (dry mass equivalent; ground using mortar and pestle) using a single-phase chloroform–methanol–phosphate buffer extraction (Bligh and Dyer 1959, White et al. 1979). Polar lipids were fractionated and separated from the neutral lipids and glycolipids using silicic acid chromatography (King et al. 1977, Guckert et al. 1985). Polar lipids were transesterified using mild alkaline methanolysis to create fatty acid methyl esters (FAMEs) (Guckert et al. 1985). FAMEs were separated using capillary gas chromatography and quantified using flame ionization detection (FID) on a 5890 Series II chromatograph (column: 40-m nonpolar, 0.2 mm ID, 0.1-μm film thickness) (Hewlett Packard, Palo Alto, California, USA). Peaks were integrated using Chrom-Perfect software (Justice Laboratory Software, Denville, New Jersey, USA). The amount (in micromoles per gram of soil) of each PLFA was calculated by comparing peak areas to an internal 9:0 (methyl nonanoate) or 19:0 (methyl nonadecanoate) FAME standard added to each sample.

Phospholipid fatty acid peaks were identified using retention times from a mixture of 32 known FAME standards and by gas chromatography–mass spectrometry (GC–MS). One PLFA sample was randomly selected from each harvest × site × N treatment combination for GC-MS analysis (42 out of 840 total samples). These samples were analyzed on an Agilent (Santa Clara, California, USA) 6890N GC with a Leco (St. Joseph, Michigan, USA) Pegasus HT time-of-flight mass spectrometer using the identical column and oven temperature parameters as on the GC-FID. In total, we identified 64 PLFAs having 14–24 carbon atoms that were used in the following analyses. These fatty acids comprised six groups: normal saturates, midchain branched saturates, terminally branched saturates, branched monoenoics, straight chain monoenoics, and polyenoics. In addition, where it could be determined, we classified monoenoic fatty acids as either the *cis* or *trans* isomer. All PLFAs were expressed in micromoles per gram relative to the total PLFAs.

Mole fractions of terminally branched, monounsaturated, and midchain branched saturated PLFAs were summed as measures of total proportional Gram-positive, Gram-negative, and actinomycete bacterial abundances, respectively (McKinley et al. 2005). The mole fractions of the PLFA markers 18:2ω6 and 18:1ω9c were summed as measures of proportional fungal abundance. Finally, we calculated the ratio of normal saturated : unsaturated PLFAs, which has been shown to increase under conditions of nutritional stress (Kieft et al. 1997).

Extracellular enzyme activity

We analyzed samples for the following extracellular enzyme activity according to the methods of Saiya-Cork et al. (2002) and Sinsabaugh et al. (1992): two enzymes involved in cellulose decomposition, β-1,4-glucosidase (BG, EC 3.2.1.21) and cellobiohydrolase (CBH, EC 3.2.1.91); two enzymes involved in decomposition of polyphenols such as lignin, phenol oxidase (POX, EC 1.10.3.2) and peroxidase (PER, EC 1.11.1.7); an enzyme involved in chitin decomposition, β-1,4-N-acetylglucosaminidase (NAG, EC 3.2.1.1.14); and an enzyme involved in mineralizing ester-bonded phosphate, acid phosphatase (AP, EC 3.1.3.2). Hydrolytic enzymes (GLU, CBH, NAG, and AP) were assayed fluorometrically using methylumbelliferone (MUB)-labeled substrates (excitation at 365 nm, emission at 450 nm). Oxidative enzymes (POX, PER) were assayed using L-3,4-dihydroxyphenylalanine (L-DOPA) and L-DOPA and hydrogen peroxide as substrates, respectively, and measuring absorbance on a microplate spectrophotometer (460 nm). Subsamples (~0.5 g) were homogenized in 125 mL of acetate buffer (50 mmol/L, pH 5.0) in a blender, and substrate suspensions were dispensed into 96-well plates using a Precision 2000 robotic pipettor (BioTek Instruments, Winooski, Vermont, USA) (16 replicate sample wells, sample solution + substrate; eight replicate blank wells, sample solution + buffer; eight negative control wells, substrate + buffer; eight quench standard wells, standard + sample solution). Plates were incubated in the dark at 20°C for 0.5–20 hours, depending on the assay. Fluorescence or absorbance

(corrected for negative controls, blanks, and quenching) was used as a measure of activity.

In addition to measuring enzyme activity of decomposing substrates, we determined whether treatment effects on enzyme activity varied seasonally by measuring extracellular enzyme activities of the native forest floor (O horizon) in all treatments in one randomly selected site (one of the oak stands) on seven dates in 2009: 23 April, 22 May, 18 June, 11 July, 13 August, 1 September, and 29 September. The O horizon was sampled from one randomly selected 20×20 cm quadrat per plot and frozen (−20°C) until assayed as described above.

Data analysis

We fit substrate proportion of mass remaining to three exponential decay models and determined the best fit among them using Akaike's Information Criteria (AIC_c), where a difference between two candidate models of >3 was used to indicate a significant difference in model fits. The three models were single exponential ($X = e^{-k_s t}$), double exponential ($X = Ce^{-k_1 t} + (1 - C)e^{-k_2 t}$), and asymptotic ($X = A + [1-A]e^{-k_a t}$) decomposition models, where X is the proportion of initial mass remaining at time t, and k_s is the decomposition constant in the single exponential model. In the double exponential model, C is the fraction of the initial mass that decomposes with decomposition rate k_1, while the remaining fraction $(1 - C)$ decomposes with rate k_2. In the asymptotic model, A is the fraction of the initial mass with a decomposition rate of zero (i.e., the asymptote), while the remaining fraction $(1 - A)$ decomposes with rate k_a. A is the same as 1 minus the "limit value," the cumulative proportional mass loss where decomposition slows to zero, modeled extensively by Berg and colleagues (e.g., Berg 2000). Although in reality decomposition rate would never actually equal zero, the asymptotic model assumes that there is a fraction of litter that decomposes so slowly that the rate is very nearly zero, so A can be thought of as the fraction of litter that decomposes very slowly, or the "slow fraction."

Because of the small number of time points measured per replicate (five), we pooled all replicates of a site–treatment–substrate combination to determine the best model fit. Subsequently, we fit each replicate individually to the best model to determine individual replicate model parameters. We compared decomposition model parameters, maximum N immobilization (grams of N per gram of initial substrate), and enzyme activities of decomposing substrates at each harvest among sites, treatments, and substrates using three-way analysis of variance (ANOVA). Enzyme activities were ln-transformed to improve normality and ANOVAs were performed with and without including proportion of initial mass lost as a covariate to control for the stage of decomposition. We also compared average decomposition parameters (k_s, k_a, A) of all substrates within replicate plots among sites and treatments using analysis of covariance, with litter pH included as a covariate, to determine whether any treatment effects on decomposition could be explained by treatment effects on pH, since addition of NH_4NO_3 is known to reduce pH. We analyzed native forest floor extracellular enzyme activity using repeated-measures ANOVA with treatment as main effect, and values were ln-transformed for analysis.

