Available online at www.sciencedirect.com
SCIENCE DIRECT®
Forest Ecology and Management 196 (2004) 159–171
Forest Ecology and Management
www.elsevier.com/locate/foreco

# Chronic nitrogen enrichment affects the structure and function of the soil microbial community in temperate hardwood and pine forests

Serita D. Frey[a,*], Melissa Knorr[a], Jeri L. Parrent[b], Rodney T. Simpson[a]

[a]*Department of Natural Resources, University of New Hampshire, Durham, NH 03824, USA*
[b]*Department of Biology, Duke University, Durham, NC 27708, USA*

**Abstract**

We examined how chronic nitrogen (N) enrichment of pine and hardwood forest stands has affected the relative abundance, functional capacity, and activity of soil bacteria and fungi. During Fall 2002 we collected one soil core (5.6 cm diameter; organic horizon plus 10 cm of mineral soil) from each of four 5 m × 5 m subplots within the control, low N (5 g N $m^{-2}$ per year), and high N (15 g N $m^{-2}$ per year) plots in both the hardwood and pine stands at the Chronic Nitrogen Amendment Study at Harvard Forest. The samples were analyzed for total and active bacterial and fungal biomass, microbial catabolic response profiles, the activities of cellulolytic and ligninolytic enzymes, and total, labile and microbially derived organic carbon (C). Live, fine roots were also collected from the control and low N pine plots and analyzed for ectomycorrhizal fungal community composition and diversity. Active fungal biomass was 27–61% and 42–69% lower in the fertilized compared to control plots in the hardwood and pine stands, respectively. Active bacterial biomass was not greatly affected by N additions, resulting in significantly lower fungal:bacterial biomass ratios in the N-treated plots. This shift in microbial community composition was accompanied by a significant reduction in the activity of phenol oxidase, a lignin-degrading enzyme produced by white-rot fungi. In the pine stand, ectomycorrhizal fungal community diversity was lower in the low N-treated plot than in the control plot. Differences in ectomycorrhizal community structure were also detected between control and fertilized pine plots, including a reduction in those species with the highest relative frequencies in the control community. Finally, N enrichment altered the pattern of microbial substrate use, with the relative response to the addition of carboxylic acids and carbohydrates being significantly lower in the N-treated plots, even after the data were normalized to account for differences in microbial biomass. These patterns are consistent with lower decomposition rates and altered N cycling observed previously at this site.


*Keywords:* Bacteria; Microbial enzyme activity; Ectomycorrhizal fungi; Microbial biomass; Nitrogen deposition; Saprotrophic fungi

## 1. Introduction

Global estimates indicate that inputs to the terrestrial N cycle have doubled in the past century due to anthropogenic activities, particularly fertilizer use and fossil fuel combustion (Vitousek et al., 1997). One consequence of this human-driven change in the N cycle is the significant increase in wet or dry N deposition in forest ecosystems. At the local and regional scale, N deposition near industrialized and agricultural areas can greatly exceed those in unpolluted areas of the world (Vitousek et al., 1997). Rates of N deposition are particularly high in the Midwestern and Northeastern US and in Europe.

* Corresponding author. Tel.: +1-603-862-3880; fax: +1-603-862-4976.
*E-mail address:* serita.frey@unh.edu (S.D. Frey).

0378-1127/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.foreco.2004.03.018

The soil biota in many terrestrial ecosystems evolved under low N conditions and thus one anticipated consequence of elevated N inputs is a change in microbial community structure and function as ecosystems move towards N saturation. Increases in N availability due to atmospheric deposition have been linked to reduced sporocarp production, root colonization, and species richness of mycorrhizal fungi (Arnolds, 1991; Dighton and Jansen, 1991; Egerton-Warburton and Allen, 2000; Lilleskov et al., 2002a,b). Microbial enzyme activities (Carreiro et al., 2000; Saiya-Cork et al., 2002; Michel and Matzner, 2003), litter decay (Magill and Aber, 1998; Hobbie, 2000), and N dynamics (Aber, 1992; Tietema, 1998; Gundersen et al., 1998) are also often altered, with the direction of response depending on site characteristics (e.g., litter quality, forest floor C:N ratio). For example, cellulase activity of decaying dogwood, maple, and oak litter was stimulated by N additions; whereas, lignin-degrading activity was significantly reduced, but only for high lignin-containing oak litter (Carreiro et al., 2000).

