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MICROBIAL ENZYME SHIFTS EXPLAIN LITTER DECAY RESPONSES TO SIMULATED NITROGEN DEPOSITION

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Abstract. Some natural ecosystems near industrialized and agricultural areas receive atmospheric nitrogen inputs that are an order of magnitude greater than those presumed for preindustrial times. Because nitrogen (N) often limits microbial growth on dead vegetation, increased N input can be expected to affect the ecosystem process of decomposition. We found that extracellular enzyme responses of a forest-floor microbial community to chronically applied aqueous NH_4NO_3 can explain both increased and decreased litter decomposition rates caused by added N. Microbes responded to N by increasing cellulase activity in decaying leaf litter of flowering dogwood, red maple, and red oak, but in high-lignin oak litter, the activity of lignin-degrading phenol oxidase declined substantially. We believe this is the first report of reduced ligninolytic enzyme activity caused by chronic N addition in an ecosystem. This result provides evidence that ligninolytic enzyme suppression can be an important mechanism explaining decreased decay rates of plant matter seen in this and other N-addition experiments. Since lignin and cellulose are the two most abundant organic resources on earth, these altered enzyme responses signal that atmospheric N deposition may be affecting the global carbon cycle by influencing the activities of microbes and their carbon-acquiring enzymes—especially the unique ligninolytic enzymes produced by white-rot fungi—over broad geographic areas.

Key words: litter decay; microbial enzymes; nitrogen deposition; white-rot fungi.

Introduction

Increased atmospheric nitrogen (N) emissions (Galloway et al. 1994, Vitousek et al. 1997) now cause natural systems near industrialized and agricultural areas to receive N inputs of up to 60 $kg \cdot ha^{-1} \cdot yr^{-1}$ (van Breeman et al. 1982, Nihlgard 1985) compared with fluxes of ~0.5 to 2 $kg\ N \cdot ha^{-1} \cdot yr^{-1}$ (Nihlgard 1985, Aber et al. 1989, Ollinger et al. 1993) at more isolated sites. Since plants and microbes of many terrestrial ecosystems have evolved under N-limiting conditions (Vitousek and Howarth 1991), these recent and large increases in N input can be expected to affect ecosystem processes like primary production and decomposition.

Because primary production in most terrestrial systems is N-limited, a prevailing paradigm for many decades has been that N must also limit microbial decay of plant tissues. This supposition has been supported by observations that plant materials with low carbon (C)-to-nitrogen or lignin:N ratios decayed more rapidly than those with high C:N or lignin:N ratios, and that recalcitrant plant materials containing high C:N ratios or high lignin content accumulated N, probably from soil and throughfall precipitation, during the early to middle stages of decay (Swift et al. 1979, Aber and Melillo 1982, Melillo et al. 1982, Dighton and Boddy 1989). Dead plant tissues are low in N because they are composed primarily of cell wall materials that in turn consist mostly of carbohydrates and aromatic C compounds. Cellulose and hemicellulose are the most abundant materials in the primary cell walls of plant tissues like leaf mesophyll, while cells having secondary walls are also impregnated with lignin, an aromatic C polymer that covers and shields cellulose and hemicellulose from microbial decay (lignocellulose) (Hall et al. 1982). Nitrogen inputs from atmospheric deposition would then be expected to accelerate litter decay rates by supplying decomposer microbes with this limiting nutrient.

However, such straightforward predictions have been complicated by many findings to the contrary, namely that N addition to soil or dead plant matter either reduced or had little effect on microbial growth and respiration, or on litter mass loss rates (Fog 1988,

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TABLE 1. Initial leaf litter quality for dogwood, red maple, and red oak.