We compared total PLFAs, total number of nonzero PLFAs, proportional abundances of the major microbial groups of PLFAs, and nutritional stress index among sites, substrates, and treatments using three-way ANOVA for each of the two harvests separately. Total PLFAs and nutritional stress were ln-transformed to improve normality for analysis. ANOVAs were performed with and without including proportion initial mass lost as a covariate to control for the stage of decomposition. Analyses were performed in JMP 8.0.1 (SAS Institute 2009).

To examine the effects of sites, substrates, treatments, and harvests on microbial community composition, we generated Euclidean distance matrices using proportional abundance data from major microbial groups of PLFAs as well as individual PLFAs. PERMANOVA analyses with 999 permutations were performed to compare sites, substrates, and treatments for each of these data sets. Nonmetric multidimensional scaling (NMDS) ordination (in two dimensions) and vector fitting with harvest, proportion of mass loss, site, treatments, and substrates were used to visualize and quantify the magnitude of effects of these factors on community composition. All community composition analyses were performed using the vegan package 1.17–3 in R (R v. 2.10.1; R Development Core Team 2009), except NMDS, which was run on R v. 2.14 with vegan package 2.0-3 (R Development Core Team 2012).

Results

Substrate characteristics

As expected, initial N concentrations were about three times higher in leaves than in litter, and were higher in leaves and litter from the unburned sites compared to the frequently burned sites (Table 1). Hereafter, for ease of reporting, we refer to leaves and litter from the frequently burned site as "lower-N" leaves and litter and from the unburned site as "higher-N" leaves and litter. However, we acknowledge that besides affecting N, both burning and senescence also affected nearly every other aspect of substrate chemistry significantly (Table 1, two-way ANOVA, with site and substrate type as main effects, analyses not shown). The magnitude of these effects varied depending on the substrate constituent: the effects of burning had modest effects on cellulose, Ca, K, and P concentrations (<10% difference between unburned and burned sites), while burning reduced initial N, hemicellulose, lignin, and Mg between 10% and 20%, reduced Mn by 55%, and increased cell solubles by 16% compared to the unburned site. The

TABLE 1. Initial concentrations (%) of nutrients and carbon fractions in litter and green leaves used in the experiment ("lower-N litter and leaves" collected from a frequently burned site; "higher-N litter and leaves" collected from an unburned site).

Substrate	Nutrients							
	Ca	K	Mg	Mn	P	N	C	Ash
Lower-N litter	1.016 (0.007)	0.377 (0.011)	0.162 (0.002)	0.063 (0.002)	0.155 (0.002)	0.63 (0.01)	48.68 (0.33)	0.85 (0.06)
Higher-N litter	1.059 (0.011)	0.231 (0.011)	0.183 (0.006)	0.130 (0.007)	0.100 (0.004)	0.82 (0.02)	47.82 (0.42)	0.76 (0.08)
Lower-N leaves	0.732 (0.063)	0.671 (0.024)	0.182 (0.006)	0.024 (0.008)	0.143 (0.005)	2.01 (0.10)	47.13 (0.13)	0.89 (0.10)
Higher-N leaves	0.845 (0.012)	0.900 (0.040)	0.240 (0.003)	0.065 (0.001)	0.173 (0.003)	2.21 (0.08)	47.45 (0.08)	0.76 (0.08)

Notes: Carbon fractions are expressed per gram on an ash-free dry mass basis. $N = 3$ for all analyses. Values are means with standard errors (in parentheses).

largest effects of senescence were to reduce N, P, K, Mg, and cellulose by 18–65%, and to increase lignin, Ca, and Mn by 30–120% in litter vs. in leaves.

Decomposition models

In the great majority of cases (58 of 84, 69%), single exponential and asymptotic models were indistinguishable in terms of fit to the data. In only 12% (10 of 84) or 16% (13 of 84) of cases did a single exponential or asymptotic model fit the data best, respectively. For the remaining cases (4%, 3 of 84), the three models were indistinguishable in terms of fit. A double exponential model was never the single best fit to the data. Therefore, we fit individual replicates with both the single exponential and asymptotic decomposition models, and present parameters from both.

Decomposition and substrate N dynamics

As expected, N generally increased initial rates of decomposition. However, in contrast to our expectations, both inorganic and organic forms of N had significant effects on initial decomposition. For k_a, all main effects were significant ($F_{2, 336} = 16.81$, $F_{6, 336} = 7.89$, and $F_{3, 336} = 169.69$, $P < 0.0001$, for site, treatment, and substrate, respectively, overall model $R^2 = 0.66$), with k_a of green leaves greater than k_a of litter (lower-N leaves > higher-N leaves > lower-N litter > higher-N litter; Tukey's HSD, $P < 0.05$) and k_a in LTN and ON significantly greater than in CONT (Fig. 1; Appendix B, Bonferroni-corrected post hoc comparisons of all treatments with CONT, the control; experiment-wise alpha = 0.05).

As hypothesized, all forms of externally supplied N (both organic and inorganic) increased the fraction of substrate whose decomposition rate was (or approached) zero (A). However, in contrast to our expectations from prior research by Berg and colleagues and our research at Cedar Creek comparing multiple species' litter (S. E. Hobbie, *unpublished data*), substrate N generally decreased A, at least when comparing leaves vs. litter. For ln-transformed A, all main effects were significant ($F_{2, 336} = 4.83$, $F_{6, 336} = 15.68$, and $F_{3, 336} = 5.11$, $P = 0.0086$, $P < 0.0001$, and $P = 0.0018$, for site, treatment, and substrate, respectively, overall model $R^2 = 0.36$), with higher-N litter having significantly greater A (i.e., a larger fraction of slowly decomposing substrate) than lower-N leaves (lower-N litter and higher-N leaves were intermediate) (Fig. 1; Appendix B). A was significantly larger in all treatments relative to CONT except CARB, which reduced A relative to CONT. Patterns for k_s generally reflected those for k_a among sites and substrates, but resembled patterns for A among treatments (Fig. 1; Appendix B).

There were significant effects of treatment and substrate, as well as a significant site by substrate interaction on the maximum amount of N immobilized ($F_{2, 335} = 4.67$, $P = 0.0001$; $F_{3, 335} = 157.05$, $P < 0.0001$; $F_{6, 335} = 3.01$, $P = 0.0071$, respectively, overall model $R^2 = 0.64$). There was a weak interaction between treatment and substrate ($P = 0.04$). In Bonferroni post hoc contrasts, only CARB was different from CONT in terms of maximum immobilization, with significantly lower maximum immobilization with added carbon (data not shown). Regardless of site, green leaves exhibited lower N immobilization when adjusted for mass remaining than did leaf litter (Appendix C), consistent with lower demand for N by N-rich substrates and with past work by Parton et al. (2007).

Extracellular enzyme activity

There were strong pairwise correlations among activities of many enzymes, with all of the hydrolytic enzymes correlated with one another ($0.48 \leq r \leq 0.95$, $P < 0.0001$ in all cases) and the two oxidative enzymes (POX and PER) correlated with one another ($0.53 \leq r \leq 0.96$, $P < 0.0001$) at all harvests. Oxidative and hydrolytic enzyme activities were uncorrelated after 0.5 year, and became correlated after 1 year, although the correlation coefficients were lower than for within-hydrolytic or within-oxidative correlations, and some pairwise relationships (POX-NAG, POX-AP, PER-GLU) were not significant at the two-year harvest.

