ATMOSPHERIC NITRATE DEPOSITION AND ENHANCED DISSOLVED ORGANIC CARBON LEACHING: TEST OF A POTENTIAL MECHANISM

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Abstract

Atmospheric NO$_3$ deposition has the potential to disrupt litter decomposition in temperate forests by suppressing enzymes responsible for lignin degradation. A reduction in phenol oxidase activity could potentially trigger an increase in soluble phenolic compounds, which in turn are known to decrease the activity of cellulytic enzymes like β-glucosidase. Our study investigated whether the inhibition of lignin degradation by experimental NO$_3$ deposition could increase soluble phenolics in soil, suppress β-glucosidase activity, and potentially explain a greater export of dissolved organic C (DOC) from northern hardwood ecosystems. We found no evidence that the suppression of phenol oxidase by NO$_3$ additions increased soluble phenolics in mineral soil, nor did we find a strong inverse relationship between soluble phenolics and β-glucosidase activity. It appears that reductions in mineral soil lignolytic activity induced by experimental deposition are not responsible for greater DOC export from soil.

Chronic atmospheric NO$_3$ deposition has the potential to directly affect microbial activity and initiate a series of physiological changes that alter the cycling and storage of C in soil (Berg, 1986; Berg and Matzner, 1997). For example, white rot Basidiomycota and xylariales Ascomycota are the primary agents of lignin degradation, and high concentrations of inorganic N can inhibit their ability to produce phenol oxidase and peroxidase (Keyser et al., 1978; Kirk and Farrell, 1987; Fog, 1988). Experimental atmospheric NO$_3$ additions in forest ecosystems, at levels already occurring in some portions of the northeastern USA and Europe (Fenn et al., 1998), have caused significant reductions in the activity of these extracellular enzymes (Carreiro et al., 2000; Sálya-Cork et al., 2002; DeForest et al., 2004a). Recently, we have observed that experimental NO$_3$ deposition also can reduce β-glucosidase activity (DeForest et al., 2004a) and increase the leaching of DOC from soil (Pregitzer et al., 2004), both of which occurred with a concomitant decline in lignolytic activity. These observations suggest that chronic atmospheric NO$_3$ deposition can set in motion a series of microbial physiological changes that diminish the degradation of lignified plant cell walls and increase microbial byproducts of plant litter degradation (e.g., DOC). An increase in the DOC leaching would likely increase the leaching of soluble phenolics, which is a component of the total DOC pool. Here, we test a potential mechanism by which chronic NO$_3$ deposition could elicit this chain of events.

Our rationale linking declines in extracellular enzyme activity to increases in soluble phenolic leaching centers on NO$_3$ deposition reducing the activity of lignolytic enzymes, resulting in the partial degradation of lignin in litter and humified compounds in soil organic matter. Such a response would be consistent with our previous observations (DeForest et al., 2004a), and it could lead to the accumulation of soluble phenolics in soil solution, which are known to inhibit the activity of extracellular enzymes responsible for cellulose hydrolysis (i.e., β-glucosidase). In wetland soils, low pO$_2$ limits phenol oxidase activity (McLatchey and Reddy, 1998), which can produce an accumulation of soluble phenolics and the suppression of β-glucosidase and other cellulytic enzymes (Freeman et al., 2001). Copolymerization and immobilization into soil organic matter are two processes by which soluble phenolics and polyphenolics (e.g., tannins) might inhibit β-glucosidase and other hydrolytic enzymes (Sarkar and Burns, 1983). We hypothesized that a similar series of events is initiated by atmospheric NO$_3$ deposition. If this mechanism was at work in our experiment, we expected to observe an inverse relationship between the activity of lignolytic enzymes and soil phenolic concentrations, as well as an inverse relationship between phenolic concentrations and β-glucosidase. We expected experimental NO$_3$ deposition would produce significantly higher concentrations of soluble phenolics and lower activity of extracellular enzymes involved with lignin and cellulose degradation.