Species	Percentage cellulose	Percentage lignin	Percentage N	C:N	Lignin:N	LCI†
Dogwood	10.81 (0.23)	6.07 (0.29)	0.67 (0.03)	68.4	9.1	0.36
Red maple	10.38 (0.35)	9.76 (0.29)	0.82 (0.04)	58.2	11.9	0.49
Red oak	20.02 (0.29)	26.07 (0.89)	0.91 (0.01)	54.7	28.7	0.57

Note: Each value is the mean (with 1 SE reported in parentheses) of six samples and reported on a percentage ash-free dry mass basis.

† The LCI (lignocellulose index) is the mass fraction, lignin/(lignin + cellulose).

Magill and Aber 1998). Fog (1988) observed that those litters with high lignin content were usually the ones in which decay was suppressed by N. At least two hypotheses have been proposed to explain this surprising effect: (1) N reduces the efficiency of extracellular microbial enzymes by randomization of chemical bond structures of organic materials and (2) inorganic N suppresses microbial production of ligninolytic enzymes (Fog 1988, Magill and Aber 1998). A few studies using pure cultures of litter-decay fungi (Keyser et al. 1978, Kirk 1987) have supported this latter hypothesis by showing that some fungal species produced ligninolytic enzymes only when their growth became N limited, and that addition of inorganic N delayed appearance of these enzymes and suppressed lignin breakdown in vitro.

Carbon quantity in relation to N is not the only determinant of decay rate that should be considered. Melillo et al. (1989) have related litter decay patterns to the quality of carbon (C) available for microbial consumption by using the lignocellulose index (LCI), the ratio of lignin mass to combined lignin plus cellulose mass. In general, the greater the relative lignin content, and hence the higher the LCI, the more slowly a material will degrade. Recently, the integrated activities of microbial extracellular enzymes that break down cellulose and lignin have been correlated with litter mass loss rates, and hence with the turnover rates of organic carbon in a variety of ecosystems (Sinsabaugh and Linkins 1993, Sinsabaugh 1994, Sinsabaugh and Moorhead 1994). These enzyme measurements permit the functional responses of the microbial community to litter quality and other environmental factors to be followed directly rather than indirectly. Here we report the results of a controlled field experiment in which we evaluated how chronic N deposition affects the C-mineralization activities of decomposer microbial communities by measuring the activities of lignin- and cellulose-degrading enzymes, and by relating the resultant enzyme responses to the decay rates of leaf litters that differ in initial lignin content and LCI. Identifying predictable patterns of enzyme response to chronic N addition and linking them to decay rates can help improve existing models that estimate soil carbon residence time for terrestrial systems that may be subjected to increasing atmospheric N deposition in the future.

METHODS

Rationale and experimental design

In this study we measured the effects of N addition (+20 and +80 kg $N \cdot ha^{-1} \cdot yr^{-1}$ above estimated ambient fluxes of 10 kg $N \cdot ha^{-1} \cdot yr^{-1}$ at the experimental site [Meyers et al. 1991, Ollinger et al. 1993]) on the leaf litter decay rates of three tree species common in deciduous forests in the eastern United States (*Cornus florida* [flowering dogwood], *Acer rubrum* [red maple], and *Quercus rubra* [red oak]). These species vary in initial foliar C:N ratios, lignin concentrations, and LCI (Table 1). To provide reasonably realistic decay responses to N, we applied aqueous solutions of N (as NH_4NO_3) monthly throughout the year rather than in the large, pulsed doses (150–600 kg N/ha, often in one annual application) used in most N fertilization experiments in the past (Fog 1988). Over a 1- to 3-yr period we followed mass loss from each litter species in a temperate forest, and the activity of five microbial enzymes that degrade lignin and cellulose, the two most abundant organic polymers in terrestrial ecosystems (Cooke and Whipps 1993, Dix and Webster 1995).

