Changes in litter quality caused by simulated nitrogen deposition reinforce the N-induced suppression of litter decay

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Abstract. Rates of nitrogen (N) deposition are increasing in industrialized and rapidly developing nations. Simulated N deposition suppresses plant litter decay rates, in particular for low quality (high lignin) litter. Litter quality is a primary driver of litter decomposition; however, it is not clear how changes in litter quality caused by long-term ecosystem exposure to chronic N additions interact with altered soil N-availability to influence litter decay dynamics. To document the effects of simulated N deposition on litter quality, we conducted a meta-analysis of available litter nutrient data from simulated N deposition experiments in temperate forests. To directly test whether changes in litter quality caused by N deposition affect decay rates, we also conducted a reciprocal litterbag study in an existing N addition experiment, where a northern hardwood forest has been exposed to simulated N deposition for more than 20 years. The experiment enabled us to disentangle the effects of N additions, litter quality, and their interactions on litter decay dynamics. We measured litter mass loss and extracellular enzyme activities after one and two years of decomposition. The meta-analysis demonstrated that simulated N deposition causes decreases of leaf Al, B, Ca, Mg, Mn, P, and Zn concentrations. Moreover, higher cumulative amounts of simulated N deposition result in greater decreases of leaf Ca and Mn concentrations. In the field experiment, litter originally collected from N-enriched plots exhibited similar N-induced nutrient changes as observed in our meta-analysis and also had lower lignolytic enzyme activities and decay rates than litter collected from control plots. The decreased litter decay rates observed with simulated N deposition were a result of the combined effects of the N fertilization itself and the historical effects of N deposition on tree litter chemistry. The data suggest that changes in litter quality caused directly by long-term N enrichment reinforce the negative effects of simulated N deposition on litter decay, particularly for high lignin species. The slowed decay associated with changes in litter quality caused by N enrichment itself may partly explain the accumulation of organic matter previously observed at ours and at other simulated N deposition experiments.

Key words: calcium; decomposition; extracellular enzymes; lignin; litter decay; litter quality; manganese; meta-analysis; nitrogen; Quercus; temperate forest.

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INTRODUCTION

Rates of nitrogen (N) deposition are increasing in industrialized and rapidly developing nations and are predicted to increase to >50 kg N ha\(^{-1}\) yr\(^{-1}\) by 2050 in many parts of the world (Galloway et al. 2008). Nitrogen enrichment affects many ecosystem processes, including plant litter decay (Zak et al. 2008, Lovett et al. 2013, Frey et al. 2014). Decomposition is a major pathway through which carbon (C) and N are cycled through ecosystems (Prescott 2010), and plant litter quality (e.g., C:N, lignin:N) is a primary control on decay rates (Prescott 2010). In particular, decomposition of low quality litter (litter with high lignin concentrations) is, on average, negatively affected by chronic N additions, while decomposition of high quality litter (litter with low lignin concentrations) is enhanced by increased N availability (Fog 1988, Knorr et al. 2005, Janssens et al. 2010). However, we do not fully understand whether decreased decay rates of high lignin litters observed under chronic N deposition are a result of the N fertilization itself, the historical effects of N deposition on tree litter chemistry, or some combination of these two mechanisms.

Changes in litter quality caused by long-term N enrichment have been reported, but previous studies have primarily focused on litter N concentrations, with the effects on other litter nutrients typically not measured (Magill et al. 2004, Pregitzer et al. 2008, Lovett et al. 2013). It is unlikely that N is the only litter nutrient affected by simulated N deposition, and while N-induced changes of other nutrients, for example calcium (Ca), magnesium (Mg), and phosphorus (P), have repeatedly been reported in tree foliage, they have primarily been considered from the perspective of their implications for plant health and physiology (Minocha et al. 2000, Lucas et al. 2011). The availability of nutrients other than N, including Ca and manganese (Mn), have been documented to impact litter decay rates (Berg et al. 2007, Davey et al. 2007, Berg and McClugherty 2014). Microorganisms require Ca and Mn for metabolism (e.g., Dominguez 2004, Plante 2007), and these nutrients are also required by saprotrophic fungi for the production of lignolytic enzymes which are responsible for the decomposition of lignin (Dashtban et al. 2010, and references within). Changes in leaf litter concentrations of Ca and Mn may thus impact the production of lignolytic enzymes, influencing litter decay dynamics. However, the effects of simulated N deposition on these nutrients in plant litter are not well documented.

To determine whether simulated N deposition affects litter nutrient concentrations, and to assess whether N-induced changes in litter quality contribute to the observed effects of simulated N deposition on decay rates of high lignin litter, we (1) synthesized the existing literature to quantify changes in litter nutrient concentrations as a consequence of simulated N deposition by conducting a meta-analysis of available litter nutrient data measured at simulated N deposition experiments in temperate forests, and (2) examined the effect of N-induced litter quality change on mass loss and microbial enzymatic capacities using a reciprocal litter transplant study conducted at a long-term (~22 years) N addition experiment. Decay dynamics of litter collected from N-amended versus control plots were compared, enabling us to document whether changes in litter decomposition dynamics were the direct result of the N addition itself, caused by the indirect effect of long-term N-induced changes in litter quality, or a result of interactions between these two mechanisms.

