Ectomycorrhizal community and extracellular enzyme activity following simulated atmospheric N deposition

Richard W. Lucas*, Brenda B. Casper

Department of Biology, University of Pennsylvania, Philadelphia, PA 19104-6018, USA

ABSTRACT

Ectomycorrhizal (EM) fungi are abundant in temperate and boreal ecosystems and are understood to be an important means whereby plants can fulfill their nutrition requirements. The extent of the EM fungal involvement in accessing organic sources of N, however, remains unknown. Some EM fungi have been found to produce lignolytic and proteolytic enzymes which are necessary to depolymerize organic substrates, but this ability varies by species. Both EM fungal communities and the activities of lignolytic and proteolytic enzymes may be sensitive to changes in inorganic N availability such as through increased atmospheric deposition. Our objectives were to simulate an ecologically relevant increase in atmospheric N deposition in areas currently receiving very little exogenous N and examine changes in EM community composition, lignin degrading enzyme activity, and soil protein depolymerization. We found a distinct shift in the EM community composition following simulated atmospheric N deposition. Likewise, we found a significant decrease in the activity of lignin degrading enzymes, which could have important implications on ecosystem N and C cycling. Contrary to our hypotheses, proteolysis increased following N addition. The fact that lignolytic and proteolytic enzymes exhibit opposite responses is counterintuitive and suggests much is yet to be learned about how N addition affects global C storage by affecting the decomposition of organic matter. Our data suggest small increases in atmospheric N deposition could produce significant changes in communities of EM fungi and N and C cycles.

© 2008 Elsevier Ltd. All rights reserved.

1. Introduction

In terrestrial ecosystems, soil organic matter (SOM) is an important source of nitrogen (N), whose availability is mediated by various processes of decomposition. SOM is primarily comprised of plant material and its decomposition depends on the action of at least two broad classes of enzymes: proteases that decompose proteins and liberate organic N; and lignolytic enzymes that facilitate the decomposition of lignin.

Although lignin is one of the most abundant organic macromolecules (Taiz and Zeiger, 1991) and is very common in SOM, it is extremely slow to decompose (Hammel, 1997), and therefore, serves as an important form of terrestrial carbon and N storage. Few enzymes are able to break down lignin (Mester and Tien, 2000) because the irregular arrangement of its repeating phenol subunits precludes the operation of the well-characterized lock-and-key mechanism found in other enzyme–substrate complexes (Allison, 2006).

There is increasing evidence that ectomycorrhizal (EM) fungi, which are abundant in temperate forests and boreal ecosystems, may play a role in the decomposition of SOM (Chen et al., 2003; Courty et al., 2006; Buee et al., 2007). It is generally understood that EM fungi sequester nutrients that otherwise would be unavailable to the host plant and transfer them to the plant in exchange for photosynthate (Smith and Read, 2002). EM fungi provide greater access to nutrients both physically, because their smaller hyphae can explore a greater volume of soil than can roots alone, and biochemically, because many species of EM fungi are thought to be able to access refractory nutrients such as N stored in organic forms (Chalot and Brun, 1998; Courty et al., 2005). Obligate decomposers such as the saprotrophic white-rot and brown-rot fungi are understood to be the more efficient, primary decomposers of organic matter (Dix and Webster, 1995), but EM fungi may be important as well (Schimel and Bennett, 2004; Buee et al., 2007). Such a role for EM fungi could have far-reaching importance for ecosystems in which they are common.

The addition of inorganic sources of N, such as from increased atmospheric N deposition, can alter the abundance or activity of lignolytic enzymes (Waldrop and Zak, 2006) and may potentially feedback to alter the community structure of the organisms that produce them (Read and Perez-Moreno, 2003). The community composition of EM fungi may be particularly sensitive to changes in the abundance of inorganic N because the ability to produce lignolytic and proteolytic enzymes likely differs among EM fungal species (Read and Perez-Moreno, 2003).

* Corresponding author. Department of Botany, University of Wyoming, Laramie, WY 82071, USA. Tel.: +1 307 766 3261; fax: +1 307 766 2851.
E-mail address: rlucas3@uwyo.edu (R.W. Lucas).

Previous research has found that the activity of two lignolytic enzymes, phenol oxidase and peroxidase, decrease with increased inorganic N availability in oak-dominated systems (Sinsabaugh et al., 2004; Waldrop and Zak, 2006). This may be due to associated changes in the composition of the decomposer community (Fog, 1988) or to changes in the chemical structure of lignin or the extracellular enzymes involved in its decomposition (Berthrong and Finzi, 2006). It is hypothesized that the activity of protease will decrease with increased inorganic N availability, since it is important in the acquisition of N from organic sources. Likewise, the few studies that have examined the community structure of EM fungi have also found changes in community composition following N addition (Kårén and Nylund, 1997; Peter et al., 2001; Avis et al., 2003; Dighton et al., 2004). Although useful, these studies have not considered the EM community and the activity of these enzymes simultaneously, and those systems have been exposed to relatively high inorganic N inputs, either from increased ambient atmospheric N deposition, or from artificially added N. In order to better understand the responses of EM fungal communities and soil enzyme activities, it is important to conduct ecologically relevant field experiments using realistic levels of N.

We conducted an N addition experiment, simulating projected N deposition levels, in forests that currently receive among the lowest levels of ambient atmospheric N deposition in the northeastern United States. These sites are hypothesized to receive around 20 kg N ha^{-1} by the year 2020, a rate currently observed in other areas of the northeastern United States (Galloway et al., 1994; NADP, 2004). Our overall goal was to determine how EM fungal communities and the activities of soil enzymes involved in organic matter decomposition respond to more realistic, predicted increases in atmospheric N deposition. We examined the EM fungal community structure as well as phenol oxidase, peroxidase, and proteolytic activity in response to simulated atmospheric N deposition. Specifically, we tested three hypotheses related to changes occurring with increasing atmospheric N deposition: (1) there would be a distinct shift in the composition of the EM community following N addition; (2) the activity of lignin degrading enzymes would decrease with added N; and (3) proteolytic activity would decrease with added N.

2. Methods and materials

2.1. Study sites and N additions

The experiment was carried out at four sites in Maine, USA (Table 1). Two were located at the Holt Experimental Forest owned by the University of Maine (hereafter referred to as the Holt sites) and two were located near the Range Pond State Park (hereafter referred to as the Park sites). These locations were chosen because ambient levels of atmospheric N deposition there are among the lowest in the northeastern United States, receiving between 3 and 5 kg N ha^{-1} y^{-1} (NADP, 2004), but are expected to rise to 15–20 kg N ha^{-1} y^{-1} or greater by 2020 (Galloway et al., 1994).