The experiment was performed in a 40-ha, mixed deciduous forest at the Louis Calder Center in Armonk, New York, USA (41° 07′ N and 73° 44′ W; elevation 192 m). Ten grams of air-dried red oak, 15 g of red maple, or 20 g of dogwood leaf litters were placed in separate 1.3-mm fiberglass mesh bags. Litter bags were pinned to the forest floor as two-bag sampling units in a randomized block design (six blocks × three N treatments). The red oak litter experiment was started on 1 November 1993; red maple on 17 October 1994; and dogwood on 23 October 1995. Thirteen collections each of red oak, red maple, and dogwood litters were made over 1111 d, 764 d, and 381 d, respectively. On each collection date, one bag pair for each litter species was collected from each treatment per block. One bag from each pair was used to estimate mass loss. Mass loss from the litter was determined after drying at 55°C and ash-free dry mass (AFDM) after loss on ignition (450°C). The initial percentage C and percentage N of the litter were measured using a Perkin-Elmer (Norwalk, Connecticut, USA) CHN analyzer, and initial lignin and cellulose content were determined using the wet chemistry, C fractionation method of Van Soest et al. (1991). Due to the C-fractionation method used in

this study, the cellulose fraction does not include hemicellulose.

Enzyme assays

Using the contents of the second litter bag from each bag pair, we quantified the activities of the following cellulolytic and ligninolytic enzymes: β-1,4-glucosidase (EC 3.2.1.21), cellobiohydrolase (EC 3.2.1.91), β-1,4-endoglucanase (endocellulase) (EC 3.2.1.4), phenol oxidase (EC 1.10.3.2 and 1.14.18.1), and phenol peroxidase (EC 1.11.1.7). β-1,4-glucosidase and cellobiohydrolase are exocellulases that remove glucan units from the ends of the cellulose chains, while β-1,4-endoglucanase is an endocellulase that disrupts bonds within the cellulose chains. Assays were performed by blending 1 g of litter in 150 mL acetate buffer (50 mmol/L, pH 5). Activities of the exocellulases (β-1,4-glucosidase and cellobiohydrolase) were measured using the substrates (*p*-Nitrophenyl-β-D-glucopyranoside and *p*-Nitrophenyl-β-D-cellobioside, respectively) bound to the chromogen *p*-nitrophenol (*p*NP) and adding them to the litter slurry, incubating the mixture for 1 to 4 h at 25°C, and spectrophotometrically measuring the optical density (OD) of *p*NP released after enzyme cleavage at 410 nm (Sinsabaugh and Linkins 1990). L-dihydroxyphenylalanine (L-DOPA) was used as substrate for both peroxidase and phenol oxidase assays (litter homogenate with and without peroxide, respectively) and the OD of the oxidized reaction product measured at 460 nm. The endocellulase assay (Almin and Eriksson 1967) is a viscometric method that uses carboxymethylcellulose as a substrate added to the litter slurry. Enzyme activities for the *p*NP and L-DOPA assays are expressed as μmol substrate converted per gram of ash-free dry mass litter per hour. Endocellulase activity is expressed as viscometric units per gram of ash-free dry mass litter per hour.

Data analyses

Cumulative enzyme activity was calculated using the trapezoidal rule to integrate the area under the curves of enzyme activity plotted against time (the respective decay periods in days for each litter type) and is expressed as cumulative enzyme activity-days. For each litter type, differences among treatments in the cumulative activities of each of the five C-acquiring enzymes were examined using two-way (treatment × block), mixed model ANOVA without replication. We estimated exponential decay rate coefficients, k, for each of the three litter species and three N treatment levels by fitting the equation $f = a\exp(-kt)$ to the data using nonlinear mixed-effects (nlme) modeling (Davidian and Giltinan 1995, Pinheiro and Bates 1995), with N treatments as the fixed effect and blocks as the random effect. The software used was the nlme procedure for S-plus (Pinheiro and Bates 1995). Relationships between cumulative enzyme activities and litter mass loss rates were explored using multiple linear regression with models selected by minimizing the Akaike information criterion (Venables and Ripley 1997). Separate decay coefficients (k's) were calculated for each block, N level, and litter species (18 k's in all for each litter species) using nonlinear regression, and then regressed on the integrated activity of each enzyme for each block. Cellobiohydrolase activity was dropped from the multiple regression analyses because it correlated highly with β-glucosidase (Pearson r values of 0.99, 0.91, and 0.76 for dogwood, maple, and oak, respectively) and β-glucosidase activity provided slightly more explanatory power. For comparisons of relative enzyme activity against initial LCI and decay rate, the cumulative activities of β-glucosidase and cellobiohydrolase were combined as an index of cellulase activity. Endocellulase was omitted for this comparison, because it was measured using a different technique and expressed in different units, making its results nonadditive with the other cellulases. For the ligninolytic enzymes, peroxidase activity was not used in this last comparison since it was found not to correlate with decay rate in the multiple linear regression.