The Chronic Nitrogen Amendment Study at Harvard Forest was established to evaluate the impact of long-term N additions on C and N dynamics in a pine plantation and in a mixed hardwood forest. Nitrogen enrichment has resulted in decreased tree growth, increased foliar N concentrations, cation imbalances, reduced litter decomposition rates, and altered N cycling (Aber et al., 1993, 1998; Magill and Aber, 1998; Magill et al., this volume; McDowell et al., this volume). Little work has been done to date to determine how the microbial community has responded to increased N availability at this site or how shifts in the community may be linked to the observed changes in tree growth and nutrient dynamics. Our objective was to examine how N enrichment has affected bacterial and fungal biomass, microbial-derived organic C, ectomycorrhizal fungal (EMF) community composition and diversity, microbial functional capacity, and the activities of cellulolytic and ligninolytic enzymes.

## 2. Materials and methods

### 2.1. Sample collection

We collected soil samples from the Chronic Nitrogen Amendment Study at Harvard Forest in central Massachusetts in October 2002. These plots, established and maintained by the Forest Ecosystems Study Group at the University of New Hampshire, were established in 1988 in two adjacent forest stands to examine the effects of N additions on the litter decay process (Magill and Aber, 1998; Magill et al., this volume). One stand is dominated by mature, even-aged red pine (*Pinus resinosa*), the other by black (*Quercus velutina*) and red (*Q. rubra*) oak mixed with black birch (*Betula lenta*), red maple (*Acer rubrum*) and American beech (*Fagus grandifolia*). Three plots, measuring 30 m × 30 m and divided into thirty-six 5 m × 5 m subplots, were established in each stand and have been treated annually with 0, 5, or 15 g N $m^{-2}$ as $NH_4NO_3$. A fourth plot in each stand has been treated with low N (5 g N $m^{-2}$) plus sulfur as $Na_2SO_4$. There is one 30 m × 30 m plot per treatment in each stand for a total of eight plots. We collected O-horizon material and 10 cm of mineral soil, using a 5.6 cm diameter corer, from each of four 5 m × 5 m subplots within the control, low N, and high N plots in both the hardwood and pine stands. We did not collect samples from the nitrogen plus sulfur plot for this study. Samples were sieved (2 mm), stored field moist at 4 °C, and analyzed within 2 weeks of collection.

For EMF community analysis we collected live, fine roots from O-horizon soils in July 2002 from the control and low N pine plots. Samples were not collected from the high N pine plot due to the high degree of host tree mortality in this plot; hardwood plots were not sampled because of time and financial constraints. The spatially heterogeneous distribution of individuals in EMF communities requires that many spatially separated samples be collected. In order to maximize plot coverage while minimizing sampling impact we did not use a core sampler to collect roots; instead, live, fine roots, each 10 cm in length, were located by hand and removed with forceps from the O-horizon soil. Two root samples were taken approximately 0.5 m from—and at opposite sides of—the bole of every live *P. resinosa* individual, for a total of 150 root samples from the control pine plot, and 148 root samples from the low N pine plot. Samples were stored at 4 °C and processed within 1 week of collection. Two live root tips were randomly chosen from each root piece and stored in microcentrifuge tubes containing 55 mM CTAB buffer solution until DNA

extraction for molecular identification could be performed.