At each site, two 20×20 m plots were delineated in stands primarily composed of northern red oak (*Quercus rubra*), which form strong associations with EM fungi and are common forest trees throughout the northeastern United States. Sites were selected based on four criteria: (1) red oak leaves comprised roughly 75% or greater of the leaf area of the canopy; (2) red oaks were at least 25 cm in diameter at breast height; (3) there was no evidence of recent disturbance (i.e. logging, fire, or agriculture); and (4) soils were similar in type and texture. Soils are loamy, mixed, active, frigid lithic Dystrudepts derived from mica schist of the Hollis and Charlton series for the Holt and Park sites respectively.

One plot at each site was randomly selected to receive N additions and the other designated as a control. A total of 20 kg N ha^{-1} was added annually to each treatment plot, applied in four monthly applications from May to August in both 2005 and 2006. The form of N was granular sodium nitrate and ammonium chloride mixed in a 17:3 ratio, similar to the mean ratio of NO_3^- and NH_4^+ detected at atmospheric N deposition monitoring stations in the surrounding area (NADP, 2004). In all cases, treatment and control plots were separated by 30–40 m.

2.2. EM community composition

EM fungal community evaluations were carried out using soil cores (10 cm deep, 5 cm dia.) collected from under five northern red oak trees in each plot, with one tree randomly selected as the focal tree. Four 5-m transects were established radiating from the base of the focal tree in the cardinal directions. Soil cores were taken at 1, 2, 3, 4, and 5 m along each transect. Four soil cores were taken at each of the other four trees, 1 m away from the base, again in cardinal directions. This yielded 36 samples per plot on each sampling date. At the Holt sites, cores were collected in May 2005 prior to fertilization and again in September 2006 following fertilization. At the Park sites, cores were collected only in September 2006 because we were unable to obtain permission for collecting plant and fungal material prior to the addition of N. Soil cores were collected using narrow-walled acrylic tubes which were then capped tightly, both top and bottom, wrapped in aluminum foil, and kept at 4 °C until EM fungal morphotype abundance could be determined.

EM fungal communities were determined from a sample of roots taken from each soil core. Forty root sections, each 2–3 cm long, were hand picked from each of three stratified soil sections: the O horizon (generally 2–3 cm deep), the top portion of the A horizon (generally 4 cm), and the bottom portion of the core (generally 4 cm). Each core section was gently washed in tap water to remove soil particles and adhering organic matter. Root tips were separated into morphotypes, yielding 200–900 root tips per core (mean: 375), approximately 13,500 root tips per plot; 130,879 root tips were evaluated over the course of the study. Because virtually all root tips were colonized by EM fungi, no effort was made to quantify the level of root colonization.

Morphological assignments of root tips were based on the following criteria: type and shape of ramification, branching pattern,

Table 1
Selected attributes for each study site in Maine

Study site	Lat (°N)	Long (°W)	Elevation (ft)	N dep (kg ha^{-1})	C (se) (mg g^{-1})	N (se) N (mg g^{-1})	C/N (se)	MAT (°C)	MAP (mm)
Holt Research Forest 1	43.800	69.750	70	5.219	1088.47 (63.62)	42.07 (3.12)	25.93 (0.48)	8.9	1541
Holt Research Forest 2	43.819	69.761	60	5.554	846.12 (130.58)	34.95 (7.64)	23.64 (1.46)	8.9	1541
State Park 1	44.034	70.345	240	4.988	1386.88 (69.14)	49.64 (6.27)	28.46 (2.13)	7.7	1042
State Park 2	43.855	70.189	160	4.722	650.43 (231.49)	36.12 (11.78)	17.24 (1.29)	8.0	1089

N dep = ambient atmospheric nitrogen deposition; mean annual temperature (MAT) and mean annual precipitation (MAP) data are taken from weather stations located at nearby Phippsburg, ME for the Holt sites, and Poland Spring and Naples, ME for each of the Park sites.

features of the mantle-surface, presence/absence of rhizomorphs, rhizomorph connection with mantle, color, and presence/absence of hyphae (Agerer, 1987–2002). Each morphotype was catalogued using a digital visual database created in Microsoft® Access with DBPix version 2.0.3, a third party image control (Ammara Digital Image Solutions, Farnham, UK). This allowed for greater documentation of each morphotype and greater precision defining and sorting the morphotypes.

2.3. Soil processes

Phenol oxidase, peroxidase, and proteolytic activities were determined from a second set of samples collected from the organic soil 1 m away from the base of the five northern red oak trees at each plot. Four soil cores (10 cm deep, 5 cm dia.) were collected from underneath each tree, one from each of the four cardinal directions relative to the tree trunk, and composited into a single sample per tree. Five other sample locations were randomly selected in each plot, at which four samples were collected from within a 1-m^2 area and composited into a single sample per location. This yielded a total of ten samples per plot. Composited samples were homogenized in the field by passing them through a 2-mm sieve, which also removed rocks and large debris. Samples were kept on ice for transportation. Processing of samples for lignolytic enzyme activities were begun within 48 h of collection and for proteolytic activity within 72 h. Samples were collected from sites in May 2005 prior to N fertilization, and again in September 2006 following N additions.

Phenol oxidase and peroxidase assays followed microplate procedures described by Saiya-Cork et al. (2002) beginning with soil suspensions created by adding 1.0 g soil (fresh weight) of each soil sample to 125 ml of 50 mM sodium acetate buffer (pH 5.0). Suspensions were incubated for 24 h using 5 mM L-3,4-dihydroxyphenylalanine (L-DOPA) as the substrate. The peroxidase assay included 25 µl of 6% H_2O_2. Enzyme activities were measured using clear polystyrene 96-well microtiter plates (Fisher Scientific, Pittsburg, PA, USA) on a spectrophotometer (Multiskan RC, Labsystems, Helsinki, Finland) using an emission wavelength of 450 nm. Sixteen analytical replicates were used per sample.

Proteolytic activity was estimated using the method described by Watanabe and Hayano (1995) and Lipson et al. (1999). This technique measures the accumulation of amino acids over time in soil suspensions in the absence of microbial uptake (Lipson et al., 1999) and is expressed as $\mu g \, N \, g^{-1}$ soil h^{-1}. Two types of proteolytic activities, 'actual' and 'potential', were determined. Actual proteolytic activity refers to measurement made when the protein present in the native soils is the only substrate available to proteolytic enzymes. In measuring potential proteolytic activity, casein, a labile substrate for proteolytic enzymes, was added to determine if the soil suspensions were substrate limited. Actual proteolytic activity was determined from soil suspensions created by adding 5.0 g soil (fresh weight) to 40 ml of 50 mM sodium citrate buffer. Potential proteolytic activity was determined by adding 5.0 g soil (fresh weight) to 20 ml of 50 mM sodium citrate buffer and 20 ml of a 0.6% solution of casein. Microbial activity was inhibited by the addition of 0.4 ml toluene to each suspension. Suspensions were subsampled before and after a 6-h incubation period at 24 °C on a shaker table. Immediately upon subsampling, a 2 ml aliquot of trichloroacetic acid solution was added to the 2 ml subsample to halt the activity of proteolytic enzymes. Subsamples were kept frozen until they could be analyzed spectrophotometrically (Multiskan RC, Labsystems, Helsinki, Finland) using a 96-well microplate version of the ninhydrin colorimetric analysis for total amino acids (Lipson and Monson, 1998). Leucine was used as the standard and microplates were read using an emission wavelength of 570 nm.