As hypothesized (and largely consistent with treatment effects on decomposition), N enrichment increased the activity of hydrolytic enzymes involved in carbon degradation early in the experiment, but decreased the activity of oxidative and N-acquiring enzymes later in the decomposition process (Fig. 2). After 0.5 year, all of the N-containing treatments, regardless of form, increased the activity of GLU and CBH, and IN and INC additionally increased the activity of NAG. These positive effects of N addition on hydrolytic enzyme

TABLE 1. Extended.

Carbon fractions			
Cell solubles	Hemicellulose	Cellulose	Lignin
50.08 (1.00)	14.63 (0.31)	17.68 (0.32)	18.05 (0.48)
44.30 (0.46)	15.64 (0.05)	18.76 (0.13)	21.72 (0.38)
56.17 (1.71)	17.81 (0.41)	15.92 (0.68)	10.53 (0.73)
47.28 (0.47)	20.9 (0.50)	17.87 (0.56)	14.36 (0.55)

activities disappeared by 1 year, and after 3 years, addition of N (in LTN and ON) decreased NAG activity. Oxidative enzyme activities at the first two harvests were too low for statistical analyses (there were many zero values). As expected, after 2 years, LTN and ON decreased POX activity, as did NON. After 3 years, LTN continued to decrease POX activity (Fig. 3). PER activity was not significantly altered by treatments. Addition of non-N nutrients decreased PHOS activity in

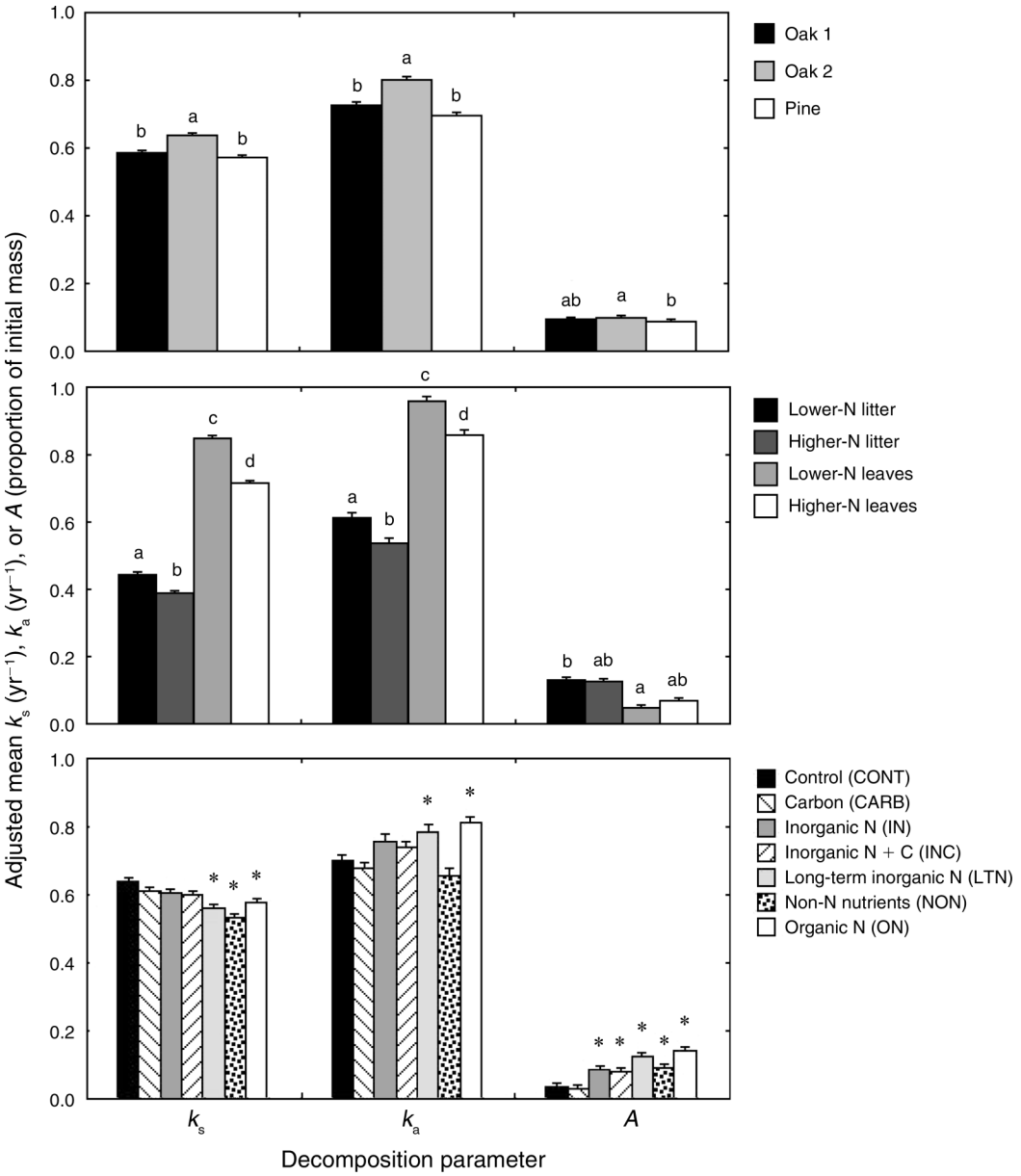

FIG. 1. Adjusted mean decomposition parameters obtained from fitting single exponential and asymptotic models to data to show differences among sites, substrates, and treatments: k_s, single-exponential model decomposition rate constant; k_a, asymptotic model decomposition rate constant; A, asymptote. Values are adjusted means + SE. Different letters within a decomposition parameter indicate significant differences for Tukey's HSD post hoc comparisons among different levels of sites and substrates ($P < 0.05$). Asterisks indicate significant Bonferroni-corrected pairwise comparisons within a decomposition parameter of all treatments with the control (experimentwise alpha = 0.05).