### 2.2. Microbial analyses

Total bacterial and fungal biomass was determined by direct epifluorescent microscopy and computer-assisted image analysis (Frey et al., 1999). Total physiologically active microbial biomass and the relative contributions of bacteria and fungi to the active biomass were measured by substrate-induced respiration (SIR) with selective inhibitors (Anderson and Domsch, 1975; Beare et al., 1991). The activities of the cellulolytic enzyme β-1,4-glucosidase and the ligninolytic enzyme phenol oxidase were determined according to Carreiro et al. (2000) to assess the functional response of the microbial community to changes in N availability. β-1,4-Glucosidase is an exocellulase that cleaves glucan units from the end of the cellulose polymer, while phenol oxidase is one of a suite of lignin-degrading enzymes. One gram of sieved soil was suspended in 60 ml of 50 mM acetate buffer, and the suspension was stirred constantly on a stir plate for 90 s. While stirring, 2 ml subsamples were pipetted from the slurry and mixed with 5 mM solutions of the substrates *p*-nitrophenyl-β-D-glucopyranoside and L-dihydroxyphenylalanine (L-DOPA) to assess β-1,4-glucosidase and phenol oxidase activity, respectively. The mixtures were incubated for 1 h at 25 °C, after which the absorbance was measured spectrophotometrically at 410 (for β-1,4-glucosidase) or 460 nm (for phenol oxidase). Enzyme activities are expressed as micromole substrate converted per gram soil organic C to account for differences among samples in organic matter quantity.

Catabolic response profiles (CRPs) were obtained by measuring the short-term respiration responses to 25 substrates following the method developed by Degens and Harris (1997). This approach provides a fingerprint of the functional potential of the microbial community. CRPs have been found to differ between species of microorganisms grown in the same soil (Degens, 1999) and for soils exposed to different environmental conditions (Degens, 1998; Degens et al., 2001). The substrates consisted of two carbohydrates (glucose, mannose), two amines (D-glucosamine, L-glutamine), six amino acids (L-arginine, L-asparagine, L-glutamic acid, L-histidine, L-lysine, L-serine), and 15 carboxylic acids (L-ascorbic acid, citric acid, fumaric acid, gluconic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, DL-malic acid, malonic acid, pantothenic acid, quinic acid, succinic acid, tartaric acid, uric acid, urocanic acid). Substrate solution concentrations were 75 mM for carbohydrates, 15 mM for amines and amino acids, and 100 mM for carboxylic acids. Two milliliter of each substrate were added to a 20 ml serum vial containing 1 g field moist, sieved soil. After a 30 min equilibration period, the vials were sealed, vortexed and incubated for 4 h at 25 °C. Evolved $CO_2$ was measured on 2 ml of headspace gas using a Licor LI-6252 infrared gas analyzer. The respiration response for each substrate was corrected for the amount of $CO_2$-C evolved from a control sample that received deionized water only. The corrected CRP data were used to calculate catabolic evenness, a measure of the variability in substrate use by the heterotrophic microbial community (Degens et al., 2001). Catabolic evenness $(E) = 1/\sum p_i^2$, where $p_i$ is summed for all substrates and $p_i = r_i/\sum r_i$ defined as the respiration response of each substrate ($r_i$) as a proportion of total respiration responses summed over all substrates $(\sum r_i)$. The maximum achievable evenness value is 25, the point at which all substrates are used equally. The lower the evenness value, the more variable (i.e., less even) is the respiratory response among the 25 substrates. We focused on evenness in our study because catabolic richness, the total number of substrates used, did not differ between soils. That is, all 25 substrates were used to some degree in every sample.

To accurately determine EMF species identity for each root sample, we used a DNA sequence based approach. DNA was extracted from each sample according to the protocol of Zolan and Pukkila (1986), and the internal transcribed spacers (ITS) and 5.8S ribosomal RNA gene were amplified by PCR and sequenced. For PCR amplification, the basidiomycete specific primer pair ITS 1F and ITS 4NA was used (Gardes and Bruns, 1993; Walker and Parrent, in press). However, the universal fungal primer pair, ITS 1F and ITS 4 (White et al., 1990), was used if the first reaction failed to amplify. PCR products were sequenced using the Big Dye kit and visualized on an ABI3700 automated sequencer (Applied Biosystems). Sequences were manually edited and queried against

both the Genbank database (NCBI) and a large in-house database of EMF ITS sequences (Duke mycology database, http://152.3.12.243/dfmo/). Sequences were classified as conspecific if they shared 98% or greater sequence similarity across the entire gene region. For sequences that could not be identified to species, they were recognized as congeneric if they shared 93–97% sequence similarity, though a more conservative designation was given in several instances where the phylogenetic classification of the group (e.g., genera in the Boletales) is known to be under revision.