METHODS AND MATERIALS

Meta-analysis of leaf nutrient concentrations in response to long-term N addition

Data on leaf nutrient concentrations measured in N addition experiments were gathered from published and unpublished sources. For published studies, we searched the literature using Web of Science in April 2014 with the search terms “nitrogen” and “forest” and refined the search using “calcium” which is one of the most commonly measured nutrients in plant litter. Studies were included in the meta-analysis if values from both control and N-addition treatments were available and if error terms (i.e., standard deviations or standard errors) and the number of sample replicates were reported. We only included published studies where at least three of the following nutrients were measured; aluminum (Al), boron (B), copper (Cu), calcium
(Ca), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), nitrogen (N), phosphorus (P), and zinc (Zn). If data were displayed in figures, values were extracted using Gsys2.4.6 (Graph Suchi Yomitori System, Ryusuke SUZUKI, Japan). Because of the low number of published simulated N deposition studies where litter nutrient concentrations were reported, we expanded our search to include unpublished data. We contacted lead scientists of established long-term N addition experiments and inquired about the availability of unpublished litter nutrient data or litter samples to analyze for nutrient concentrations.

In addition to the nutrient data, study metadata (N addition rates, length of experiment, tree type) were extracted from the published studies or obtained from site collaborators and used in the meta-analysis to determine potential predictor variables. A previous meta-analysis of simulated N deposition effects on litter decomposition revealed that litter quality, as defined by lignin concentration and the amount of N fertilizer applied at a site were important predictor variables influencing rates of litter decomposition (Knorr et al. 2005). Therefore, we used the following categorical variables within our meta-analysis: litter lignin concentration (low: <15%, intermediate: 16–25%, high: >25%), amount of simulated N deposition (low: ≤50, intermediate: 51–100, high: >100 kg N ha⁻¹ yr⁻¹), length of experiment (short: <5, intermediate: 6–10, long: >10 years), and tree type (conifer vs. hardwood species). In addition we analyzed the influence of a continuous variable, the cumulative amount of N fertilizer added at the time of sampling (kg N ha⁻¹), which takes into account both the amount of simulated N deposition (kg N ha⁻¹ yr⁻¹) and the length of the experiment (in years). Because of the low number of studies that measured nutrient concentrations in senesced leaf litter we also included studies with measurements of green foliage (typically dried for analysis). Throughout the manuscript we use ‘litter’ to refer to senesced leaf litter (after abscission) and ‘foliage’ to refer to green leaves (before abscission), and we use the term ‘leaf’ to refer to both ‘litter’ and ‘foliage’. Nutrient concentrations differ between foliage and litter because of nutrient retranslocation at leaf senescence and also due to the extraction procedure used for foliage (exchangeable nutrients expressed on fresh weight basis vs. total expressed on dry weight basis). For these reasons, we made no direct comparisons in our meta-analysis between the two leaf types or extraction method, but rather evaluated the relative change in nutrient concentrations for each individual study in response to N additions. To understand if simulated N deposition affected litter or foliage nutrients differentially, we also included leaf type (i.e., litter or foliage) as a categorical variable within our meta-analysis.

The effect size (lnR) for each observation (k) was calculated as the natural log of the response ratio $R = \frac{X_i}{X_c}$, where $X_i$ and $X_c$ are the mean of the N treatment and control treatment, respectively (Hedges et al. 1999). Some studies had multiple N addition levels or included multiple tree species or multiple sample years. Within each study the multiple N addition levels and tree species were treated as separate observations (k), resulting in more than one response ratio for some studies. To ensure observation independence, multiple sample years of the same study were not included in the effect size calculations, except for the effect size calculations with the categorical variable “length of experiment (time)” and the continuous variable “cumulative amount of N addition.” We calculated R and bias-corrected 95% confidence intervals (CI) with 9999 iterations for each observation using the program MetaWin version 2.0 (Hedges et al. 1999, Rosenberg et al. 2000). R was defined as percent change of the measured variable with N treatment compared to the control treatment after back transformation of the response ratio ($e^{\text{lnR}}$). The effect of simulated N deposition was considered to be significant at $\alpha < 0.05$ if the 95% CI of the response ratio did not overlap with zero, while nutrient response ratios between two categories were considered significantly different if their 95% CIs did not overlap. To examine which categorical variables influenced the magnitude of a nutrient response to N addition, we partitioned the total variance of a group of observations ($Q_v$; total heterogeneity) into within-group ($Q_w$) and between-group heterogeneity ($Q_b$) (Hedges and Olkin 1985). Categorical variables with a higher and significant $Q_b$ were considered better predictors of the nutrient responses to N addition compared to variables
with a lower $Q_b$. Within a meta-analysis this is similar to using partitioning of variance in ANOVA, and $Q_b$ is similar to the model sums of squares and $Q_w$ to the error sums of squares (Hedges and Olkin 1985).