Materials and Methods

Study Area and Field Sampling

To determine soil phenolic concentrations and their potential to suppress β-glucosidase activity, we studied soil in four Acer saccharum Marsh.—dominated northern hardwood sites in Michigan with similar stand composition, history, structure, and soil development (Fig. 1; Burton et al., 1991). Soils at the three northern sites (A, B, and C) were sandy, mixed, frigid Typic Haplorthods and sandy, mixed, frigid Alfic Haplorthods. At site D, soils were sandy, mixed, mesic Entic Haplorthods. These study sites are located along a 500-km climatic and N
Fig. 1. Distribution of the four northern hardwood sites along a 500-km climatic and NO\textsubscript{3} deposition gradient in Michigan, USA. These stands span the geographic distribution of sugar maple dominated northern hardwood forests in the upper Great Lake States region.

deposition gradient where the northernmost Site A receives the least amount of N deposition (6.8 kg N ha\textsuperscript{-1} yr\textsuperscript{-1}), followed by Site B at (9.1 kg N ha\textsuperscript{-1} yr\textsuperscript{-1}), Site C and Site D around (11.7 kg N ha\textsuperscript{-1} yr\textsuperscript{-1}). These sites represent a common forest ecosystem found throughout the Upper Great Lakes region. Within each study site, six 30 m x 30 m experimental plots were established. Three plots receive ambient levels of N deposition and served as a control. Since 1994, the other three plots received 30 kg NO\textsubscript{3}–N ha\textsuperscript{-1} yr\textsuperscript{-1} in addition to ambient N deposition. We broadcast six equal applications of dry NaNO\textsubscript{3} over the forest floor during the growing season to simulate atmospheric NO\textsubscript{3} deposition. The amount and type of N added is similar to atmospheric deposition in forests near industrial regions of the northeastern USA (Fenn et al., 1998).

We sampled the soil six times during the 2002 growing season (May to October) to assess the influence of experimental NO\textsubscript{3} deposition on soluble soil phenolics and extracellular enzyme activity. On each sampling date, we collected eight soils cores (2-cm diam. and 10 cm deep) in plots receiving ambient and elevated atmospheric NO\textsubscript{3} deposition. The surface organic horizons (O\textsubscript{i} and O\textsubscript{e}) were removed and the soil cores contained a small amount of the Oa horizon (<0.5 cm) and surface mineral soil (0–10 cm) of A and E horizon. The eight soil samples from each plot were composited, and all subsequent analyses were performed on the composite samples. Samples were kept on ice and processed within 48 h of field collection.

**Extracellular Enzyme Activity**

We used three subsamples of each composite sample to assay the activity of phenol oxidase, peroxidase, and \( \beta \)-glucosidase. The enzyme assays were prepared by mixing 2 g of soil in 150 mL of acetate buffer (50 mM, pH 5). \( \beta \)-glucosidase activity was fluorometrically measured in 96-well plates using 4-methylumbelliferone-\( \beta \)-d-glucoside as a substrate (Saiya-Cork et al., 2002). At the termination of the assay, we added 25 \( \mu \)L of NaOH (0.2 M), to each well and then measured fluorescence using a F-max fluorometer (Molecular Devices Corp., Sunnyvale, CA); excitation energy was 355 nm and emission was measured at 460 nm. We measured peroxidase and phenol oxidase activity using \( L \)-dihydroxyphenylalanine (Saiya-Cork et al., 2002). After incubating the samples at 25°C for 18 h in 96-well plates, the optical density (460 nm) of the oxidized reaction product was measured on a spectrophotometer (Bio-Tek Instruments, Winooski, VT). All enzyme activities are expressed as nanomoles of substrate cleaved per gram dry mass of soil per hour (nmol g\textsuperscript{-1} soil h\textsuperscript{-1}).

**Soluble Soil Phenolics**

Soluble soil phenolic concentrations were determined by comparing soil solution with a standard mixture of phenolic compounds. This standard mixture contained 50 \( \mu \)mol L\textsuperscript{-1} each of ferulic, \( p \)-coumaric, \( p \)-hydroxybenoic, vanillic, and syringic acid. We adjusted the standard mixture to pH 6 and then diluted the standard to have a graduated range of phenolic concentrations from 3 to 250 \( \mu \)mol L\textsuperscript{-1}, which encompasses a common range of phenolic compounds and concentrations in soil (Sposito, 1989). To extract the soluble phenolics from soil, we agitated 5 g of soil and 25 mL of water on an orbital-shaker for 18 h. The samples were centrifuged (900 \( \times \) g) and the supernatant was passed through a 0.45-\( \mu \)m nylon filter (Ohno and First, 1998). We combined 5 mL of filtered supernatant, or standard phenolic mixture, with 0.75 mL of Na\textsubscript{2}CO\textsubscript{3} (1.9 M) and 0.25 mL of Folin-Ciocalteu reagent (Ohno and First, 1998). Deionized water was used as a negative control. After 1-h incubation at 25°C in the dark, absorbance was measured at 750 nm using a Spectronic 20 Genesix spectrophotometer (Spectronic Instruments, Rochester, NY). A regression of absorbance and standard phenolic concentration was determined (\( r^2 = 0.99 \)), and the absorbance of samples was adjusted to represent phenolic concentrations (\( \mu \)mol C g\textsuperscript{-1} of soil).