### 2.3. Chemical analyses

The amino sugars *N*-acetylmuramic acid, *N*-acetylgalactosamine and *N*-acetylglucosamine were measured to quantify bacterial and fungal contributions to the production of microbially derived organic matter (Zhang and Amelung, 1996; Guggenberger et al., 1999; Simpson et al., in press). Muramic acid derives exclusively from bacterial cell walls, while glucosamine is present in fungal cell walls, bacterial cell walls, and microarthropod exoskeletons. Bacterial cell walls contain peptidoglycan, a molecule composed of alternating monomers of *N*-acetylglucosamine and *N*-acetylmuramic acid. We corrected the soil glucosamine concentrations for the glucosamine present in bacterial cell walls by assuming that the ratio of glucosamine to muramic acid in bacteria is 1:1 (Brock and Madigan, 1988). Thus the glucosamine concentrations reported here represent fungal-derived glucosamine plus an unknown contribution from microarthropods. The contribution of microarthropods to the total soil glucosamine concentration is likely minimal since microarthropod biomass is typically less than 0.5% of fungal biomass (Beare, 1997). We therefore assume that the glucosamine concentrations reported here derive primarily from fungi. The source of galactosamine is less well defined than for glucosamine and muramic acid, but available evidence indicates a predominantly bacterial origin (W. Amelung, pers. comm.). Amino sugar analysis followed the procedure of Zhang and Amelung (1996). Soil samples containing approximately 0.3 mg N were hydrolyzed with 6 M HCl. The hydrolysates were purified, freeze dried and the amino sugars transformed to aldononitrile derivatives before separation and quantification by gas liquid chromatography.

Total organic C and N were determined on finely ground subsamples by dry combustion on a Perkin-Elmer 2400 series CHN analyzer. Labile soil C was estimated by incubating 10 g of field moist, sieved soil with 20 ml deionized water in sealed 250 ml Erlenmeyer flasks. The soil slurries were incubated at 25 °C on a rotary shaker for 30 days. Evolved $CO_2$ was measured every 2–4 days using a Licor LI-6252 infrared gas analyzer, after which the flasks were flushed with compressed air and resealed.

### 2.4. Statistical analyses

Two-way analysis of variance was performed using rank analysis of variance (PROC RANK, PROC GLM, SAS Institute, 1999) to determine the effect of N treatment (control, low N, high N) and stand type (hardwood versus pine) on microbial community structure and function. A non-parametric procedure was selected because the data violated the assumptions of normality and homogeneity of variance for standard analysis of variance. The Ryan–Einot–Gabriel–Welsch multiple range test was used to determine significant differences among means at $P < 0.05$. Principle components analysis (PCA) was performed on the catabolic response profile data to determine if the CRP patterns differed among N treatments and forest stands. Prior to PCA, the CRP patterns were standardized by dividing by the average substrate response for each soil to reduce bias associated with differences in microbial biomass among soils (Degens, 1998).

Simpson's $D$ $\left(D = 1 - \sum P_i^2\right)$ and Shannon's $H'$ $\left(H' = -\sum P_i \ln P_i\right)$ diversity indices were calculated for EMF samples to evaluate the influence of N addition on EMF community diversity. To assess precision of the $D$ and $H'$ estimates calculated from the observed data, and whether there were statistically significant differences in diversity between unfertilized and fertilized (low N) pine plots, bootstrap confidence intervals (95%) were estimated and randomization tests were performed. To obtain the bootstrap distribution for each plot, 1000 bootstrap replicates were generated. For each replicate, samples were drawn at random with replacement from the observed community data to achieve a sample size