**Litter decay experiment to investigate the interactive effects of litter quality and N additions on decomposition dynamics**

To complement our meta-analysis, we conducted a litterbag experiment at the Chronic Nitrogen Amendment Study at the Harvard Forest Long-term Ecological Research (LTER) site in Petersham, Massachusetts, USA (42°28’ N, 72°10’ W). This simulated N deposition experiment is located in a mixed hardwood forest dominated by black and red oak (*Quercus velutina* and *rubra*), red and striped maple (*Acer rubrum* and *pennsylvanicum*), black birch (*Betula lenta*), and American beech (*Fagus grandifolia*), with the two oak species representing ~85% of total annual litter fall. The experiment was established in 1988 and consists of three 30 m plots which receive either ambient N deposition (control, N0), or an additional 50 (N50) or 150 (N150) kg N ha$^{-1}$ yr$^{-1}$ as an aqueous solution of NH$_4$NO$_3$ applied over six equally spaced intervals during the growing season (Magill and Aber 1998). Each 30 × 30 m plot is subdivided into 36 5 × 5 m subplots. Ambient N deposition at the site was estimated to be 8–10 kg N ha$^{-1}$ yr$^{-1}$ in 2010, with levels up to 15 kg N ha$^{-1}$ yr$^{-1}$ in 2002 (Schwede and Lear 2014). The N50 treatment is representative of amounts of N deposition predicted for some areas of the world by 2050 (Galloway et al. 2008), while the N150 treatment represents a space for time substitution meant to push the ecosystem toward N saturation (Aber et al. 1993).

We implemented a reciprocal litter transplant experiment using oak litter collected from each of the three N addition treatments where the trees had been exposed to 22 years of ambient or elevated N conditions, leading to potential differences in litter quality. Initial quality of the collected litter was measured as described below. The term “litter origin” will be used throughout the manuscript to denote the N treatment (N0, N50, or N150) where the oak litter was collected, and the term “decay environment” will be used to denote the N treatment in which decay occurred.

Litter was collected in litter traps placed in each of the three N treatment plots during leaf abscission in the fall of 2010. Litter traps were suspended off the ground to prevent contamination by soil borne microorganisms. The collected litter was air-dried at room temperature and the oak leaves were sorted out, cut (following stem removal) into approximately 3 × 3 cm pieces, and homogenized by N treatment. Litterbags (20 × 20 cm) were made of 0.3 mm nylon mesh and filled with 10 g of processed litter. Each bag was sewn closed and the litter spread evenly within the bag. Initial weight of litter in each bag was corrected for remaining moisture using the mean moisture content of five subsamples of the processed litter obtained by oven-drying at 60°C for 48 hours.

Two replicate bags of each litter origin (N0, N50, N150) were placed in each of five subplots within each N decay environment in early December 2010. Outer subplots of each decay environment were avoided to minimize edge effects. This design resulted in a total of 30 oak litterbags per N decay environment (10 N0 bags, 10 N50 bags, and 10 N150 bags) with five replicate bags for each litter origin per N decay environment at each harvest time. The bags were placed on top of the forest floor beneath the recent litter fall and pinned to the ground with two U-shaped gardener’s pins.

The first set of litterbags (5 N0 bags, 5 N50 bags, and 5 N150 bags per N decay environment) was harvested in October 2011 (after ~10 months), and a second set was collected in November 2012 (after ~23 months). At each harvest, litter and other debris attached to the outside of the bags was removed, and the wet weight of each bag was recorded. Each litterbag was sealed inside a Ziploc bag, placed on ice, and transported to the University of New Hampshire, where they were stored at 4°C until further processing. Within three days of harvest the litter was removed from the bags and subsampled for subsequent analyses. Subsamples (~1 g) were oven-dried (60°C for 48 hours) to determine moisture content and ashed (5 hours at 450°C) to assess potential mineral contamination. Another 1 g subsample was stored at 4°C for microbial enzyme activity analysis, and the remaining litter was frozen at −80°C, freeze-
dried, ground, and analyzed for total C and N concentrations.

Enzyme analyses were done according to methods outlined in Saiya-Cork et al. (2002) and DeForest (2009). Activities of the hydrolytic enzymes cellulohydrolase (CBH), acid phosphatase (PHOS), N-acetyl-β-glucosaminidase (NAG), and β-glucosidase (BG) were assayed using the methylumbelliferyl-β-D-glucoside linked substrates (200 μM) β-D-cellobiosidase, phosphate, N-acetyl-β-D-glucosaminide, and β-D-glucopyranoside, respectively. The hydrolytic enzyme leucine aminopeptidase (LAP) was assayed using a 7-amido-4-methylcoumarin (AMC)-linked substrate L-Leucine (200 μM). Activities of oxidative enzymes phenol oxidases (OX1 and OX2) and peroxidases (PER1 and PER2) were assayed using the substrates L-3,4-dihydroxyphenylalanine (L-DOPA, 25mM), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, 3mM), L-DOPA+H₂O₂ (0.3% hydrogen peroxide), and 3,3′,5,5′-Tetramethylbenzidine (TMB + H₂O₂) (Johnsen and Jacobsen 2008), respectively.