2.4. Statistical analyses

Differences in EM fungal community structure among treatment and control plots, both before and after N addition, were analyzed with non-metric multidimensional scaling (NMS) using the PC-ORD software package (MjM Software Design, version 5.0, Gleneden Beach, OR, USA). NMS ordinations are essentially maps reflecting ecological dissimilarity between sampling units. Points closer to each other on the ordination have more similar EM fungal communities than points farther apart. NMS is well suited to ecological data, which are typically non-normal, because it utilizes the rank order of among-sample dissimilarities and thereby avoids assumptions about underlying sampling distributions or about linear relationships among variables (Clarke, 1993). EM fungal morphotypes occurring in soil cores three times or less were eliminated from the analysis to reduce noise in the data and avoid disproportionate effects of rare morphotypes. Two hundred and fifty runs on real data, using the Sørensen distance measure and a maximum of 250 iterations, starting from random configurations, were performed and the strength of the resulting patterns were checked using 250 Monte Carlo runs. Diagnostic (scree) plots suggested a three-dimensional solution was optimal. The solution with the lowest stress was selected as the starting configuration for the final NMS analysis reported here. Stress in this context is a measure of departure of fit between the ordination and the data (McCune and Grace, 2002). Differences among EM fungal communities were tested using multiresponse permutation procedures (MRPP) using Sørensen's distance measure within the PC-ORD software package.

The responses of EM fungal morphotype richness, morphotype diversity (calculated using the inverse of Simpson's diversity index [McCune and Grace, 2002]), phenol oxidase activity, peroxidase activity, and proteolysis to N addition were examined using ANOVA, with treatment and time (year of collection) as fixed variables. Plots (random variable) were nested within N treatment. When there was a significant treatment by time interaction, Tukey's HSD was used to make specific post-hoc comparisons. The Park sites were not included in the morphotype richness or diversity analyses because of the lack of EM fungal community data prior to N fertilization. Reduced major axis (RMA) regression was used to examine the relationship between phenol oxidase and protease. Significance level was set at $\alpha = 0.05$. All analyses were performed using SAS version 9.1 (SAS, 2003).

3. Results

3.1. EM community

We found a total of 85 different morphotypes over the course of the study. Of those 85, fourteen morphotypes were unique to control plots or plots surveyed before the N additions, and 3 were unique to the N treatment plots. The relative abundances of all morphotypes occurring in at least 3 soil cores and in total representing between 97 and 100% for each plot, are reported in Table 2. The most common morphotypes present before N additions were IBDYO, *Cenococcum geophilum*, and ISYOL (see Table 2 for abbreviations). Following two years of N additions, the most common morphotypes in the N treated plots were *C. geophilum*, SBLYS, and IBRLR. In general, we observed an increase the abundance of *C. geophilum* following N addition.

Our results support the hypothesis that EM fungal communities are affected by increased levels of inorganic N. The richness of EM fungal morphotypes on a per core basis significantly decreased ($P < 0.01$) following two years of N additions (Fig. 1). Morphotype diversity in the treatment plots after N addition was also significantly lower than that of the control plots, but was not significantly

Table 2
Short description of each morphotype occurring in at least 3 soil cores and its corresponding relative abundance (no. root tips colonized/total no. root tips examined per plot) in each plot before simulated N deposition and following two years of N deposition