Table 1
Total and active microbial biomass, active fungal:bacterial biomass ratio (F:B) and catabolic evenness for soil collected from hardwood and pine stands at the Chronic Nitrogen Amendment Study at Harvard Forest[a]

| Stand/plot | Total microbial biomass | | Active microbial biomass | | F:B | Catabolic evenness[b] |
|---|---|---|---|---|---|---|
| | Fungi (μg C $g^{-1}$ soil) | Bacteria (μg C $g^{-1}$ soil) | Fungi (μg $CO_2$-C $g^{-1}$ soil) | Bacteria (μg C $g^{-1}$ soil) | | |
| Hardwood | | | | | | |
| Control | 501 (41) | 33 (4.0) | 7.5 (0.9) | 3.8 (0.4) | 2.1 (0.4) | 12.59 (1.1) |
| Low N | 365 (71) | 28 (2.8) | 5.5 (0.6) | 3.8 (0.3) | 1.5 (0.2) | 12.15 (0.5) |
| High N | 637 (53) | 34 (1.7) | 2.9 (0.6) | 3.2 (0.9) | 1.1 (1.6) | 8.80 (0.5) |
| Pine | | | | | | |
| Control | 407 (60) | 24 (0.1) | 6.2 (1.5) | 1.7 (0.3) | 3.7 (0.5) | 10.23 (0.9) |
| Low N | 406 (50) | 24 (1.3) | 3.6 (0.5) | 1.1 (0.3) | 4.1 (0.5) | 7.71 (0.9) |
| High N | 433 (48) | 36 (1.5) | 1.9 (0.9) | 1.1 (0.1) | 1.6 (0.8) | 6.85 (0.5) |

[a] Total fungal and bacterial biomass were determined by direct epifluorescent microscopy. Active microbial biomass was estimated by substrate-induced respiration with selective inhibitors. Values represent the mean of four replicates ± one standard error.

[b] Catabolic evenness $(E) = 1/\sum p_i^2$, where $p_i$ is summed for all substrates and $p_i = r_i/\sum r_i$ defined as the respiration response of each substrate ($r_i$) as a proportion of total respiration responses summed over all substrates ($\sum r_i$). The maximum achievable evenness value is 25, the point at which all substrates are used equally. The lower the evenness value, the more variable (i.e., less even) is the respiratory response among the 25 substrates.

equal to the number of actual samples generated for that plot; $D$ and $H'$ were then calculated. Confidence intervals (95%) were calculated from the 1000 bootstrap replicates for each plot and compared to the diversity indices calculated from the actual data. For randomization tests, samples were randomly assigned to either the control or low N plot, keeping the sample sizes the same as the actual sample size for each plot, $D$ and $H'$ were calculated for each plot, and the difference between control and low N diversity indices were determined. This was iterated for 1000 replicates, yielding a distribution of differences in diversity between plots. The 95% confidence intervals were then determined, and if the observed difference in diversity between plots was outside the 95% CI, it was considered statistically significant. Fisher's exact tests were calculated to detect significant differences in the relative frequencies of individual EMF species between plots.

## 3. Results and discussion

### 3.1. Microbial biomass and C pools

Total fungal and bacterial biomass ranged from 365 to 637 and 24–36 μg C $g^{-1}$ dry mass soil, respectively (Table 1). Differences between N treatments ($P = 0.086$) or stands ($P = 0.173$) for total fungal biomass were not significant. Compton et al. (this volume) also reported no significant N treatment effect for fungal biomass as measured by direct microscopy. However, microbial biomass determined by chloroform fumigation was significantly reduced by N additions, particularly in the high N plots. Unlike total fungal biomass, we found active fungal biomass to be greatly affected by N addition, being 27–69% lower in the N-treated compared to control plots in both forest stands ($P < 0.001$). While fungal biomass was significantly reduced by low N addition in both stands, there was a greater effect in the pine plot (42% versus 27% reduction). Both stands showed a similar response (61–69% reduction) to the high N addition.

Total bacterial biomass did not differ between stands ($P = 0.098$), but was higher in the high N compared to low N and control plots ($P = 0.001$). Active bacterial biomass, however, was similar across the N gradient in both stands, resulting in approximately 50% lower fungal:bacterial biomass ratios in the N-treated plots ($P = 0.022$). Bossuyt et al. (2001) observed a similar response in a laboratory incubation where fungal:bacterial biomass ratios were approximately 3.0 and 0.3 for soil amended with low quality (C:N = 108) and high quality (C:N = 20) plant litter,

respectively. When mineral N was added to the soil containing low quality material, fungal biomass was significantly reduced while bacterial biomass was not. Fungal:bacterial ratios have also been shown to decrease in N-fertilized pastures (Bardgett et al., 1996, 1999; Bardgett and McAlister, 1999). Thus, fungi appear more sensitive than bacteria to soil N enrichment.