Briefly, ~0.5 g of field-moist litter was homogenized in 125 ml sodium acetate buffer (50 mM, pH = 4.7, average pH of the litter) for 30 seconds using a Magic Bullet (Homeland Housewares LLC). Sample homogenate (200 μl) was transferred to a 96-well microplate followed by the addition of 50 μl substrate. Microplates were incubated for 15 min to 18 hours depending on the enzyme substrate. After incubation, fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 450 nm (hydrolytic enzymes) and absorbance was measured at 450 nm (OX1, PER1, PER2) or 420 nm (OX2) on a Biotek HT plate reader (Biotek, Winooski, USA). All enzyme assays were done with eight or sixteen replicate wells per sample and corrected for background fluorescence or absorbance of substrate (negative control). For hydrolytic enzymes, the conversion of fluorescence was based on a standard line of the reference standard MUB (BG, CBH, NAG, PHOS) or AMC (LAP). Conversion of OX1 and PER1 was determined based on an empirically determined extinction coefficient of ε₄₅₀ = 59,000 M⁻¹ cm⁻¹ (Josephy et al. 1982) and ε₄₂₀ = 36,000 M⁻¹ cm⁻¹ (Bourbonnais and Paice 1990), respectively. Final enzyme activity was calculated using formulas outlined in DeForest (2009) and expressed as μmol of substrate converted per hour per g litter dry mass (μmol h⁻¹ g⁻¹).

Litter C and N concentrations were measured by dry combustion using a CN Analyzer (Perkin Elmer 2400 Series II). Acid unhydrolyzable residue (AUR) and cellulose concentrations were analyzed at Cumberland Valley Analytical Services (Hagerstown, Maryland, USA) using the acid detergent fiber (ADF) procedure (Goering and Van Soest 1970). Total Al, B, Ca, Cu, Fe, K, Mg, Mn, P, and Zn concentrations were determined by dry ashing followed by dissolution in 50% hydrochloric acid (Kalra and Maynard 1991) and inductively coupled plasma atomic emission spectrophotometry (ICP-AES) at the University of Maine’s Analytical Laboratory (Orono, Maine, USA).

Effects of N decay environment and litter origin on all variables (mass loss, decay rate, moisture, N (% and net change), %C, C to N ratio, nutrients, and potential enzyme activities) were tested using two-way ANOVA in R version 3.0.1 (R Core Team 2013). If needed, variables were transformed to ensure a normal distribution and homogeneity of variance. Effects of N decay environment and litter origin on patterns in enzyme activities were further explored with Permutational Multivariate Analysis of Variance (PERMANOVA; Anderson 2001) using the adonis function in R vegan package version 2.0–10 (Oksanen et al. 2013) and Nonmetric Multidimensional Scaling (NMDS) using Bray Curtis distance matrix. For PERMANOVA and NMDS analyses, the enzyme activities were relativized (expressed as a proportion of the highest activity measured for each enzyme).

**RESULTS**

**Meta-analysis of leaf nutrient concentrations in response to long-term N addition**

Our literature search, and the data collected from unpublished N addition experiments, resulted in a total of 637 observations from 16 different publications or experiments, ranging from 27 to 90 observations per nutrient. Five of
the 16 studies were based on senesced litter, while the remaining targeted green foliar measurements (Appendix: Table S1). Overall, simulated N deposition caused a decrease in leaf nutrient concentrations, with P, Ca, Mg, Mn, B, Al, and Zn being significantly decreased by 7%, 20%, 11%, 24%, 22%, 6% and 15%, respectively (P < 0.05, Fig. 1). The one exception to the trend was N, which significantly increased by 27% with N addition (P < 0.05, Fig. 1). The N addition level (low: ≤ 50, intermediate: 51–100, high: >100 kg N ha⁻¹ yr⁻¹), tree type (conifer vs. hardwood), leaf lignin concentration (low: <15%, intermediate: 16–25%, high: >25%), length of experiment (short: ≤ 5, intermediate: 6–10, long: >10 years), and leaf type (foliar vs. litter) all significantly influenced the magnitude of the response to N addition for one or more nutrients (Table 1, Fig. 2).

Leaf Ca and Mn concentrations decreased more in studies using intermediate and high N addition levels, compared to low N addition levels (Fig. 2A, Table 1). Moreover, leaf Ca and Mn response ratios were negatively correlated with the cumulative amount of N deposition (Fig. 3). That is, a higher total N loading caused a greater decrease in leaf Ca and Mn concentrations (R² = 0.24 and P < 0.001, R² = 0.37 and P < 0.001, respectively, Fig. 3). Leaf B, P and Zn concentrations decreased more in studies with high N addition compared to low N addition levels (Fig. 2A). Simulated N deposition increased leaf N concentrations and decreased Zn concentrations more in conifers compared to hardwoods, while P decreased more in hardwoods compared to conifers (Fig. 2B, Table 1). Leaf N concentrations were increased more by simulated N deposition in leaves with a high lignin concentration compared to leaves with a low lignin concentration (Fig. 2C, Table 1). In general, green foliar nutrient responses were similar to senesced litter nutrient responses, with litter concentrations on average showing larger decreases than foliar concentrations in response to N additions, except for B and Mg, which were significantly more decreased in foliage (Fig. 2D, Table 1).