Abbreviation short description		Before				Control				N addition			
		Holt plots				Holt plots		Park plots		Holt plots		Park plots	
		H1	H2	HA	HB	H1	H2	P1	P2	HA	HB	P1	P2
CenGeo	*Cenococcum geophilum*	16.19	9.02	10.92	5.64	20.45	24.76	14.36	19.73	19.03	16.66	18.14	33.61
DBYCM	Dichotomous, bent, yellow, cottony mycelium	0.09	0.09	0	0.01	0.09	0.33	0.26	0.32	0.05	0.28	0.27	0.06
DSGRP	Dichotomous, straight, greyish red purple	0.73	2.67	1.73	4.16	0.55	0.72	1.50	1.84	1.34	2.59	0	0
DTSSDBB	Dichotomous, tortuous, short-spiny, dark blackish brown	0.51	0.85	0.82	2.17	0	0	2.28	0.27	1.08	0.67	0	0
DWLRRP	Dichotomous, whispy long ramifications, red purple	0.30	0.01	0	0.04	0.84	3.10	0.60	0.16	0.42	0.91	5.24	0.82
IBBRO	Irregular, bent, brown red orange	0.53	2.49	1.73	2.54	0	0	1.26	0	0.79	2.05	1.30	0
IBCDRT	Irregular, bent, cream with dark red tip	1.01	0.84	0.53	0.31	0.23	0.79	0.16	0.16	0.23	0.03	0	0
IBDYO	Irregular, bent, darker yellowish orange	10.45	10.18	23.26	19.32	3.36	1.50	5.44	5.88	3.11	5.12	7.01	2.92
IBFLW	Irregular, bent, frail long white	0	0	0	0	0	0.11	1.29	0.17	0.01	0.09	1.88	1.45
IBO	Irregular, bent, orange	2.21	4.85	5.04	4.76	2.25	3.11	8.20	4.95	1.93	7.88	3.11	0
IBRLR	Irregular, bent, red, long ramifications	2.74	6.16	4.43	1.35	10.14	8.01	4.41	4.79	5.11	6.95	9.58	8.48
IBROPC	Irregular, bent, red orange purple, cottony	4.24	4.88	4.08	5.13	0	0	4.03	3.92	5.72	5.73	0	0
IBRS	Irregular, bent, red and shrivelled	1.95	0.65	3.28	1.19	3.33	2.00	0.42	0	0.12	1.37	0.43	0
IBRYR	Irregular, bent, red, yellow rhizomorph	0	0.62	0.77	3.05	0	0	0	0	0	0	0	0
IBWBYR	Irregular, bent, whispy bright yellow, rhizomorph	0.35	0.23	0	0.07	1.27	3.01	1.08	1.59	1.16	0.26	3.73	3.42
IBYBI	Irregular, bent, yellow brown, inflated	0.64	0.28	3.75	2.34	0	0	0.89	0.83	1.19	1.43	0.59	0
IFS	Irregular, fusiform, smooth	0.11	0.35	0	0.02	0	0	0.51	0.38	0.28	0.21	0.20	0
ISGO	Irregular, straight, grey ochre	0.98	0.66	0.53	0.73	0.48	0.46	1.76	0.74	0.96	0.64	1.55	0.81
ISOY	Irregular, straight, opaque yellow	0.20	0.05	0.85	0.03	0.23	0.35	1.15	1.40	0.54	0.84	1.92	0.76
ISROW	Irregular, straight, reddish orange, wrinkly	0	0	0	0	0	0	0	0	0	0	2.67	2.42
ISYOL	Irregular, straight, yellowy orange, lactating	7.26	5.63	7.19	5.20	18.24	14.17	7.28	9.50	12.28	7.19	1.86	5.16
ITBR	Irregular, tortuous, brown w/rhizomorph	3.40	8.65	3.71	1.23	0	0	0	0	0.09	0.11	0	0
ITBWW	Irregular, tortuous, bright white, woolly	0.14	0.22	0	0.08	0.42	0.32	0.48	0.36	0.68	1.02	0.50	0.40
ITDB	Irregular, tortuous, dark brown	0	0	0	0.83	0.10	0.15	1.13	0.14	0.33	0.22	1.17	0.16
ITGRBR	Irregular, tortuous, grey red brown, rhizomorph	0.34	0.19	0.37	0.26	0.40	0	0	0	0	0	0	0
ITOB	Irregular, tortuous, orangey black	3.34	5.39	2.86	1.37	0	0.29	0.23	0	0.32	2.71	0.60	0
ITOTCBW	Irregular, tortuous, ocre, thick cottony brilliant white	0.23	2.52	0	1.10	0	0.32	0.05	0.13	0.19	0.03	0.62	0.47
PBBRYP	Pinnate, brownish red yellow, pubscent	0.67	0.12	0.08	0.33	2.31	0.19	0.65	0.65	2.08	0.34	0	0
PBGLYR	Pinnate, bent, greyish light yellow, rhizomorph	0	0	0	0	0.60	7.50	1.89	1.67	1.93	3.55	1.10	0.33
PBLCRS	Pyramidal, beaded, light creamy yellow, smooth	2.96	0.78	0.08	3.48	0.73	0	1.89	0.43	0.52	0.44	4.62	0.47
PBRYBHM	Pinnate, bent, green yellow brown with hyphal mat	0.05	0.14	0	0	0	0	0.64	0.14	0.09	0	0	0
PBYBL	Pinnate, bent, yellow brown, lactating	0	0	0	0.39	0	0	0.21	0.14	0	0	0	0
PIDRY	Pyramidal, inflated, dirty red yellow	0.30	0.54	0.10	0.55	0	0	0	0	0.33	0.04	0	0
PSBYH	Pinnate, straight, almost black with yellow highlights	0.55	0.26	0.40	2.88	0	0	3.31	4.82	2.78	2.22	0.59	0.29
PSBYYR	Pinnate, straight, bright yellow, yellow rhizomorph	0	0	0	0	0.11	0.01	0.51	0.47	0.50	0.37	0.52	0.82
PSDBR	Pyramidal, straight, dark brown, rhizomorph	1.41	0.76	3.00	0.63	0.03	0	0.18	1.04	0.78	1.40	0.19	0
PSDDR	Pinnate, straight, dirty dark red	0.58	0.27	1.12	0.54	0	0	0.36	0	0	0	0.38	0
PSDGR	Pinnate, straight, deep greyish red	0	0.95	0	1.19	0	0.11	0.01	0.19	0.32	0.17	0.01	0.22
PSGRCWM	Pinnate, straight, greyish red, cottony white mycelium	10.52	2.10	1.68	3.83	0	0	0.20	0.19	0.24	0.98	0.20	0.16
PSLYL	Pyramidal, straight, light yellow, lactates	0.10	0.01	0	0	1.93	0.85	0.72	0.50	0.10	0.72	0.80	0.46
PSLYS	Pinnate, straight, light yellow, smooth	0	0.07	0	0.08	1.04	0.60	0.17	0.16	0.30	0.32	2.64	2.60
PSOB	Pyramidal, straight, orangey black	0.53	0.81	0	0.50	0	0	0	0	1.19	0.07	0	0
PSRPSR	Pinnate, straight, red purple, sticky and rhizomorph	1.39	0.44	2.30	0.23	2.96	1.03	0.70	1.18	1.71	0.88	1.55	2.21
PSYS	Pyramidal, straight, yellowy salmon	0.47	0.12	0.57	0.55	0	0	0.60	0.14	0.81	2.52	0.19	0
SBDRPS	Simple, bent, dirty red purple, shrivelled	2.77	2.76	0.35	4.55	7.44	9.48	5.39	5.11	8.80	4.95	0	0
SBDRR	Simple, bent, dark red, rhizomorph	6.38	5.99	5.26	4.44	1.80	0.97	7.30	10.35	3.87	3.26	3.15	3.67
SBLYS	Simple, beaded, light yellow, smooth	0.48	0.01	0	0	6.60	4.98	0.46	0.12	0	0.21	15.68	14.52
SBOS	Simple, bent, orange, almost smooth	1.64	1.33	0.97	0.91	0	0	0.59	0.35	1.08	1.25	0	0
SSBGR	Simple, straight, bluish–greenish, rhizomorph	0.02	0.14	0.02	0	0	0.87	0	0	0	0	0	0
SSDTY	Simple, straight, dirty thin yellow	0	0	0	0	2.10	3.97	0.53	1.68	1.53	3.04	0.86	0.75
SSFLOR	Simple, slightly fusiform, light orange, rhizomorph	1.03	2.00	0.08	0	0	0	0	0	0.09	0.05	0	0
SSLCC	Simple, straight, light cream, cottony	0.14	0.17	0.17	0.06	0.35	0	0	0	0	0.24	0	0
SSO	Simple, straight, orange	0.39	0	1.42	0	0.20	0	0	0	0	0	0	0
SSOLR	Simple, straight, orangey, lactating, rhizomorph	3.24	0.56	0.90	0.48	1.31	0	0	0	0.03	0	0	0
SSYSAK	Simple, straight, yellow, smooth with apical knob	0.75	6.53	2.18	7.27	4.48	3.67	7.18	8.48	3.84	3.79	2.06	12.22
SUO	Simple, unramified, orange	0.66	0.05	0.72	0.21	0.16	0	0	0	1.14	0	0	0
TO	Twisty, orange	1.68	3.03	1.07	2.00	0.99	0	1.03	0	0.24	1.01	1.60	0
USDB	Unramified, straight, dark brown	0.05	0.38	0.45	0.43	1.04	0.30	3.98	3.09	4.29	1.90	0	0
USDGB	Unramified, straight, darkish grey brown	0	0.16	0	0.59	0.68	0.46	1.44	1.67	2.41	0.49	0.13	0.09
USY	Unramified small yellow	0	0	0	0	0.62	0.17	0.05	0.10	0.33	0.21	0.05	0.12

different ($P = 0.07$) from morphotype diversity before N additions in the treatment plots.