The amino sugars glucosamine, galactosamine, and muramic acid are components of microbial cell walls, and have been used to assess the microbial contribution to soil organic matter accumulation and turnover in soils (Zhang and Amelung, 1996; Chantigny et al., 1997; Simpson et al., in press). Total soil amino sugar concentrations are typically 1–2 orders of magnitude higher than those estimated to be present in intact microbial cells, because this organic matter pool includes both cell wall components of living microbes as well as decomposing microbial residues (Guggenberger et al., 1999). The size of the amino sugar C pool is the balance between the production of new microbial cells and the decomposition rate of dead microbial material, the latter being dependent on both the chemical composition of the compounds and their degree of physical and/or chemical protection within the soil matrix. At Harvard Forest, fungal-derived glucosamine increased 13–19% in the high N plots, following an increase, though not statistically significant, in total soil C (Table 2). Glomalin, a glycoprotein thought to be produced by arbuscular mycorrhiza, was also observed to increase with N availability in these plots (Robinson, 2002). Glomalin concentrations were 95–125 g C $m^{-2}$ in the control plot compared to 140–177 and 176–226 g C $m^{-2}$ in the low and high N plots, respectively. Any decline in the production of fungal material as a result of reduced fungal activity is apparently being balanced by slower decomposition rates, allowing these compounds to remain in the soil and possibly even accumulate over time. Simpson et al. (in press) observed that amino sugars, especially fungal-derived glucosamine, accumulate preferentially in soil microaggregates (53–250 μm) contained within macroaggregates (>250 μm). Although we observed that Harvard Forest soil is fairly coarse-textured with little macroaggregation, we hypothesize that fungal products are being sequestered in microaggregate-sized structures.

### 3.2. Ectomycorrhizal fungal community diversity and composition

Increased N availability through deposition or fertilization has been shown to influence EMF communities in a variety of systems. Two general conclusions can be drawn from previous studies. First, N addition reduces the number, species richness and composition of EMF fruiting bodies (Wallenda and Kottke, 1998 and references therein; Lilleskov et al., 2001). Second, while total belowground species richness in the EMF community may either show no change (Peter et al., 2001) or a decline (Kårén and Nylund, 1997; Lilleskov et al., 2002a), some individual species typically

Table 2
Carbon pools isolated from soil collected from hardwood and pine stands at the Chronic Nitrogen Amendment Study at Harvard Forest[a]

| Stand/plot | Total soil C (μg C $g^{-1}$ soil) | Labile C (μg $CO_2$-C $g^{-1}$ soil) | Amino sugar C[b] | |
|---|---|---|---|---|
| | | | Fungal (μg C $g^{-1}$ soil) | Bacterial (μg C $g^{-1}$ soil) |
| Hardwood | | | | |
| Control | 6864 (611) | 147 (6) | 63.4 (5.2) | 27.0 (2.5) |
| Low N | 6509 (228) | 102 (11) | 63.4 (4.6) | 28.6 (3.2) |
| High N | 7208 (448) | 117 (10) | 71.4 (3.8) | 32.0 (3.9) |
| Pine | | | | |
| Control | 6283 (408) | 102 (5) | 56.0 (8.4) | 29.3 (2.2) |
| Low N | 7861 (443) | 96 (14) | 92.9 (5.6) | 31.0 (2.0) |
| High N | 8088 (338) | 107 (5) | 66.9 (3.0) | 32.4 (4.2) |

[a] Values represent the mean of four replicates ± one standard error.

[b] Fungal-derived amino sugar C = (total glucosamine − bacterial-derived glucosamine (equal to muramic acid concentration)) × 0.4022; bacterial-derived amino sugar C = (total muramic acid × 0.4303) + (total galactosamine × 0.4202) + (bacterial-derived glucosamine × 0.4022).

decline in relative frequency on root tips, while others increase.