Litter decay experiment to investigate the interactive effects of litter quality and N additions on decomposition dynamics

The effect of simulated N deposition on the chemistry of the oak litter used in our litterbag experiment was similar to effects observed in our meta-analysis. Oak litter collected from the N50 and N150 treatments had higher initial N concentrations compared to litter collected from the control (N0) treatment by 8% and 27%, respectively, and C:N ratios were decreased by 5% and 19%, respectively (Table 2). However, these changes were only significant for the N150 litter (Table 2). Litter collected from the N addition plots also had significantly lower B, Ca, Mg, Mn, and P compared to litter collected from the N0 treatment (Table 2). However, there were no significant differences in initial lignin (Acid unhydrolyzable residue; AUR) and cellulose contents, LCI (Lignocellulose index, Melillo et al. 1989), or the AUR:N ratio (Table 2). Our data are measurements of leaf nutrient concentrations, but can be used as proxies for total nutrient contents, as we did not observe changes in litter fall (Frey et al. 2014) or specific leaf area (Wicklein et al. 2012) as a consequence of N addition at our field experiment.

Litter mass loss ranged from 23% to 41% in the first year of the study and 51% to 71% over two
years of decomposition (Appendix: Table A2 and A3). During the first year, there were no main effects of litter origin or decay environment on decay rate (Table 3). However, there was an interaction between litter origin and decay environment; litter decay rates of litter originating from the high N treatment (N150) was significantly lower in the high N environment compared to the control (N0) and N50 environments ($P < 0.01$), while litter originating from the N0 and N50 treatments did not differ in decay rates among N decay environments (Fig. 4A, Table 3; Appendix: Table A2). In the second year, there were significant effects of both litter origin and decay environment, but no interaction (Table 3). The litter originating from the N amended plots (both N50 and N150 treatments) decomposed more slowly compared to control (N0).

Table 1. Between-group variability ($Q_b$) for each categorical variable among $k$ observations, suggesting the potential for each variable to predict leaf nutrient responses to simulated N deposition; from the meta-analysis.

<table>
<thead>
<tr>
<th>Response variable</th>
<th>Nitrogen addition level</th>
<th>Tree type: conifer vs. hardwood</th>
<th>Lignin level</th>
<th>Length of experiment (time)</th>
<th>Leaf type: litter vs. foliage</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>52 (76)</td>
<td>4.9†</td>
<td>2.1</td>
<td>0.20</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>53 (73)</td>
<td>145‡</td>
<td>7.2</td>
<td>151</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>57 (82)</td>
<td>0.01‡</td>
<td>4.5</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Ca</td>
<td>61 (90)</td>
<td>3.2‡</td>
<td>18</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>Mg</td>
<td>61 (90)</td>
<td>8.00</td>
<td>3.9</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Mn</td>
<td>39 (58)</td>
<td>0.22</td>
<td>0.48</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>34 (39)</td>
<td>1.1</td>
<td>4.7</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Al</td>
<td>36 (44)</td>
<td>0.05</td>
<td>0.76</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>Cu</td>
<td>22 (27)</td>
<td>0.36</td>
<td>1.3</td>
<td>0.79</td>
<td></td>
</tr>
<tr>
<td>Zn</td>
<td>24 (29)</td>
<td>2.3</td>
<td>6.5</td>
<td>2.3</td>
<td></td>
</tr>
</tbody>
</table>

† Number of observations ($k$), and in parentheses the total number of observations, which includes multiple time points at one study site.
§ Categorical variables with a large $Q_b$ are a better predictors of variation than variables with a small $Q_b$.
* $P < 0.05$, **$P < 0.01$, ***$P < 0.001$, statistical significance of $Q_b$.

Table 2. Initial quality of oak litter collected from the different N treatment plots of the Chronic Nitrogen Amendment Study at the Harvard Forest LTER, and percent change from the control treatment (means with standard errors in parentheses, $n = 3$). Means not sharing the same letter are significantly different from each other ($P < 0.05$, Tukey post-hoc test).

<table>
<thead>
<tr>
<th>Component</th>
<th>Litter origin</th>
<th>Percent change from N0</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUR† (%)</td>
<td>N0</td>
<td>N50</td>
</tr>
<tr>
<td>Cellulose (%)</td>
<td>29.3 (1.7)</td>
<td>29.5 (1.1)</td>
</tr>
<tr>
<td>LCI§</td>
<td>13.8 (0.7)</td>
<td>17.5 (1.5)</td>
</tr>
<tr>
<td>N (%)</td>
<td>0.90 (0.06)*</td>
<td>0.97 (0.02)*</td>
</tr>
<tr>
<td>C:N</td>
<td>58.6 (3.5)*</td>
<td>55.4 (1.2)*</td>
</tr>
<tr>
<td>AUR+N</td>
<td>32.9 (2.9)</td>
<td>30.5 (1.2)</td>
</tr>
<tr>
<td>Ca (mg/g)</td>
<td>5.40 (0.01)*</td>
<td>4.72 (0.03)*</td>
</tr>
<tr>
<td>K (mg/mg)</td>
<td>3.00 (0.06)*</td>
<td>3.63 (0.11)*</td>
</tr>
<tr>
<td>Mg (mg/g)</td>
<td>1.17 (0.01)*</td>
<td>1.29 (0.01)*</td>
</tr>
<tr>
<td>P (mg/g)</td>
<td>1.17 (0.01)*</td>
<td>1.08 (0.01)*</td>
</tr>
<tr>
<td>Mn (mg/g)</td>
<td>3.73 (0.08)*</td>
<td>2.25 (0.02)*</td>
</tr>
<tr>
<td>Al (mg/kg)</td>
<td>56.4 (2.8)</td>
<td>59.1 (3.7)</td>
</tr>
<tr>
<td>B (mg/kg)</td>
<td>27.5 (0.3)*</td>
<td>25.4 (0.5)*</td>
</tr>
<tr>
<td>Fe (mg/kg)</td>
<td>95.8 (20.1)</td>
<td>57.9 (8.1)</td>
</tr>
<tr>
<td>Cu (mg/kg)</td>
<td>3.49 (0.04)</td>
<td>3.52 (0.08)</td>
</tr>
<tr>
<td>Zn (mg/kg)</td>
<td>32.2 (0.4)*</td>
<td>31.6 (0.6)*</td>
</tr>
</tbody>
</table>