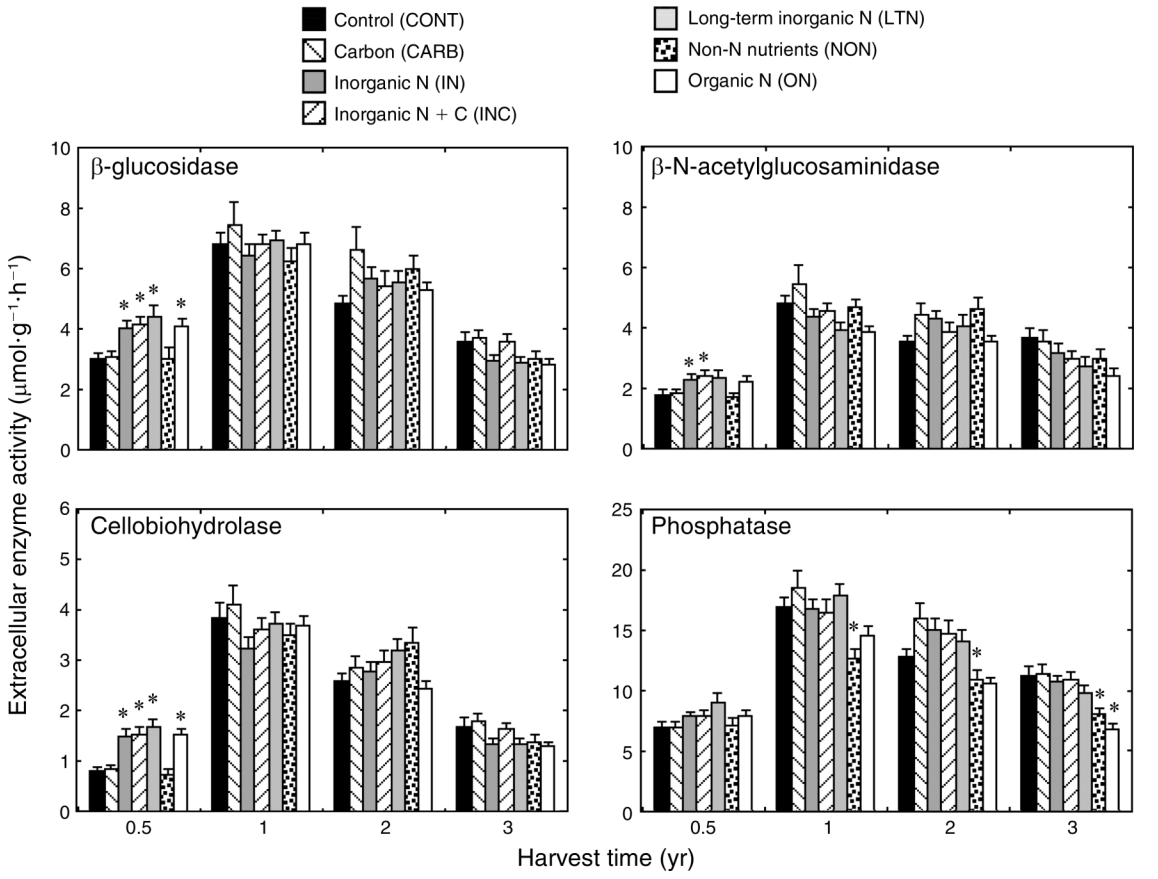

FIG. 2. Hydrolytic enzyme activity on substrates harvested during the first three years of the experiment by treatment (averaged over sites and substrates). An asterisk indicates that a particular treatment differed significantly from the control treatment at a particular harvest time (experimentwise alpha = 0.05). Values are means + SE. Overall model R^2 values from three-way ANOVAs including treatment, site, and substrate as main effects and done separately for each harvest date for the hydrolytic enzymes ranged from 0.25 to 0.58 for GLU, from 0.24 to 0.69 for CBH, from 0.21 to 0.8 for NAG, and from 0.28 to 0.56 for PHOS. Enzyme abbreviations are defined in *Methods: Extracellular enzyme activity*.

years 1–3. Including the proportion of initial mass lost as a covariate in the ANOVA models, to control for the stage of decomposition, did not alter treatment effects on enzyme activities, indicating that treatments were affecting microbial communities more than just by changing decomposition rate and thus the stage of decomposition (analyses not shown).

Enzyme activities differed among substrates (Appendix D), with differences depending on harvest and enzyme identity, and there were no consistent differences in enzyme activity between leaves and litter, except that at some harvests litter had lower activities of polysaccharide-degrading enzymes and higher activity of POX than did leaves. Enzyme activities also differed significantly among sites, but differences changed depending on harvest and enzyme identity (data not shown).

Total carbon-degrading enzyme activity (the sum of the activity of GLU, CBH, NAG, POX, and PER, with the value at each harvest weighted by the duration of the interval preceding that harvest) was positively related to cumulative mass loss at all harvests except the first one, when the two variables were significantly negatively related ($P < 0.0001$ in all cases; $r = -0.38$, 0.34, 0.43, and 0.30 for 0.5, 1, 2, and 3 years, respectively).

At the one site (Oak 2) where we measured extracellular enzyme activities of the forest floor (the O horizon), there were no significant effects of treatment or treatment by time for any of the hydrolytic enzymes measured except for PHOS, for which time ($P < 0.0001$) and treatment by time ($P < 0.05$) were significant (data not shown). When we analyzed each of the seven time points separately, there were significant treatment effects at time points 3, 5, 6, and 7 ($P < 0.05$), with IN increasing PHOS compared to CONT at time point 3, and NON decreasing PHOS compared to CONT at time points 5–7 (Bonferroni-corrected post hoc comparisons, alpha = 0.05) (data not shown). Oxidative enzyme activities were not detectable for the first four time points (median activities of POX and PER were zero), so analyses were restricted to time points 5, 6, and 7, and treatment and treatment by time effects were not significant (data not shown).

O horizon pH was significantly higher in the ON treatment compared to the control (by approximately 0.4 pH units, $P < 0.001$). O horizon pH was unrelated to k_s, k_a, and A in ANCOVA (analyses not shown).

There were no significant effects of treatment on fungal : bacterial ratios. However, treatments affected bacterial PLFAs, and the effects changed after one vs. four years of decomposition. After one year, green leaves had higher total abundance and richness (number of nonzero) of PLFAs than did leaf litter, as well as greater relative abundance of Gram-positive and Gram-negative organisms (Tukey's HSD $P < 0.05$; Table 2, Fig. 4). Organic N addition (ON) increased, while NON nutrient addition (NON) decreased, Gram-negative and Gram-positive organisms relative to CONT, the control treatment (Bonferroni-corrected post hoc contrast) (Table 2, Fig. 5). There was no significant difference between the control and any other treatment for PLFAs after one year.

After four years, the treatment effects on PLFAs had changed: there were no longer significant differences among substrates in the total number or richness of PLFAs or in the proportional abundance of Gram-positive or Gram-negative organisms (Table 2, Fig. 5). While the overall treatment effect on Gram-positive and Gram-negative organisms remained significant, it was now driven by reduced proportional abundances of these organisms in NON, IN, and INC treatments (for Gram-positives) and the LTN and INC treatments (for Gram-negatives) relative to the control, CONT (Bonferroni-corrected contrast) (Fig. 5).

Including the proportion of initial mass lost as a covariate in the ANOVA models, to control for the stage of decomposition, did not alter treatment effects, indicating that treatments were affecting microbial communities more than just by changing decomposition rate and thus the stage of decomposition (analyses not shown). By contrast, differences between leaves and litter after one year were largely explained by leaves being further along in the decay process, and more heavily colonized by decomposers as a result, as including proportion of initial mass lost as a covariate weakened the significance of substrate effects (analyses not shown).