We sampled root tips from the control and low N pine plots to assess the effects of N addition on belowground EMF community diversity and composition. DNA was extracted from 150 root tips in the control pine plot and 148 root tips from the low N pine plot. Of these 298 samples, we were able to successfully sequence and analyze 152 samples, 119 from the control stand and 33 from the low N stand. From these samples we detected 19 EMF species; 19 species were found in the control stand and 10 species in the low N stand. Simpon's ($D$) and Shannon's ($H'$) community diversity indices, which consider both richness and evenness in their assessment of diversity, were lower in the low N than in the control plot. Bootstrap distributions for each plot were centered on the observed value, and the 95% confidence intervals for the two plots were non-overlapping, demonstrating that EMF community diversity was significantly decreased with N fertilization (Table 3). Randomization test results found the difference in diversity to be statistically significant (observed, $D$ low N $-$ $D$ control $= -0.14$; 95% CI for 1000 randomization test replicates $= -0.08 - 0.3$).

Despite the equal sampling effort for both control and low N pine plots, a disproportionately low number of samples from the fertilized plot were successfully analyzed. Samples had to be omitted from our analysis due to either: (1) unsuccessful PCR amplification (the vast majority of the cases), or (2) sequences were identified as soil contaminants or root pathogens rather than EMF. For samples that did not amplify, PCR reactions were repeated with the same primers using different DNA concentrations, and additional PCR reactions were performed using the more general fungal ITS primers, ITS 1F and IT S4. Despite the additional effort, PCR amplification was still unsuccessful for these samples. Microscopic examination of cross-sections from colonized root tips revealed a much thinner mantle structure on root tips from the fertilized plot than those in the control plot. The much lower amplification and sequencing success from the low N pine plot compared to the control pine plot, 22.3 and 79.3%, respectively, in conjunction with the observation of the thinner mantle structure on EMF in low N plots, suggests a decrease in EMF biomass with N addition, and likely a decrease in EMF root tip colonization as well.

EMF community composition was also influenced by N fertilization. Shifts in the relative frequencies of several EMF community members were significantly correlated with N fertilization, including those species with the highest relative frequencies in the control community (Fig. 1). For example, *Lactarius theiogalus* was the dominant EMF species in the control plot, colonizing 21% of the root tips sampled, but was completely absent in the low N plot ($P = 0.003$, Fisher's exact test). Conversely, the relative frequency of the dominant species in the N amended plot, *Piloderma* sp. 2, decreased from 33.7% to less than 12% in the control plot ($P = 0.001$).

This individualistic response of different EMF species to N fertilization is consistent with the findings of other studies (Fransson et al., 2001; Peter et al., 2001; Lilleskov et al., 2002a). One notable difference between the findings of this study and that of Lilleskov et al. (2002a) is the response of *L. theiogalus* to N addition. In contrast to the decline of *L. theiogalus* with N addition found at Harvard Forest, samples collected from root tips of white spruce hosts in Alaska (*Picea glauca*) showed increases in *L. theiogalus* from 10 to >50% relative frequency with increased N availability (Lilleskov et al., 2002a). This variation in response of the same EMF species to N addition in different systems may be attributable to

Table 3
Ectomycorrhizal fungal community diversity for control and low N pine stands

| | Samples | Richness | Simpson's | | Shannon's | |
|---|---|---|---|---|---|---|
| | | | $D$ | 95% bootstrap CI | $H'$ | 95% bootstrap CI |
| Control | 119 | 19 | 0.90 | 0.86–0.91 | 2.51 | 2.26–2.57 |
| Low N | 33 | 10 | 0.75 | 0.57–.83 | 1.71 | 1.25–1.98 |

Confidence intervals (CI) for Simpson's and Shannon's diversity indices were generated from 1000 bootstrap replicates for each plot. Richness is the total number of observed species per plot.