† Acid Unhydrolyzable Residue (AUR).
§ LCI (lignocellulose index) is equal to AUR/(AUR + cellulose).
* $P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 
litter, irrespective of decay environment ($P < 0.05$, Fig. 4B, Table 3; Appendix: Table A3). In addition, all litter, regardless of origin, decomposed more slowly in the highest N decay environment (N150) compared to the other two environments (N0, N50; $P < 0.001$).

In both years, final N concentrations in decomposed litter were significantly affected by litter origin, but a significant litter origin by decay environment interaction was only observed in the first year (Table 3). While all litter had similar N concentrations after one year of decomposition in the N150 decay environment, in the control (N0) and N50 environments, litter originating from the N150 treatment had higher N concentrations compared to litter originating from the N0 and N50 treatments ($P < 0.001$, Table 3; Appendix: Table A2). After two years of decomposition, litter originating from the N150 treatment had a higher N concentration in all
decay environments compared to the litter originating from the N0 and N50 treatments ($P < 0.01$). Regardless of the higher initial N concentration of the litter originating from the N150 treatment, all litter had net increases of $\sim 20\%$ in absolute N content in all decay environments after one year of decomposition (Table 3; Appendix: Table A2). However, after two years of decomposition, N was no longer immobilized and a net loss of absolute litter N content was observed in the control and N50 decay environments, while litter in the N150 decay environment had no net change in absolute N content (Table 3; Appendix: Table A3). After one and two years of decomposition, the C:N ratio of litter originating from the N150 treatment was significantly lower than that of litter originating from the N0 and N50 treatments, irrespective of decay environment ($P < 0.05$, Table 3; Appendix: Tables A2, A3).

The activities of the lignolytic enzymes peroxidase and phenol oxidase were significantly affected by litter origin in the first year, while in the second year, N decay environment was a significant factor, and no interactions were observed in either year. This resulted in more than a $50\%$ reduction in peroxidase activities in litter originating from the N50 and N150 treatment compared to activities in control litter after one year of decomposition, irrespective of the N decay environment (Tukey HSD, $P < 0.01$; Fig. 5a, Table 3; Appendix: Table A2). Phenol oxidase (OX2) activity was reduced by $\sim 57\%$ in litter originating from the N150 treatment compared to the control litter, irrespective of N decay environment (Tukey HSD, $P < 0.01$; Fig. 5C, Table 3; Appendix: Table A2). In contrast, in the second year all litter had reduced peroxidase (PER1) activity in the N150 decay environment (Fig. 5B), and phenol oxidase (OX2) activity was reduced in the N50 decay environment (Fig. 5D) compared to control environment (Tukey HSD, $P < 0.01$; Table 3; Appendix: Table A3).

Phosphatase and cellobiohydrolase activities were significantly increased in the N150 decay environment compared to the control environment after two years of decomposition (Tukey HSD, $P < 0.05$), while N-acetyl-$\beta$-glucosaminidase was significantly increased in the N50 decay environment compared to the control (Tukey HSD, $P < 0.05$; Table 3; Appendix: Table A2). No significant decay environment or litter origin effects were observed for these enzymes after one year of decomposition, except a significant
increased phosphatase activity in the N50 compared to control decay environment (Tukey HSD, \( P < 0.05 \); Table 3; Appendix: Table A2). No significant interactions between litter origin and decay environment were observed for any enzymes (Table 3; Appendix: Table A2).

Patterns in enzyme activities were primarily affected by litter origin \( (P < 0.05) \) in year one and N decay environment in year two \( (P < 0.001) \), while no interactions between litter origin and decay environment were observed in either year (Table 3, Fig. 6). In both years, litter originating from the N150 treatment that was decomposed in the control environment had enzyme activities