Both substrate and treatment significantly altered the ratio of normal saturated/monounsaturated PLFAs, an index of nutritional stress (Table 2). After one year, this index was higher (indicating nutritional stress) for leaf litter than for green leaves (Tukey's HSD $P < 0.05$, data not shown). After four years, substrate no longer significantly affected this nutritional stress index. Among treatments, after one year, the index declined significantly with organic N addition compared to the control (Bonferroni-corrected contrast, $P < 0.05$, Fig. 6). After four years, the index was significantly increased in the LTN and INC treatments (Bonferroni-corrected contrast, $P < 0.05$).

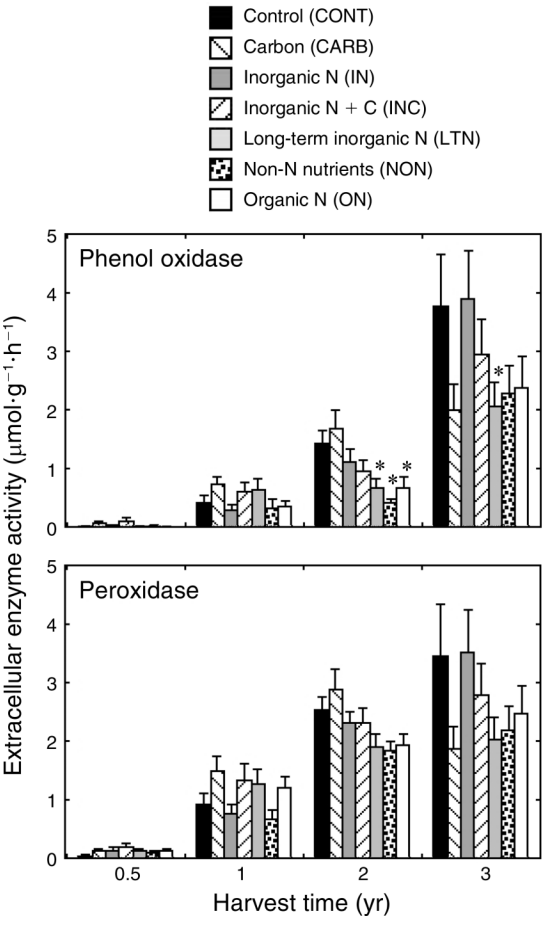

FIG. 3. Oxidative enzyme activity on substrates harvested during the first three years of decomposition by treatment (averaged over sites and substrates). Statistical comparisons were only done for the two- and three-year harvests because of high numbers of zero values in prior harvests. An asterisk indicates that a particular treatment differed significantly from the control treatment at a particular harvest time (experiment-wise alpha = 0.05). Values are means + SE. Overall model R^2 values for the oxidative enzymes from three-way ANOVAs including treatment, site, and substrate as main effects were 0.34 and 0.24 for POX (phenol oxidase) and 0.22 and 0.23 for PER (peroxidase) after two and three years of decomposition, respectively.

When all mole fractions of PLFAs were analyzed in a single community analysis using PERMANOVA, there were significant main effects of treatment, site, substrate, and harvest ($P < 0.001$ in all cases) and significant site by harvest and substrate by harvest interactions ($P < 0.001$) on community composition. These results were robust across dissimilarity indices and were similar regardless of whether proportional abundances of each individual PLFA or the major groups of soil microbes (e.g., Gram positive, Gram negative, actinomycetes, fungi) were analyzed. Vector-fitting of NMDS ordination on individual PLFAs or on groups yielded significant relationships with harvest and proportion mass loss and site, treatments, and substrates ($P < 0.001$

TABLE 2. Results of three-way ANOVAs for each major group of PLFAs comparing site, treatment, and substrate effects, analyzed for each harvest interval separately (see *Methods*).

Harvest and effects	Total PLFAs	Total number of PLFAs	Gram-positive/ total PLFAs	Gram-negative/ total PLFAs	Actinomycete/ total PLFAs	Fungal/ total PLFAs	Normal saturated/ monounsaturated PLFAs
One-year harvest							
Site (S)	†	†	***	**			
Substrate (Sub)	***	***	***	***			***
Treatment (T)		†	***	***			***
S × Sub							
S × T		*		*			
Sub × T			*				
S × Sub × T				†		†	
Four-year harvest							
Site (S)	**	*		***	***	***	***
Substrate (Sub)						†	
Treatment (T)		†	***	**			***
S × Sub						†	
S × T		*	†				
Sub × T							
S × Sub × T							

Note: Empty cells indicate nonsignificant results.
* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; † $P \leq 0.10$.

in all cases). Communities differed significantly in response to harvests, treatments, sites, and substrates; differences were large between harvests, but were small among treatments (Appendix E), sites, and substrates (not shown), regardless of whether NMDS was done for the major groups of soil microbes (Appendix E) or individual PLFAs (not shown).

DISCUSSION

By fitting asymptotic decomposition models to decomposition data (sensu Berg 2000), we were able to examine effects of substrate and externally supplied N on both initial rates of decomposition and on the value of asymptotic mass remaining, the fraction of very slowly decomposing litter. From these analyses, we conclude that both substrate and externally supplied N, regardless of form, stimulated initial rates of decomposition, while externally supplied N led to a larger, slowly decomposing litter fraction, and substrate N was associated with a smaller, slowly decomposing fraction. Below we explore these key findings in more depth, relating them to treatment effects on microbial community structure and function, and addressing underlying mechanisms and implications for ecosystem carbon cycling.

Decomposition and microbial responses to nitrogen addition

Across three forested sites, addition of externally supplied N had contrasting effects on initial vs. later stages of decomposition, speeding up decomposition initially, but resulting in greater asymptotic mass remaining, essentially a larger, slowly decomposing fraction (or lower "limit value" sensu (Berg and Ekbohm 1991). These contrasting effects of N occurred regardless of whether N was applied as inorganic N (NH_4NO_3) or as a mixture of labile organic N forms, and whether N addition was initiated 0.5 vs. 5 years prior to the beginning of the decomposition study. Although we have found such contrasting effects previously (Hobbie 2005, 2008), fitting the mass loss data to an asymptotic model made this contrast particularly clear. To our knowledge, few other studies have examined the effects of externally supplied N on decomposition using asymptotic models; those few studies found mixed responses of the asymptote (or limit value) to N fertilization that depended on litter species identity (Berg 2000). Differing responses to N addition initially vs. later in decomposition may help explain some of the variation in N effects among studies, although very few studies of N fertilization effects on decomposition are sufficiently long to allow estimation of asymptotes/limit values (Knorr et al. 2005).

The contrasting effects of externally supplied N early vs. late in decomposition were clearly linked to dynamics in extracellular enzyme activity. Very early in decomposition, N addition, regardless of form, increased activity of enzymes associated with polysaccharide degradation (β-1,4-glucosidase, cellobiohydrolase, and β-1,4-N-acetylglucosaminidase) at the same time that N increased decomposition rates. Later in decomposition, some N-containing treatments were associated with lower activity of the lignin-degrading enzyme phenol oxidase, consistent with a larger slow fraction (*A*) in these treatments.