The EM fungal community composition defined by the relative abundance of morphotypes significantly changed following simulated N deposition (Fig. 2A). The NMS ordination shows the EM fungal communities in the plots receiving N additions were more ecologically similar to each other than to plots that did not receive

N additions. Following two years of N fertilization, EM fungal communities in the treatment plots were significantly different from communities in control plots or from initial communities in the Holt plots before fertilization ($P < 0.01$, $P < 0.01$ respectively). The three-dimensional NMS solution explained 62% of the observed variation in the EM fungal communities (axis 1 = 19%, axis 2 = 21%, and axis 3 = 22%). The final stress in the analysis was
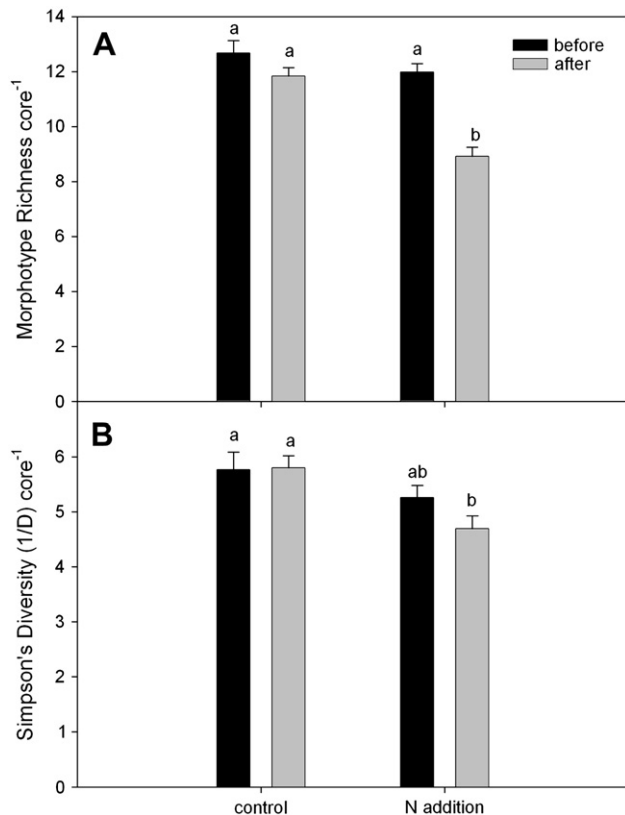
Fig. 1. Ectomycorrhizal morphotype (A) richness and (B) diversity (calculated as the inverse of Simpson's Diversity Index) in soil cores from four sites in Maine following two years of N addition. Error bars represent standard error of the mean. Bars sharing the same letter are not significantly different at $\alpha = 0.05$. $n = 10$.
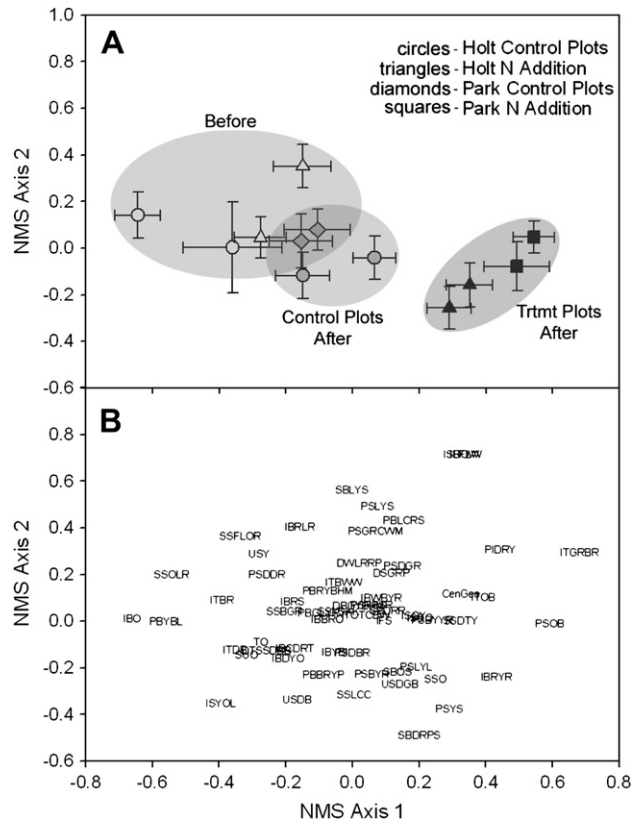

Fig. 2. Ordination of (A) ectomycorrhizal communities and (B) ectomycorrhizal morphotypes in Maine before and after two years of N addition using non-metric multi-dimensional scaling (NMS). The first and second axes explain 19 and 21% of the observed variation. Error bars represent the standard error of the mean. State Park regulations prevented the sampling of ectomycorrhizal communities at the Park sites prior to N fertilization. Light grey circles and triangles represent Holt communities before simulated atmospheric N deposition (Before). Dark grey circles and diamonds represent communities of the control plots of the Holt and Park sites after fertilization treatments (Control Plots After). The black triangles and squares represent the communities of the Holt and Park sites following simulated atmospheric N deposition (Trtmt Plots After). The cloud of points labeled Trtmt Plots After is significantly different from the clouds labeled Before and Control Plots After ($P < 0.01$, $P < 0.01$ respectively).

22.21. The Monte Carlo test indicated that the minimum stress solution was lower than would be expected by chance alone ($P = 0.02$). The instability of the final solution was 0.0005. The morphotype ordination is reported in Fig. 2B.

3.2. Soil processes

Lignolytic enzyme activity was significantly affected following two years of N addition. Following N amendment, there was a significant decline in the activity of phenol oxidase ($P = 0.05$, Fig. 3A) in treatment plots. The activity of peroxidase in plots amended with N did not decline relative to measurements before fertilization but was lower than peroxidase activity in control plots ($P = 0.05$, Fig. 3B) that increased during the study. These data support the hypothesis that lignin decomposition decreases in response to inorganic N. Prior to N fertilization, there were no significant differences between the control and N treatment plots for either enzyme.

In contrast, proteolysis did not decrease following N fertilization as hypothesized (Fig. 4). Prior to fertilization, levels of proteolysis did not differ between control plots and treatment plots. After fertilization, both actual and potential proteolysis had declined in the control plots but remained unchanged in the N-amended treatment plots. After fertilization actual proteolysis was significantly greater in the N-amended plots than in the control plots ($P = 0.01$). An increase observed in the potential proteolysis was not statistically significant ($P = 0.06$).

The opposite responses of proteolysis and lignolytic enzyme activity to simulated N deposition are surprising. Regressing actual proteolysis on phenol oxidase activity indicates that a negative relationship ($r^2 = 0.31$, $P < 0.001$) exists between these two ecosystem functions (Fig. 5).