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**Table 3.** F-values from an analysis of variance of N decay environment (E) and litter origin (L) effects on decay parameters, moisture, litter chemistry, enzyme activities, and PERMANOVA§ of E and L effects on potential enzyme activities; all statistics from field experiment.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Year 1</th>
<th>Year 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass loss (%)</td>
<td>1.22</td>
<td>9.47***</td>
</tr>
<tr>
<td>Decay rate</td>
<td>2.15</td>
<td>8.71***</td>
</tr>
<tr>
<td>Moisture</td>
<td>0.20</td>
<td>1.82</td>
</tr>
<tr>
<td>Nitrogen (%)</td>
<td>4.10*</td>
<td>2.77</td>
</tr>
<tr>
<td>Nitrogen (% net change)</td>
<td>0.62</td>
<td>7.44**</td>
</tr>
<tr>
<td>Carbon (%)</td>
<td>4.55*</td>
<td>4.89*</td>
</tr>
<tr>
<td>C:N</td>
<td>4.26*</td>
<td>4.86*</td>
</tr>
<tr>
<td>LAP†</td>
<td>2.88</td>
<td>2.10</td>
</tr>
<tr>
<td>CBH†</td>
<td>0.84</td>
<td>5.69**</td>
</tr>
<tr>
<td>BG†</td>
<td>0.35</td>
<td>3.07</td>
</tr>
<tr>
<td>PHOS†</td>
<td>3.28*</td>
<td>4.37*</td>
</tr>
<tr>
<td>NAG†</td>
<td>0.14</td>
<td>4.40*</td>
</tr>
<tr>
<td>OX1†</td>
<td>1.05</td>
<td>4.90*</td>
</tr>
<tr>
<td>PER1†</td>
<td>2.32</td>
<td>6.38**</td>
</tr>
<tr>
<td>OX2†</td>
<td>1.29</td>
<td>5.87**</td>
</tr>
<tr>
<td>PER2†</td>
<td>1.03</td>
<td>1.06</td>
</tr>
<tr>
<td>PERMANOVA§</td>
<td>0.97</td>
<td>4.04***</td>
</tr>
</tbody>
</table>

† Abbreviations for enzymes: LAP, leucine aminopeptidase; CBH, cellobiohydrolase; BG, \( \beta \)-glucosidase; PHOS, acid phosphatase; NAG, N-acetyl-\( \beta \)-glucosaminidase; OX1, phenol oxidase (L-DOPA as substrate); OX2, phenol oxidase (ABTS as substrate); PER1, gross peroxidases; (L-DOPA+ H2O2 as substrate) PER2, peroxidase (TMB + H2O2 as substrate).

§ Permutational multivariate analysis of variance.

*P < 0.05, **P < 0.01, ***P < 0.001.
more similar to litter decomposed in the N50 and N150 environment than litter decomposed in the control environment (Fig. 6). In addition, even when control litter was placed in the N150 decay environment for one or two years of decomposition, the patterns in enzyme activities were similar to activities of control litter decomposed in the control environment (Fig. 6).

DISCUSSION

Our meta-analysis of temperate forest N addition experiments demonstrates that simulated N deposition results in changes in nutrient concentrations of foliage and litter, with consequences for litter quality. As litter quality is one of the primary controls on rates of decay, these N-induced changes in litter quality may have large consequences for litter decay rates in high N deposition areas. That is, the historical effects of N deposition on litter quality may contribute considerably to the slowed rates of decomposition often observed for high lignin species exposed to enriched N conditions. This is supported by the results of a reciprocal litterbag study with a high lignin species (Quercus spp.) where we found that external N (simulated N deposition) had a greater inhibitory effect on the decay of litter collected from plots exposed to high levels of N addition over the long-term, compared to litter of the same species collected from plots exposed over the same time period to ambient N deposition. This clearly indicates that there were feedbacks between changes in litter...
quality caused by N deposition and the direct effects of N addition on litter decay. Moreover, lignolytic enzyme activities were also lower in litter collected from the N-enriched plots compared to litter collected from control plots, irrespective of the N environment where the litter was decomposed. Thus, the effects of historical N addition realized in tree litter chemistry reinforced the negative effects of simulated N deposition on litter decay dynamics.

As expected, one of the major nutrients affected by simulated N deposition (external N) was nitrogen, with an overall greater increase in leaf N concentration in high lignin compared to low lignin leaves. High litter N (internal N) generally accelerates decay at early stages of decomposition (Melillo et al. 1982, Hobbie 2005, Hobbie et al. 2012), however, we did not observe this effect in our litterbag experiment. One explanation may involve a threshold level of litter lignin: when concentrations of litter lignin are high, increased litter N may actually result in a suppressive effect. In support of this hypothesis, Berg et al. (2000) and Perakis et al. (2012) found that initial litter N concentration was negatively correlated with decay in experiments targeting coniferous litter (Picea abies and Pseudotsuga menziesii, respectively) with high lignin concentrations (>25%), at both early- and late-stages. Nonetheless, the exact mechanisms of how high internal N, in combination with high lignin, affects decomposition rates are still unclear, and both chemical and biological mechanisms have been suggested (Berg and McClaugherty 2014, and references within).