These results are consistent with some, but not all, previous research on enzyme activity at this site and other similar forest sites. For example, Carreiro et al. (2000) observed similar patterns in decomposing litter of a closely related oak species, *Quercus rubra*. However, in the present study and in other studies of forest stands dominated by oaks as well as both coniferous and other

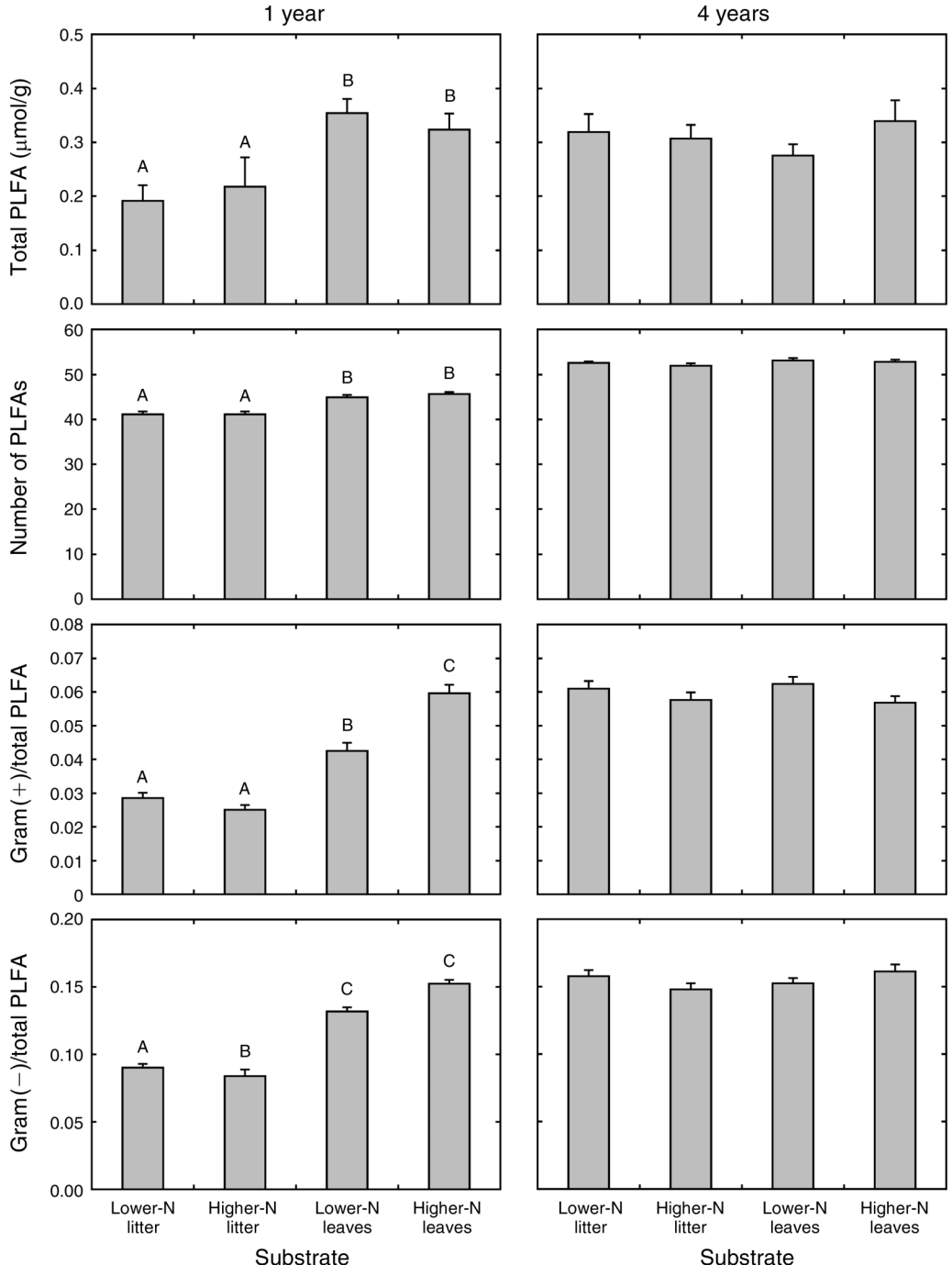

FIG. 4. Total abundance and number of phospholipid fatty acids, PLFAs (see *Methods*), and abundance of Gram-positive and Gram-negative organisms on different substrates after one and four years (averaged over treatments and sites). Different letters indicate significant differences among substrates (Tukey's HSD, $P < 0.05$). Values are means + SE.

hardwood species, there was no effect of added N on extracellular enzyme activity in the bulk O horizon (Blackwood et al. 2007, Keeler et al. 2009). In still other studies, effects of added N depended on species composition in the overstory (Waldrop et al. 2004*a*, Weand et al. 2010), with oak, mixed hardwood, and pine stands sometimes exhibiting increased hydrolytic but reduced phenol oxidase activity, responses similar to those shown here (Frey et al. 2004, Waldrop et al. 2004*a*). Taken together, these results suggest that the response of extracellular enzyme activity to added N helps explain the effects of N on decomposition.