RESULTS

Nitrogen addition affected the decay rate of leaf litter (Table 2), but both the magnitude and direction of the N effect varied with initial litter lignin content and initial LCI. For the low-lignin dogwood litter, both the high and low N additions increased the litter decay-rate coefficient by 26% compared to the control litter. In contrast, for the high-lignin oak litter, the low and high N treatments depressed the decay rate coefficient by 15% and 25%, respectively, increasing the litter mean residence time from 3.4 yr to 4.0 yr and 4.5 yr. For maple litter, the exponential decay rate coefficients were not strongly affected by N addition, but there were indications that the high N treatment reduced the decay rate coefficient slightly.

The responses of the five microbial enzymes that degrade either cellulose or lignin in the leaf litter provide mechanistic explanations for these seemingly counterintuitive results. For dogwood litter, N greatly stimulated microbial cellulase activity. The responses of all three cellulases to the N additions were monotonic and large, with the high N treatment stimulating β-glucosidase, cellobiohydrolase, and endocellulase by 66%, 100%, and 42%, respectively, over those in control litter receiving ambient N levels (Fig. 1A). Although the response was not statistically significant ($P = 0.1$), N also increased the cumulative activity of phenol oxidase, an enzyme involved in lignin breakdown (Fig. 1A). The combined increase in activity of all cellulases and one of the ligninolytic enzymes by N can, therefore, explain to a large extent the accelerated mass loss rates of dogwood litter.

For maple litter, with moderate lignin content, N increased the cumulative activity of two of the cellulases

TABLE 2. Exponential decay rate coefficients for leaf litter of three species receiving three levels of NH_4NO_3 treatment.

	Mass loss rates (d^{-1}) ($\times$ 10^4)†			
Species	Ambient N	+20 kg N·ha^{-1}·yr^{-1}	+80 kg N·ha^{-1}·yr^{-1}	P
Dogwood	−25.7 (1.83) [1.07]	−32.4 (1.97) [0.85]	−32.5 (1.97) [0.84]	0.0004
Maple	−13.6 (0.74) [2.02]	−14.1 (0.76) [1.95]	−12.0 (0.72) [2.28]	0.10
Oak	−8.15 (05.2) [3.36]	−6.92 (0.51) [3.96]	−6.11 (0.50) [4.48]	<0.0001

Notes: P values were derived from likelihood ratio tests for differences in slopes among treatments. There were 13 sample dates for each litter species. Duration of the decay periods are: dogwood, 381 d; maple, 764 d; and oak, 1111 d. All mass loss data were on an ash-free dry mass basis and were based on six litter bags per treatment per collection date for each species.

† Means (and standard errors) of the decay rate coefficients were determined by a nonlinear mixed effects model (Lindstrom and Bates 1990, Davidian and Giltinan 1995). Turnover time (mean residence time) of litter in years appears in brackets.