**Statistical Analyses**

In a previous study, we found that soil water content had a significant influence on enzyme activity (J. DeForest, unpublished data, 2003), and we used it in this analysis to adjust for any differences in field soil water contents among plots and stands. All values presented for enzyme activities are least square means adjusted for differences in soil water content. We used a two-way repeated measures ANOVA, with soil water as a covariate, to investigate the influence of sample date, site and NO\textsubscript{3} deposition treatment on soluble phenolics concentrations in mineral soil. We also explored the relationship between enzyme activity and phenolic concentrations using linear regression analysis. Significance for all statistical analysis was accepted at \( \alpha = 0.05 \).

**Results**

Experimental NO\textsubscript{3} deposition significantly decreased the activity of phenol oxidase for the two northern sites (A and B) when averaged across sampling dates (Table 1; Fig. 2). Although experimental NO\textsubscript{3} deposition reduced the activity of \( \beta \)-glucosidase and peroxidase, these differences were not statistically significant (Table 1); this contrasts with our previous observations of significant declines in activity due to experimental NO\textsubscript{3} deposition (DeForest et al., 2004a). Sampling date had a significant (\( p < 0.001 \)) influence on \( \beta \)-glucosidase and peroxidase activity, with the greatest enzyme activity oc-
Table 1. Repeated measures analysis of covariance for \( \beta \)-glucosidase, peroxidase, and phenol oxidase activity; soil water content was the covariate in this analysis. Soluble phenolic concentrations were analyzed using a repeated measures analysis of variance.

<table>
<thead>
<tr>
<th>Source of variance</th>
<th>df</th>
<th>( \beta )-glucosidase</th>
<th>Peroxidase</th>
<th>Phenol oxidase</th>
<th>Phenolic conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Within subject</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO\textsubscript{3} deposition treatment</td>
<td>1</td>
<td>0.441</td>
<td>0.089</td>
<td>0.007</td>
<td>0.536</td>
</tr>
<tr>
<td>Site</td>
<td>3</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.006</td>
<td>0.026</td>
</tr>
<tr>
<td>NO\textsubscript{3} deposition treatment ( \times ) Site</td>
<td>3</td>
<td>0.074</td>
<td>0.450</td>
<td>0.112</td>
<td>0.700</td>
</tr>
<tr>
<td>Between subject</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time ( \times ) NO\textsubscript{3} deposition treatment</td>
<td>5</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.059</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time ( \times ) Site</td>
<td>15</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.004</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time ( \times ) NO\textsubscript{3} deposition treatment ( \times ) Site</td>
<td>15</td>
<td>0.058</td>
<td>0.960</td>
<td>0.412</td>
<td>0.843</td>
</tr>
</tbody>
</table>

Owing in late June. Study sites also were a significant \((p < 0.01)\) influence on enzyme activity (Fig. 2).