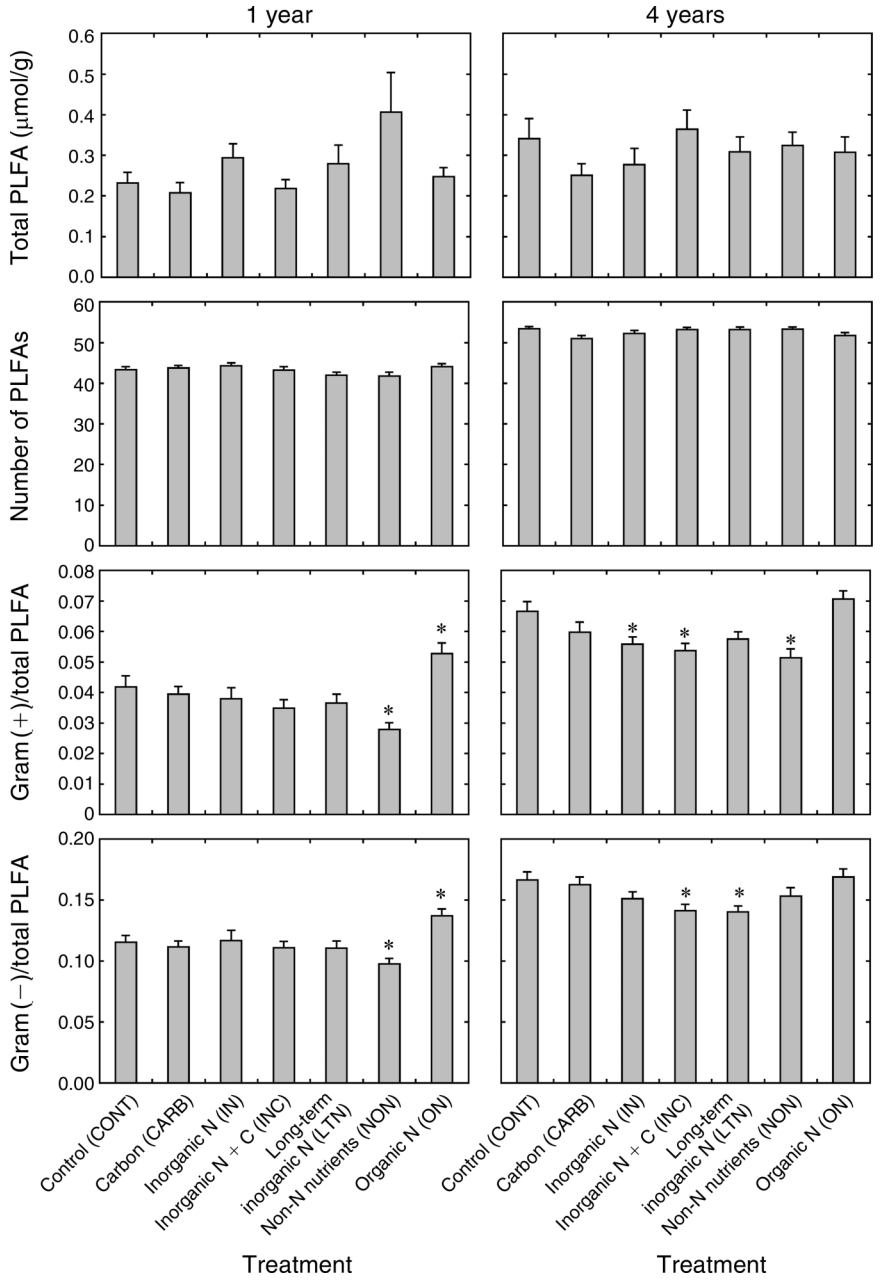

FIG. 5. Total abundance and number of PLFAs (see *Methods*) and proportional abundance of Gram-positive and Gram-negative organisms on litter decomposed in the different treatments after one and four years (averaged over substrates and sites). An asterisk indicates that a particular treatment differed significantly from the control treatment (experimentwise alpha = 0.05). Values are means + SE.

However, such activity can vary on small spatial scales within the forest floor, being isolated to particular types of litter and particular stages of litter decay, likely making these effects variable and difficult to detect in bulk forest floor samples.

In contrast to enzyme activities, patterns of N effects on decomposition were mirrored only in part by contrasting effects of N addition on decomposer community structure initially vs. later on. After one year of decomposition, only organic N addition altered microbial communities, increasing relative abundances of Gram-positive and Gram-negative bacteria and reducing nutritional stress. In contrast, all treatments containing N increased initial decomposition rates, even inorganic ones that did not alter microbial communities. Later in decomposition, all treatments containing N increased the size of the slow fraction (reduced the limit value), but only treatments containing inorganic N

appear to have created an inhospitable or competitive environment for certain microbes, reducing the relative abundances of Gram-positive and Gram-negative bacteria, and inducing nutritional stress.

A growing number of studies have shown idiosyncratic effects of added N on soil and litter microbial communities. In contrast to our results for litter, on average N reduces microbial biomass in soils (Treseder 2008). Sometimes added N increased (Weand et al. 2010) and other times decreased (Frey et al. 2004) fungi relative to bacteria (Cusack et al. 2011). Added N has also been shown to increase actinomycete abundance in soil, but not litter (Eisenlord and Zak 2010). In other studies, added N altered abundances of individual (Gallo et al. 2004) and total (DeForest et al. 2004) PLFAs, but had no effect on relative abundances of the major groups of soil organisms (i.e., Gram-negative bacteria, Gram-positive bacteria, actinomycetes, fungi). Our results indicate that such contrasting responses to added N also can occur within the same substrate over the course of decomposition.

In summary, regardless of site and substrate, the effects of externally supplied N shifted over the course of decomposition, with N accelerating the initial rate of decomposition, but ultimately resulting in a larger slow fraction. These patterns were mirrored by shifting effects of N on extracellular enzyme activity, whereby N increased activity of polysaccharide-degrading enzymes initially, but reduced rates of lignin-degrading phenol oxidase later on, a mechanism that has been reported previously. We cannot rule out that added N also slowed decomposition in the later stages by increasing the formation of recalcitrant organic compounds (Fog 1988).

Substrate vs. externally supplied nitrogen

In contrast to externally supplied sources of N, substrate N was associated with faster decomposition both initially and in the later stages of decay, as leaves had more rapid initial decomposition rates and smaller slow fractions (higher limit values) than did litter. These results contrast other studies demonstrating that N-rich leaves had slow fractions that were similar to or even larger than senesced leaves (Berg et al. 1982, Berg et al. 1996, Berg and Meentemeyer 2002, Berg et al. 2010), including a study of a broader diversity of substrates decomposed at sites including those studied here (Appendix A). Although many of the studies by Berg and colleagues lasted longer than the four years of this study, the levels of asymptotic mass loss in the present study were ~13% mass remaining for litter and ~5–7% mass remaining for leaves (limit values of 86% and 93–95%, respectively), indicating that decomposition was quite far along and well into the lignin decomposition stage (Berg et al. 1996) where negative effects of substrate N might be expected to occur.

The considerably lower initial lignin concentrations in leaves compared to litter likely negated any potential

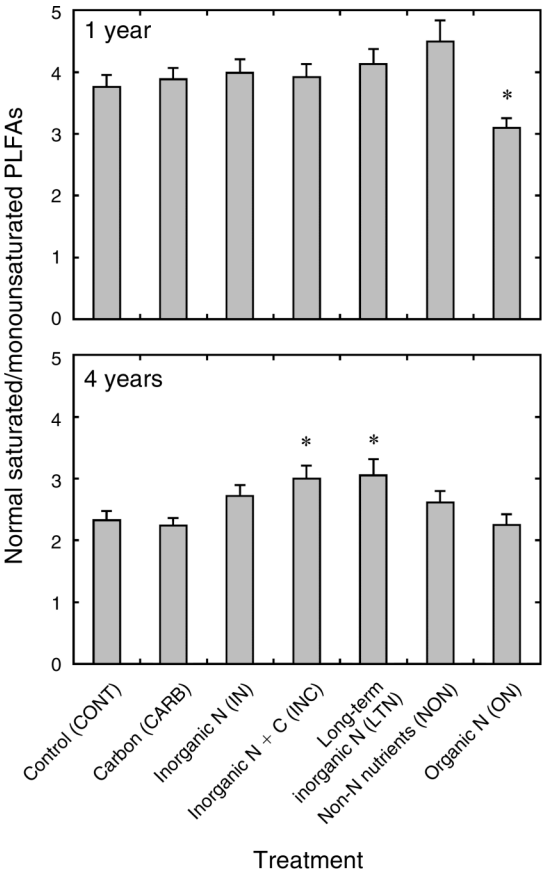

FIG. 6. The ratio of normal saturated/monounsaturated PLFAs (see *Methods*) on litter decomposed in the different treatments after one and four years (averaged over substrates and sites). An asterisk indicates that a particular treatment differed significantly from the control treatment (experiment-wise alpha = 0.05). Values are means + SE.

negative effects of initial N on late-stage decomposition, as decomposition rates often are negatively related to lignin concentrations and lignin : N ratios (Meetenmeyer 1978, Melillo et al. 1982, Aerts 1997, Cornwell et al. 2008). This makes sense particularly if the primary mechanism for negative effects substrate N is inhibition of oxidative enzyme activity, which should have larger effects on decomposition of substrates with higher lignin concentrations; low-lignin leaves would likely show little effects of altered oxidative enzyme activity. Concentrations of other nutrients besides N that differed between leaves and litter likely did not contribute to differences between leaf and litter decomposition, since addition of non-N nutrients did not indicate limitation by other nutrients besides N; however, at various time points, the NON treatment caused higher values of asymptotic mass remaining and negatively impacted hydrolytic and oxidative enzyme activities and relative abundances of some groups of microorganisms, perhaps because micronutrients reached toxic levels in the NON treatment. As a result, this treatment may not have been an

effective method for assessing effects of micronutrients in litter.