4. Discussion

Our results indicate that relatively small inorganic N inputs have the potential to alter EM fungal communities and some of the ecosystem functions in which they may be involved. These results are consistent with our hypothesis and with other studies that have examined EM fungal responses to increased N, but our study highlights three important points. First, applying N at a rate that is consistent with predicted levels of atmospheric N deposition in the year 2020 to a system where N deposition from anthropogenic sources is currently low (Galloway et al., 1994; NADP, 2004) yielded clear changes in the EM fungal community structure and the activity of enzymes involved in lignin and protein degradation. Second, the responses of the EM fungal morphotypes were fairly quick. And third, the relationship between lignin degrading and protein degrading enzymes is not what has been hypothesized.

EM fungal communities may be particularly sensitive to changes in the availability of inorganic N. Any changes in the availability of inorganic N may favor those species that can effectively utilize inorganic N (Wallenda and Kottke, 1998; Lilleskov et al., 2002). This is consistent with other studies showing that increased inorganic N can cause shifts in EM fungal communities. These include investigations of EM fungal community responses in coniferous forests across N deposition gradients covering 7 km (Lilleskov et al.,
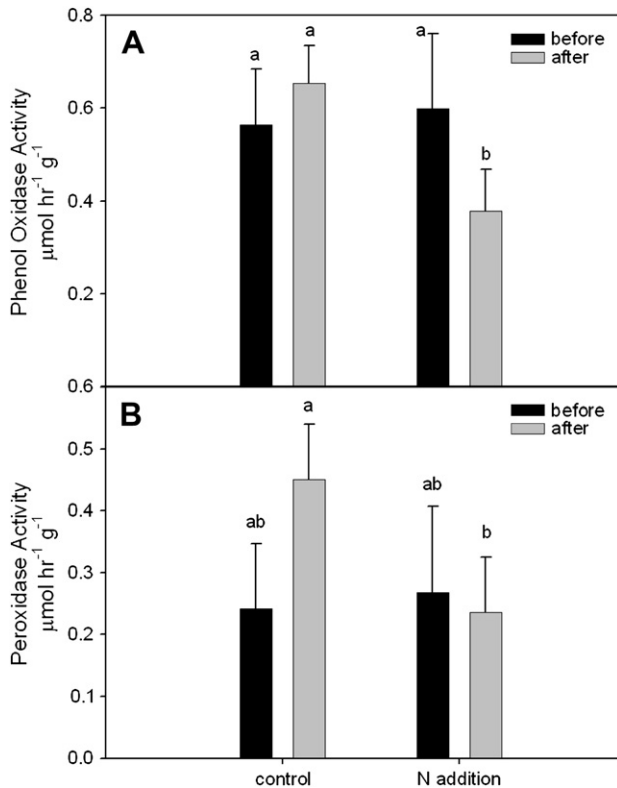
Fig. 3. Extracellular enzyme activity in Maine following two years of N addition. (A) Phenol oxidase activity, (B) peroxidase activity. Error bars represent the standard error of the mean. Bars sharing the same letter are not significantly different at $\alpha = 0.05$. $n = 8$–9.

Fig. 4. Rates of (A) actual and (B) potential proteolysis in Maine following two years of N addition. Error bars represent standard error of the mean. Bars sharing the same letter are not significantly different at $\alpha = 0.05$. $n = 10$.

2002) and 70 km (Dighton et al., 2004). Both found significant community differences with increasing ambient inorganic N. Lucas (2007) examined EM fungal communities over an atmospheric N deposition gradient from Maine to western Pennsylvania and found that N deposition was the strongest correlate of the community ordination. Frey et al. (2004) similarly observed changes in EM fungal communities in a study simulating atmospheric N deposition in which 5 kg N ha^{-1} was added to a pine forest over a period of 14 years. Given these findings, we can expect important changes in the EM fungal community should atmospheric N deposition continue to produce even small increases in inorganic N availability.

The responses in the EM fungal community took place over a period of two years, a rate of change that is fairly rapid given the two to five year life span of many EM fungal root tips (Treseder et al., 2004). One morphological character that exhibits a fast response time to changing environmental conditions is the number of rhizomorphs formed (Wallenda and Kottke, 1998). Rhizomorphs are bundles of extraradical hyphae and are hypothesized to be important structures in nutrient acquisition (Smith and Read, 2002). If inorganic N availability increases, there may be less need for rhizomorphs to help in acquiring the necessary N resources. Other studies of N polluted sites in boreal ecosystems in Alaska and Europe have shown that rhizomorph abundance decreased with increased inorganic N (Taylor et al., 2000; Lilleskov et al., 2002). In our study, we did not specifically quantify the number of rhizomorphs formed, but our data do indicate the frequency of morphotypes forming rhizomorphs increases with increased inorganic N availability. Four out of the six most abundant morphotypes in the control and treatment plots prior to N addition form rhizomorphs, whereas only one of the six most abundant morphotypes in the N treatment plots after N addition form rhizomorphs. Given
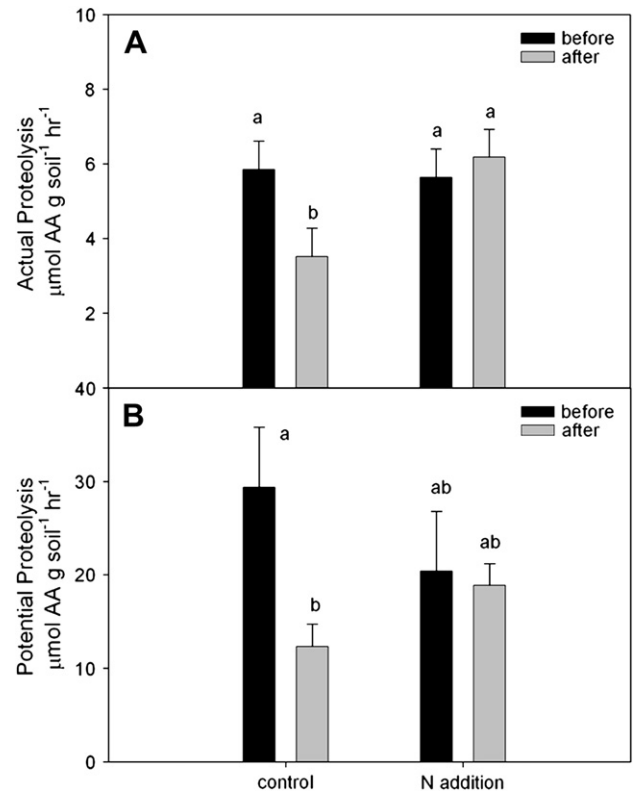
that rhizomorphs abundance has been linked to the utilization of complex organic substrates (Wallenda and Kottke, 1998), these findings suggest that the belowground fungal community experiences altered biochemistry following increased inorganic N. Additionally, we observed an increase the abundance of *C. geophilum* following N addition. Treseder et al. (2004) found that *C. geophilum* may be more responsive to increased N than other morphotypes because it may have a shorter lifespan or faster turnover time.