Over the growing season, we observed similar concentrations of soluble phenolics in ambient and elevated NO\textsubscript{3} deposition treatments (Fig. 3), which produce a nonsignificant interaction between these two factors. As a main effect, NO\textsubscript{3} deposition had no influence on soluble phenolics (Table 1), with equivalent concentrations occurring under ambient and elevated NO\textsubscript{3} deposition for Sites B, C, and D (Fig. 2). Elevated NO\textsubscript{3} deposition significantly \((p > 0.001)\) reduced soluble phenolics by 35\% at the most northern site (A; Fig. 2). Sampling date and study site had significant influences on phenolic concentrations (Table 1). Soluble phenolic concentrations were generally highest in the spring and fall and lowest in the summer. For example, concentrations peaked in June at 121.5 \( \pm \) 9.6 \( \mu \)mol C g\(^{-1}\) (mean \( \pm \) SE) and decreased throughout the growing season to a minimum in August (86.2 \( \pm \) 5.9 \( \mu \)mol C g\(^{-1}\)); by September, phenolic concentrations increased to 111.6 \( \pm \) 8.3 \( \mu \)mol C g\(^{-1}\). Additionally, phenolic concentrations were highest at the two northern Sites A & B (approximately 126.8 \( \mu \)mol C g\(^{-1}\)) and lowest at Sites C and D (approximately 82.3 \( \mu \)mol C g\(^{-1}\)). We found a significant negative relationship between phenol oxidase activity and soluble phenolics, but phenol oxidase activity accounted for a small proportion of the variation in soluble phenolic across sites and sampling dates \((n = 144; p < 0.001; r^2 = 0.09)\). We also found a significant, negative relationship between soluble phenolics and \( \beta \)-glucosidase activity, but this relationship also was very weak \((n = 144; p < 0.001; r^2 = 0.18)\).

**Discussion**

Experimental NO\textsubscript{3} deposition produced a significant reduction in phenol oxidase activity; however, we found no effect of NO\textsubscript{3} deposition on soluble soil phenolics, indicating that declines in phenol oxidase activity likely did not produce higher concentrations of these compounds. The fact that soluble phenolic concentrations displayed a very weak inverse relationship with phenol oxidase activity provides further support for this contention. Experimental NO\textsubscript{3} deposition produced a nonsignificant decline in \( \beta \)-glucosidase activity and the activities of this enzyme also displayed a weak inverse relationship with soluble phenolic concentrations in soil. Thus, it appears that reductions in lignolytic activity due to NO\textsubscript{3} deposition are unlikely to cause increased soluble phenolic concentration in mineral soil, which could potentially re-

![Fig. 2](image-url)
It is plausible that declines in lignolytic capacity limit not likely the source of DOC leaching from our NO$_3$-cally derived from cellulose (Desphande et al., 1978). It suggests that the production of these compounds is 2003), which represent a substantial input of substrate this ecosystem. In summary, NO$_3$-tration of sugar maple fine roots (25–36%; Parsons et al.,resulted in threefold increase in DOC leaching from /H9252 have documented in our experiment (DeForest et al.,Pregitzer, 1993). A decline in oxidative capacity would also have previously observed in mineral soil (DeForest et al.,2004a). Moreover, the seasonality of leaf litter fall and DOC export also coincides with temporal patterns in soluble phenolics concentrations (Fig. 2). Given the high concentrations of phenolics in sugar maple leaves (Lindroth et al., 1993) and large decreases in oxidative enzyme activity in leaf litter (DeForest et al., 2004a), microbial activity in forest floor may hold the key to understanding why NO$_3$- deposition has increased DOC production and export. It is clear that high concentrations of soluble phenolics are not accumulating in the mineral soil of our NO$_3$- deposition treatment, relative to those occurring in the mineral soil of our ambient treatment.

In conclusion, decreases in phenol oxidase activity did not result in higher concentrations of soluble soil phenolics in mineral soil receiving experimental atmospheric NO$_3$- deposition. Soluble phenolics also displayed a weak negative relationship with β-glucosidase activity, suggesting that soluble phenolics exert minimal influence the activity of this cellulolytic enzyme. Moreover, a relatively low concentration of soluble phenolics in mineral soil suggests that the production of these compounds is not likely the source of DOC leaching from our NO$_3$- deposition treatment. Although we did not measure forest floor (Oi & Oe) in this study, changes in enzyme activity and DOC production in organic horizons may hold the key to understanding why NO$_3$- deposition has resulted in threefold increase in DOC leaching from this ecosystem. In summary, NO$_3$- deposition did not alter phenolic concentrations in mineral soil, nor were phenolic concentrations strongly related to lignolytic or cellulolytic extracellular enzyme activity. Thus, we have no evidence to support our initial hypothesis that reductions in phenol oxidase due to NO$_3$- deposition would increase soluble phenolics in soil solution, which in turn would inhibit β-glucosidase and stimulate DOC leaching.

Acknowledgments

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References