(β-glucosidase and cellobiohydrolase) (Fig. 1B), but had little effect on ligninolytic enzymes. In oak, N addition stimulated β-glucosidase and cellobiohydrolase activity by 15% for the low and 32% for the high N treatment, which was not as great as the effect for dogwood (Fig. 1C). However, contrary to effects seen in dogwood litter, both levels of N addition strongly suppressed phenol oxidase activity in the high-lignin oak litter (Fig. 1C), with the high N treatment reducing phenol oxidase activity by 54%. We believe this is the first report of reduction in activity of a ligninolytic enzyme by exogenous N addition in a natural system. As explained below, the large depression in activity of this lignin-degrading enzyme can explain much of the reduction in the decay rate of oak litter due to N addition.

To determine how strongly the differential enzyme responses to added N were linked to variation in litter decomposition rate, we estimated the specific first-order decay coefficients (k) for each litter type and treatment by nonlinear regression for each of the six blocks. The coefficients were then modeled as linear functions of the integrated activities of each of the five cellulolytic or ligninolytic enzymes to estimate which enzyme or enzyme subset was best related to the mass loss rates obtained. For dogwood litter, only two enzymes were retained using multiple linear regression. The relationship relating the variation in k (fraction lost per day) due to N treatment for dogwood litter can be described by the equation

$$k = 0.00165 + (2.77 \times 10^{-8})G + (6.56 \times 10^{-11})E \quad (1)$$

where G = cumulative ß-glucosidase activity-days and E = cumulative endocellulase activity-days (adjusted R^2 = 0.662). The units for the regression coefficients are litter mass fraction per day per enzyme activity-day.

For maple litter, no enzyme singly or in combination usefully explained the variation in k due to N treatment. For oak, variation in phenol oxidase alone was associated with nearly 51% of the variance in the decay rate coefficients, and addition of other enzymes to the model contributed no additional explanatory power. The resultant equation was

$$k = 0.00049 + (1.072 \times 10^{-8}) P \quad (2)$$

where P = phenol oxidase activity-days (adjusted R^2 = 0.507).

In addition to linking litter decay responses to N to the activities of microbial C-acquiring enzymes, this experiment also demonstrates that the magnitude and direction of the N effect on litter decay rates can be related to the absolute and relative amounts of lignin and cellulose in the plant tissue. As initial LCI increased across litter types, the net effect of N on litter mass loss shifted direction from accelerated to reduced decay rates. This shift can be explained by the differential effects of N on cellulase and phenol oxidase activity and the amounts of cellulose and lignin in the litters (Fig. 2 A,B). Since N stimulated cellulase activity in all three litter types, the decay rates of litter with a high proportion of cellulose relative to lignin (low LCI) should also increase with added N (e.g., dogwood in Fig. 2A). Thus, for litter types that normally decay quickly, additional N may further accelerate mass loss. In oak litter, with a high proportion of lignin, the added N greatly reduced the activity of the ligninolytic enzyme, phenol oxidase (Fig. 2B). Therefore, for litter types that normally decay slowly due to high lignin content, exogenous N may depress mass loss rates further.

DISCUSSION

This experiment demonstrates the utility of using microbial enzyme activities to monitor directly the functional responses of microbial communities to natural or anthropogenic changes in their environment. In this study, the responses were those involving microbial breakdown and acquisition of specific C resources.