While EM fungi likely contribute to the soil processes we measured (Schimel and Bennett, 2004), we cannot specifically link the biochemical responses to changes in the EM fungal community. Saprotrophic fungi are also involved in these same processes, but we presently have little information about how N addition will
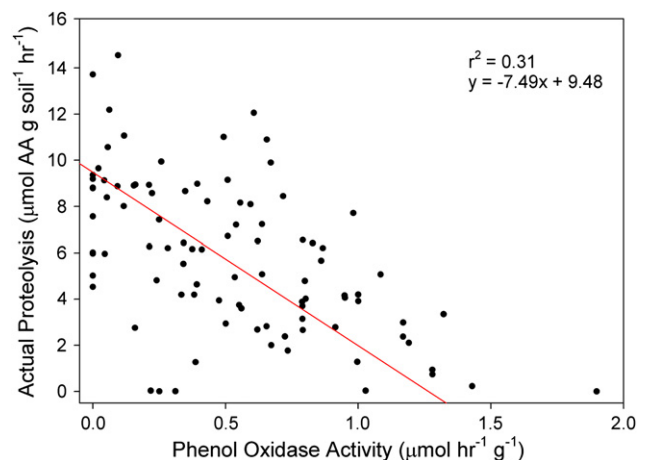
Fig. 5. Relationship of phenol oxidase and actual proteolysis reduced major axis regression.

change the relative roles of EM and saprotrophic fungi in such ecosystem functions as lignin decomposition and C storage. Saprotrophic fungi have traditionally been understood to be principally responsible for the decomposition of organic matter (Schimel and Bennett, 2004), but the traditional distinction between mutualist EM fungi and saprotrophic fungi may not be as clear as we once thought (Read and Perez-Moreno, 2003; Schimel and Bennett, 2004). EM fungi may be important contributors to the decomposition of SOM as well.

Changes in the activities of extracellular enzymes involved with lignin decomposition corresponded with differences in the EM fungal community. The decreased activity of phenol oxidase and peroxidase is consistent with decreases in these same enzymes over the Maine to western Pennsylvania N deposition gradient (Lucas, 2007) and with other N addition studies in oak-dominated forests (DeForest et al., 2004; Waldrop and Zak, 2006). These results suggest that lignin decomposition is slowing in oak-dominated ecosystems in response to increasing atmospheric N deposition (Neff et al., 2002), with potentially important consequences for soil carbon storage (Neff et al., 2002; Mack et al., 2004).

In contrast to our predictions, proteolysis was significantly greater in plots treated with N than in control plots, indicating that protein catabolism increases in response to increased inorganic N availability. This contradicts a common understanding in the literature that proteins and other organic sources of N should be less important sources of N in a high inorganic N environment (Wallenda and Kottke, 1998). We expected to see a decrease in proteolytic activity following atmospheric N deposition because the inhibition of lignolytic enzymes following N addition increases the presence of phenolic compounds that complex with proteins in the soil matrix (Gramss et al., 1999; Hättenschwiler and Vitousek, 2000). Similarly, other studies have observed an increase in peptidase activity following N fertilization (Michel and Matzner, 2003; Stursova et al., 2006). Measured increases in proteolytic activity, concurrent with a decrease in phenol oxidase and peroxidase activity, suggests current conceptual models of polyphenol-protein complexes and their roles in terrestrial ecosystem nutrient cycling need to be refined or revised (Hättenschwiler and Vitousek, 2000).

It is possible that high inorganic N availability stimulates protein production or biomass turnover in the soil microbial community and thus explains the observed relationship between lignolytic and proteolytic enzymes. Soil microbial biomass in N-amended plots was observed to be 27–61% and 42–69% lower than control plots in hardwood and pine stands (Frey et al., 2004). It is also possible that the phenolic by-products of phenolic oxidation may themselves complex with proteins and inhibit proteolytic activity during times of increased phenol oxidase and peroxidase activity. Lignin is decomposed by a non-specific, radical-based oxidation mechanism (Kirk and Farrell, 1987). When the by-products of phenolic oxidation are released, which are phenolic subunits, they may form covalent or hydrogen bonds to proteins, forming polyphenol-protein complexes, which can inhibit further decomposition (Hättenschwiler and Vitousek, 2000). Further work is necessary to understand the relationships among these biochemical processes.

Protease activity also exhibited temporal variation independent of N addition as evidenced by changes over time in the control plots. This may reflect either interannual variation in protease activity or seasonal variation as soil samples taken prior to N fertilization were collected in May, while samples collected following two years of N addition were collected in September. The fact that proteolytic activity remained the same over time in N addition plots instead of decreasing as in controls, support the idea that N elevates proteolytic activity.

In conclusion, a continued increase in atmospheric N deposition may produce significant changes in communities of EM fungi and carbon and N cycles. The fact that lignolytic and proteolytic

enzymes exhibit opposite responses, however, is counterintuitive. Much is yet to be learned about how N addition affects global C storage by affecting the decomposition of organic matter.

Acknowledgements

References

Agerer, R., 1987–2002. Colour Atlas of Ectomycorrhizae. Einhorn-Verlag, Schwabisch Gmund, Germany.

Allison, S.D., 2006. Brown ground: a soil carbon analogue for the green world hypothesis. The American Naturalist 167, 619–627.

Avis, P.G., McLaughlin, D.J., Dentinger, B.C., Reich, P.B., 2003. Long-term increase in nitrogen supply alters above- and below-ground ectomycorrhizal communities and increases the dominance of *Russula* spp. in a temperate oak savannah. New Phytologist 160, 239–253.

Berthrong, S.T., Finzi, A.C., 2006. Amino acid cycling in three cold-temperate forests of the northeastern USA. Soil Biology & Biochemistry 38, 861–869.

Buee, M., Courty, P.E., Mignot, D., Garbaye, J., 2007. Soil niche effect on species diversity and catabolic activities in an ectomycorrhizal fungal community. Soil Biology and Biochemistry 39, 1947–1955.

Chalot, M., Brun, A., 1998. Physiology of organic nitrogen acquisition by ecto-mycorrhizal fungi and ecto-mycorrhizas. FEMS Microbiology Reviews 22, 21–44.

Chen, D.M., Bastias, B.A., Taylor, A.F.S., Cairney, J.W.G., 2003. Identification of laccase-like genes in ectomycorrhizal basidiomycetes and transcriptional regulation by nitrogen in *Piloderma byssinum*. New Phytologist 157, 547–554.

Clarke, K.R., 1993. Non-parametric multivariate analyses of changes in community structure. Australian Journal of Ecology 18, 117–143.

Courty, P.E., Pritsch, K., Schloter, M., Hartmann, A., Garbaye, J., 2005. Activity profiling of ectomycorrhiza communities in two forest soils using multiple enzymatic tests. New Phytologist 167, 309–319.

Courty, P.-E., Pouysefur, R., Buee, M., Garbaye, J., 2006. Laccase and phosphatase activities of the dominant ectomycorrhizal types in a lowland oak forest. Soil Biology & Biochemistry 38, 1219–1222.