Unlike patterns of decomposition among treatments, decomposition was less clearly related to enzyme activity among substrates. After one year, some hydrolytic enzymes were higher for leaves than for litter at some time points, although leaves and litter were less clearly distinct from one another for enzyme activities than they were for mass loss. For oxidative enzymes, the differences between leaves and litter changed between two and three years: while N-rich leaves had lower phenol oxidase activity than litter after two years, as did the N-addition treatments, in the following year, leaves actually had higher activity than litter.

Patterns of decomposition among substrates corresponded in part to patterns of microbial community composition. After one year of decomposition, more rapidly decomposing leaves also had higher microbial abundance (as measured by total PLFA), higher relative abundance of Gram-positive and negative bacteria, and lower nutritional stress. These differences between leaves and litter disappeared by four years.

In summary, by examining early vs. late stages of decomposition separately using an asymptotic model, we showed that multiple forms of N (substrate N and externally supplied organic and inorganic N) sped up the initial stages of decomposition, with no readily apparent difference between organic and inorganic forms of N, in contrast to our expectations. Thus, the energetic costs of assimilating these different forms of N may not be different enough to translate into differences in early carbon use. However, in contrast to our expectations (and to results of others), substrate N decreased, while externally supplied N increased, levels of asymptotic mass loss, likely because of corresponding lower lignin levels in leaves relative to litter.

Implications for carbon storage

These results suggest that elevated atmospheric N deposition in these forested ecosystems may have contrasting effects on the dynamics of different carbon pools in the soil, decreasing mean residence times of active fractions comprising fresh litter, while increasing those of more slowly decomposing fractions including more processed litter. However, a caveat to that conclusion is that other studies have found evidence that consequences of added N on decomposition and enzyme activity for forest floor and mineral soil carbon stocks are system and horizon specific (Waldrop et al. 2004*b*, Blackwood et al. 2007, Zak et al. 2008). Of course, the net effect of added N on carbon pools depends on the relative stimulation of net primary production and initial decomposition vs. inhibition of later decomposition stages (and soil organic matter decomposition) by N. Such effects can be explored most effectively using models. To date, models have rarely considered effects of external N on decomposition, let alone differential effects of external and internal N (positive vs. negative) on different decomposition processes and carbon pools; considering litter decomposition as a multi-stage process, differentially affected by N, may prove insightful. One exception is the work of Gerber et al. (2010); they modeled decomposition of the "light fraction" of soil organic matter (i.e., litter) as an N-limited process, but modeled accelerated movement of litter into the more recalcitrant "heavy fraction" under conditions of higher soil N availability. Incorporation of these different mechanisms of N effects on decomposition processes had the net effect of modestly increasing total carbon stocks in their simulations, compared to a baseline case without these processes included. (Simulations were done with pre-industrial N deposition levels.) Gerber et al. point out that this is an active area of research that has been considered very little by such models. The results presented here add to growing evidence that carbon stabilization into more slowly decomposing fractions may occur because of N inhibition of oxidative enzyme activity and support the inclusion of such effects in simulation models.

Future research needs

The results of this research highlight the need for studies of N effects on litter decomposition that encompass the later stages of mass loss and model mass loss accordingly. Presently, the number of longer-term studies is limited, impeding meta-analytical efforts aimed at elucidating general patterns regarding N effects on decomposition (Knorr et al. 2005). In addition, in most decomposition studies, data are fit to a single exponential decay model (Adair et al. 2010); however, here we show that multi-pool models (asymptotic in this case) can be equal or better fits to the data. Fitting decomposition data to such models can be particularly informative in elucidating differential N effects on different litter stages and pools. More studies that consider N effects on multiple stages of decay (e.g., by comparing fits of different exponential models to data from N-enrichment studies) would allow the quantification of how and whether N effects differ depending on the litter fractions that are decomposing, as they did here.

In addition, studies of N effects on litter decomposition should be conducted in a wider range of sites (grasslands as well as forests, tropical as well as temperate sites) and should be coupled with measurements aimed at elucidating mechanisms that underlie N effects, particularly negative ones (e.g., measurements of extracellular enzyme activities and microbial community composition). Such studies would reveal whether there are systematic differences in decomposition responses to N depending on the type of system. For example, they would allow researchers to test the hypothesis that litter decomposition should be less responsive to added N in sites where the oxidative activity of the decomposer communities is less sensitive to added N (e.g., in grasslands where fungal communities are dominated

by Ascomycota and Glomeromycota compared to forests where Basidiomycota dominate) (Sinsabaugh 2010).

Finally, the contrasting effects of substrate and externally supplied N on different stages of decomposition should be more widely considered in biogeochemical models, as we are aware of only one such effort (Gerber et al. 2010), despite long-standing evidence that both may alter decomposition of litter and soil organic matter (Berg 1986, Fog 1988, Berg and Matzner 1997, Knorr et al. 2005, Keeler et al. 2009, Janssens et al. 2010). Considering such effects would inform modeling efforts that aim to determine the effects of anthropogenic N deposition on terrestrial carbon sequestration. Further, modeling efforts could help to scale experimental results in terms of potential impacts on regional and global carbon cycling, providing much-needed context and perspective for empirical studies of the effects of N on decomposition.

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Supplemental Material

Appendix A

Figure showing the relationship between the initial decay rate and the proportion of initial mass in the slowly decomposing fraction plotted against initial N concentration for seven leaf and litter substrates (data from Hobbie [2008]) (*Ecological Archives* M082-013-A1).

Appendix B

Parameters obtained by fitting decomposition data to asymptotic and single exponential decay models to data showing treatments, sites, and substrates (*Ecological Archives* M082-013-A2).

Appendix C

Figure showing proportion of initial mass remaining vs. proportion of initial N for green leaves vs. litter (*Ecological Archives* M082-013-A3).

Appendix D

Extracellular enzyme activities on substrates harvested during the first three years of the experiment by substrate (averaged over sites and treatments) (*Ecological Archives* M082-013-A4).

Appendix E

Results of NMDS ordinations of PLFA groups (Gram-positive bacteria, Gram-negative bacteria, actinomycetes, fungi) (*Ecological Archives* M082-013-A5).

Data Availability

Data associated with this paper have been deposited in the Cedar Creek LTER website: www.cedarcreek.umn.edu/research/data/