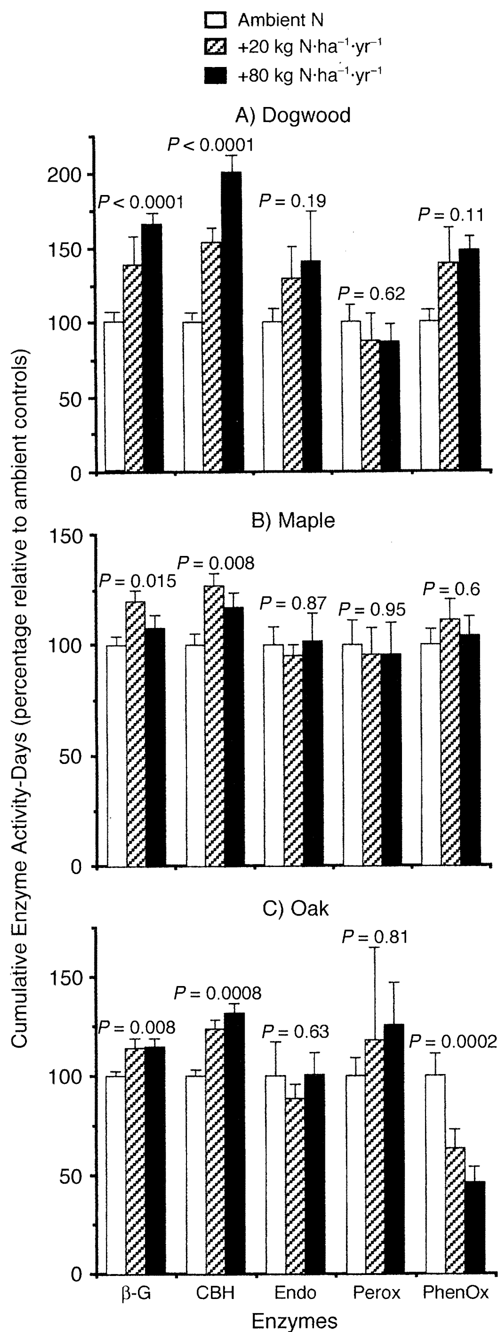

FIG. 1. Effects of two levels of N addition on the cumulative integrated activities of five microbial enzymes that degrade either cellulose (β-glucosidase, cellobiohydrolase, endocellulase) or lignin (peroxidase and phenol oxidase) in decaying dogwood, red maple, and red oak leaf litters relative to controls receiving ambient levels of N. All controls equal 100%. P values are derived from one-way analyses of variance for each enzyme–litter species combination. Data are based on six litter bags collected per treatment over 13 collection dates for each litter species.

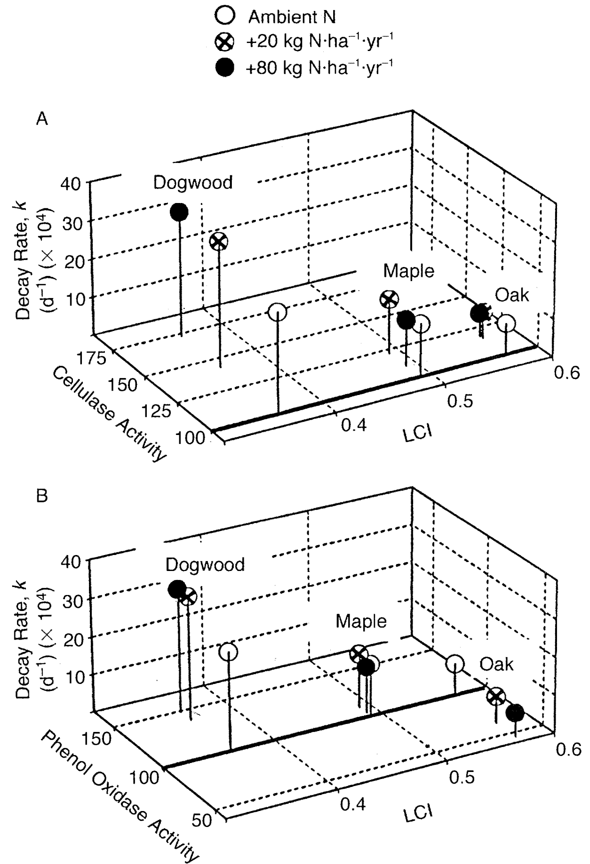

FIG. 2. Effects of two levels of N addition on the exponential decay rate coefficients (k) of dogwood, red maple, and red oak leaf litters and their relationships with litter LCI (lignocellulose index) and integrated enzyme activities relative to control levels (ambient N): (A) cellulase activity (combined activities of β-glucosidase and cellobiohydrolase); (B) activity of phenol oxidase, a ligninolytic enzyme. Solid bold lines represent the enzyme activities of ambient control levels, defined as 100%. Note especially the switch in direction of the N effect on phenol oxidase activity in the species with the highest LCI (i.e., lignin /[lignin + cellulose]).