DeForest, J.L., Zak, D.R., Pregitzer, K.S., Burton, A.J., 2004. Atmospheric nitrate deposition and the microbial degradation of cellobiose and vanillin in a northern hardwood forest. Soil Biology & Biochemistry 36, 965–971.

Dighton, J., Tuininga, A.R., Gray, D.M., Huskins, R.E., Belton, T., 2004. Impacts of atmospheric deposition on New Jersey pine barrens forest soils and communities of ectomycorrhizae. Forest Ecology and Management 201, 131–144.

Dix, N.J., Webster, J., 1995. Fungal Ecology. Chapman and Hall, London, UK, 549 pp.

Fog, K., 1988. The effect of added nitrogen on the rate of decomposition of organic matter. Biological Reviews 63, 433–462.

Frey, S.D., Knorr, M., Parrent, J.L., Simpson, R.T., 2004. Chronic nitrogen enrichment affects the structure and function of the soil microbial community in temperate hardwood and pine forests. Forest Ecology and Management 196, 159–171.

Galloway, J.N., Levy II, H., Kasibhatala, P.S., 1994. Year 2020: consequences of population growth and development on deposition of oxidized nitrogen. Ambio 23, 120–123.

Gramss, G., Ziegenhagen, D., Sorge, S., 1999. Degradation of soil humic extract by wood- and soil-associated fungi, bacteria, and commercial enzymes. Microbial Ecology 37, 140–151.

Hammel, K.E., 1997. Fungal degradation of lignin. In: Cadish, G., Giller, K.E. (Eds.), Driven by Nature: Plant Litter Quality and Decomposition. CAB International, Wallingford, England, pp. 33–45.

Hättenschwiler, S., Vitousek, P.M., 2000. The role of polyphenols in terrestrial ecosystem nutrient cycling. Trends in Ecology & Evolution 15, 238–243.

Kåren, O., Nylund, J.-E., 1997. Effects of ammonium sulphate on the community structure and biomass of EMF in a Norway spruce stand in southwestern Sweden. Canadian Journal of Botany 75, 1628–1642.

Kirk, T.K., Farrell, R.L., 1987. Enzymatic 'combustion': the microbial degradation of lignin. Annual Review of Microbiology 41, 465–505.

Lilleskov, E.A., Fahey, T.J., Horton, T.R., Lovett, G.M., 2002. Belowground ectomycorrhizal fungal community change over a nitrogen deposition gradient in Alaska. Ecology 83, 104–115.

Lipson, D.A., Monson, R.K., 1998. Plant-microbe competition for soil amino acids in the alpine tundra: effects of freeze-thaw and dry-rewet events. Oecologia 113, 406–414.

Lipson, D.A., Schmidt, S.K., Monson, R.K., 1999. Links between microbial population dynamics and nitrogen availability in an alpine ecosystem. Ecology 80, 1623–1631.

Lucas, R.W., 2007. Peeking into the Black Box: The Structure and Function of Soil Microbial Communities in Response to Increasing Nitrogen Availability.

Dissertation. Department of Biology. University of Pennsylvania, Philadelphia, PA. 157.

Mack, M.C., Schuur, E.A.G., Bret-Harte, M.S., Shaver, G.R., Chapin III, F.S., 2004. Ecosystem carbon storage in arctic tundra reduced by long-term nutrient fertilization. Nature 431, 440–443.

McCune, B., Grace, J.B., 2002. Analysis of Ecological Communities. MjM Software Design, Gleneden Beach, OR.

Mester, T., Tien, M., 2000. Oxidation mechanism of ligninolytic enzymes involved in the degradation of environmental pollutants. International Biodeterioration & Biodegradation 46, 51–59.

Michel, K., Matzner, E., 2003. Response of enzyme activities to nitrogen addition in forest floors of different C-to-N ratios. Biology and Fertility of Soils 38, 102–109.

NADP, 2004. National Atmospheric Deposition Program 2003 Annual Summary. In: NADP Data Report 2004-01. Illinois State Water Survey, Champaign, IL.

Neff, J.C., Townsend, A.R., Gleixner, G., Lehman, S.J., Turnbull, J., Bowman, W.D., 2002. Variable effects of nitrogen additions on the stability and turnover of soil carbon. Nature 419, 915–917.

Peter, M., Ayer, R., Egli, S., 2001. Nitrogen addition in a Norway spruce stand altered macromycete sporocarp production and below-ground ectomycorrhizal species composition. New Phytologist 149, 311–325.

Read, D.J., Perez-Moreno, J., 2003. Mycorrhizas and nutrient cycling in ecosystems – a journey towards relevance? New Phytologist 157, 475–492.

Saiya-Cork, K.R., Sinsabaugh, R.L., Zak, D.R., 2002. The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. Soil Biology and Biochemistry 34, 1309–1315.

SAS, 2003. SAS Software. SAS Institute, Inc., Cary, NC.

Schimel, J.P., Bennett, J., 2004. Nitrogen mineralization: challenges of a changing paradigm. Ecology 85, 591–602.

Sinsabaugh, R.L., Zak, D.R., Gallo, M., Lauber, C., Amonette, R., 2004. Nitrogen deposition and dissolved organic carbon production in northern temperate forests. Soil Biology & Biochemistry 36, 1509–1515.

Smith, S.E., Read, D.J., 2002. Mycorrhizal Symbiosis. Academic Press, San Diego, CA.

Stursova, M., Crenshaw, C.L., Sinsabaugh, R.L., 2006. Microbial responses to long-term N deposition in a semiarid grassland. Microbial Ecology 51, 90–98.

Taiz, L., Zeiger, E., 1991. Plant Physiology. Benjamin Cummings Publishing Company, Inc, Redwood City, CA.

Taylor, A.F.S., Martin, F., Read, D.J., 2000. Fungal diversity in ectomycorrhizal communities of Norway spruce [*Picea abies* (L.) Karst.] and beech (*Fagus sylvatica* L.) along north-south transects in Europe. In: Detlef Schulze, E.D. (Ed.), Carbon and Nitrogen Cycling in European Forest Ecosystems. Springer-Verlag, Heidelberg, Germany, pp. 343–365.

Treseder, K., Masiello, C.A., Lansing, J.L., Allen, M., 2004. Species-specific measurements of ectomycorrhizal turnover under N-fertilization: combining isotopic and genetic approaches. Oecologia 138, 419–425.

Waldrop, M.P., Zak, D.R., 2006. Response of oxidase enzyme activities to nitrogen deposition affects soil concentrations of dissolved organic carbon. Ecosystems 9, 921–933.

Wallenda, T., Kottke, I., 1998. Nitrogen deposition and ectomycorrhizas. New Phytologist 139, 169–187.

Watanabe, K., Hayano, K., 1995. Seasonal variation of soil protease activities and their relation to proteolytic bacteria and *Bacillus* spp in paddy field soil. Soil Biology & Biochemistry 27, 197–203.