While N is the traditional focus of experiments documenting effects of changes in litter quality on decomposition, both the meta-analysis and our field experiment indicate that chronic N enrichment causes reductions of other leaf nutrient concentrations, including Mn and Ca, and the field experiment suggests the reduction of these nutrients limits the production of lignolytic enzymes. Mn-dependent and versatile peroxidases are dominant in the environment (e.g., Hofrichter et al. 2010) and have high redox potentials relative to other known lignolytic enzymes (Dashtban et al. 2010, Zavarzina et al. 2011). The catalytic activities of these enzymes depends on (Mn-dependent peroxidase) or is enhanced by (versatile peroxidase) Mn, which is ubiquitous in both soil and litter, albeit at varying concentrations (Hofrichter 2002). Manganese is also important for the regulation of the lignolytic enzyme phenol oxidase (laccase; Archibald and Roy 1992, Dashtban et al. 2010). Higher litter Mn concentrations have been found to accelerate litter decay in the late, lignin-dominated phase (Berg et al. 2000, 2007, Davey et al. 2007, Perakis et al. 2012, Berg and McClaugherty 2014), with a few studies showing this response in the early stage, when other, more labile C polymers (e.g., cellulose) are still abundant (Berg et al. 2000,

In addition to Mn, initial litter Ca concentrations can also be a significant positive influence on litter decay and lignolytic enzymes (Berg 2000, Davey et al. 2007), as Ca is needed by some fungal species for production of lignolytic enzymes (Dashtban et al. 2010). Both the meta-analysis and our field experiment suggest N deposition causes reductions in leaf Ca concentrations, and this reduction may thus partially explain reduced lignolytic enzyme activities and decreased decay rates. Berg (2000) found that in various studies with deciduous litter, initial Ca concentration had a stronger correlation with litter decay than Mn and N concentrations.

The apparent importance of N, Mn and Ca availability for the formation of lignolytic enzymes suggests that the changes in their concentrations (increased N and decreased Ca and Mn) caused by long-term exposure to high N levels is responsible, at least in part, for the suppressed lignolytic enzyme activities observed in our field experiment for oak litter collected from the N addition plots. In the first year of the experiment, this suppression of lignolytic enzymes was observed regardless of the N environment into which the litter was transplanted. However, in the second year, N addition significantly suppressed lignolytic enzyme activities regardless of litter origin. Other studies have also found that at later stages of decomposition, external N addition reduces the production of lignolytic enzymes (e.g., Carreiro et al. 2000, DeForest et al. 2004, Frey et al. 2004). However, to our knowledge this is the first study to demonstrate a suppressive effect of high internal N, combined with low Ca and Mn, on lignolytic enzyme activities during an early stage of decomposition, particularly relevant because we did not observe a simultaneous negative effect of externally applied N. Various mechanisms may explain why studies to date have not documented the same effect, including (1) low lignolytic enzyme activities (i.e., below detection level) at the early stages of decomposition in specific experiments (e.g., Keeler et al. 2009, Hobbie et al. 2012), and/or (2) the inclusion of different plant species with variable lignin concentrations in a single experiment, potentially confounding species effects with differences in leaf tissue nutrient concentrations (e.g., Carreiro et al. 2000, Keeler et al. 2009). The other leaf nutrients (Al, B, Mg, P, and Zn), for which we found N-induced changes among the studies used in the meta-analysis, are not known to be cofactors of lignolytic enzymes (Dashtban et al. 2010). However, these nutrients are nonetheless important for various microbial and plant physiological processes (e.g., Plante 2007) and could thus have an indirect effect on C cycling within these ecosystems.

In our meta-analysis we found N-induced decreases in leaf Ca, Mg and Mn concentrations, and this may also have implications for nutrient levels in soils exposed to high levels of N addition over the long-term. Support for this hypothesis is the reduced availability of Ca, Mg, Mn, and K found in bulk soil at N deposition experiments, including our site, resulting in an overall reduced availability of these important nutrients under simulated N deposition (Minocha et al. 2000, Lucas et al. 2011). More specifically, at our study site, Mn concentrations in the organic soil horizon were lower in the N addition treatments compared to the control (Minocha et al. 2000, Turlapati et al. 2013). This observation may help explain the reduced activity of Mn-dependent and other lignolytic enzymes we have observed for soils collected previously from our site (Frey et al. 2004). Observed changes in enzyme activity in both soil and litter and the reduced litter decay rates in our field experiment are associated with increased soil C storage, the persistence of lignin, and a higher lignin:phenol ratio in the organic soil horizon (Frey et al. 2014). Increased lignin:phenol ratios suggest that C remaining in the N addition plots is made up of a higher percentage of unaltered lignin (Frey et al. 2014). Based on the results we present here, the higher percentage of unaltered lignin in the N addition plots may be explained by low lignolytic enzyme activities in the litter originating from the N addition plots caused by high N and low Ca and Mn availability.

In summary, changes in litter quality brought about by exposure of trees to long-term N deposition appears to be a widespread effect, as demonstrated by the results of our meta-analysis, and our field experiment indicates that these changes in litter chemistry can directly impact both early- and late-stage decomposition dynamics for high lignin litter. The negative effect of
simulated N deposition on lignolytic enzyme activities and litter decay in our field experiment was reinforced by higher litter N and the coincident decrease in litter Ca and Mn concentrations as a consequence of exposure to high levels of N addition over the long-term. Slower litter decay under simulated N deposition is likely to have contributed to the increased soil C storage observed in the N addition plots at our study site (Frey et al. 2014), as well as at other N deposition experiments (Zak et al. 2008, Lovett et al. 2013).

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SUPPLEMENTAL MATERIAL
ECOLOGICAL ARCHIVES

The Appendix is available online: http://dx.doi.org/10.1890/ES15-00262.1.sm