Production of enzymes targeting cellulose and lignin, two major compounds in plant tissues, were differentially affected when the availability of exogenous inorganic N to microbial communities increased. Therefore the net effects of N on mass loss were related to variation in the relative abundance of cellulose and lignin in the different litter species.

In addition, the differences in community-level functional response may reveal contrasting effects of increasing N on different microbial taxa. Cellulases, which are produced by a large number of bacterial and fungal species, were stimulated in all of the litters. However, phenol oxidase, an important ligninolytic enzyme produced only by a small group of fungi (the white rot fungi) in the Basidiomycota and xylariaceous Ascomycota (Dix and Webster 1995), was greatly reduced by increased N availability in oak litter. We

therefore hypothesize that N deposition may be having a negative impact on the ligninolytic activity and perhaps abundance of white rot fungi, which are known to colonize recalcitrant litters like oak (Dix and Webster 1995). While some bacteria and microfungi can produce a limited subset of ligninolytic and lignin-modifying enzymes, white rot fungi are the only microbes known to produce phenol oxidase and all the other enzymes that are essential for degrading lignin completely. Since white rot fungi constitute a small proportion of saprotrophic macrofungal species, there exists comparatively little taxonomic and ecological redundancy in terrestrial systems for completing all aspects of this critical C-cycling function. Since fungal biomass was similar in oak litters in all three N treatments (M. M. Carreiro, R. L. Sinsabaugh, and D. A. Repert, *unpublished data*), reduced phenol oxidase activity in N-treated oak litter may have resulted from either suppressed white rot production of this enzyme, and/or from reduced competitiveness and hence reduced abundance of white rot fungi relative to other fungi in N-enriched litter. The possibility that N deposition may be decreasing the abundance or changing the activity of white rot fungal species is critical to investigate further, particularly in forested ecosystems, because of the long-term implications this phenomenon could have for extending C residence times in soil and wood, and for changing other ecological relationships in soils. We also suggest that the different directional effects of N on phenol oxidase activity (higher in dogwood, lower in oak) may be due to differences in lignin chemistry between litters, and/or to the extent to which these different litters are colonized by microbial species whose ligninolytic enzymes are suppressed rather than stimulated or unaffected by inorganic N (Kirk 1987, Buswell et al. 1995, Kaal et al. 1995). This suggests that the direction of N effects on C mineralization processes in litter may depend on which microbial species are present in different ecosystems, as well as the chemical composition of the substratum colonized.

Several implications at the forest-stand level can be inferred from this study. First, much of the enzyme and decay-rate response to the different N treatments resulted from addition of only 20 kg $N \cdot ha^{-1} \cdot yr^{-1}$ to the litter. Thus, large increases in N flux to forests in the northeastern United States in the future are apparently not necessary to alter decomposer dynamics and litter decay rates substantially. Second, the directional effect (faster or slower) of N on decay rates in forest stands will likely be related to the litter lignin content (or LCI) of the most abundant tree species and should be most easily detected in stands dominated by a single litter type. Third, interactions between N deposition and atmospheric CO_2 on litter decay rates would be expected should rising CO_2 levels increase either the C:N ratio or the lignin fraction in leaves. Such litter quality changes in response to CO_2 have been observed in deciduous and coniferous litters (Coûteaux et al. 1991, Cotrufo et al. 1994) with concomitant reductions in their decay rates (Cotrufo et al. 1994). Atmospheric N deposition may then interact with rising atmospheric CO_2 to increase the long-term C-storage potential of forest soils by decreasing the decay rate of an enlarged, recalcitrant organic matter pool.

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