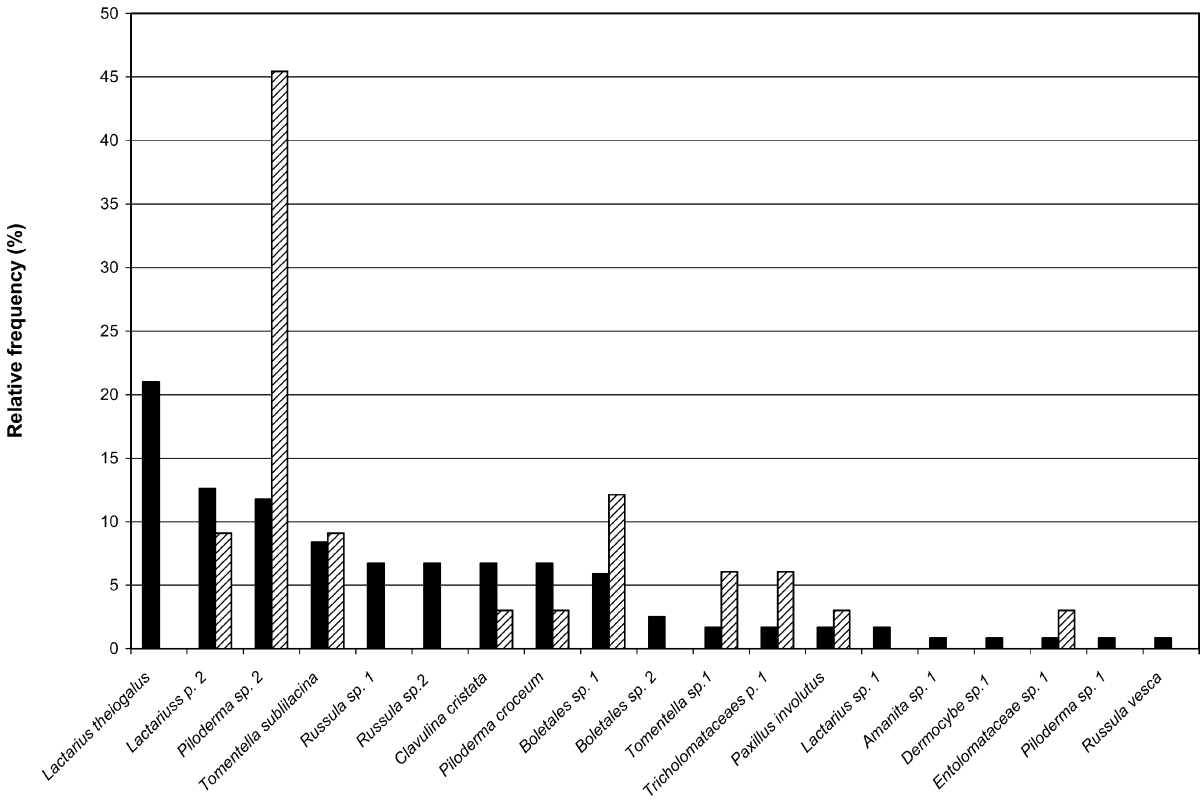


Fig. 1. Ectomycorrhizal fungal community structure in the pine stand. Solid bars are relative frequencies of EMF species in the control plot; hatched bars are relative frequencies of EMF species in the low N plot.

different abiotic factors in the Harvard forest plots than that of the high N Alaska plots; alternatively, since these ecosystems differ in tree species, biotic interactions between *L. theiogalus* and its host, or competitive interactions between this and other EMF species that differ in their response to N fertilization, may instead be important in determining the response of this species to elevated N conditions. Additional studies examining the ecological mechanism(s) responsible for EMF community shifts in response to N addition are needed.

### 3.3. Microbial community function

Magill and Aber (1998) observed that decomposition rates of red pine, red maple, yellow birch, and black oak litters declined 20–50% in the N-treated plots at Harvard Forest, accompanied by a significant increase in lignin accumulation. This response was particularly pronounced during the later stages of decay in the hardwood stand where overall N availability is lower despite the same N fertilization rates. In the present study, we assessed the effects of N addition on the functional capacity of the decomposer community by measuring enzyme activities and the respiratory response of soil to the addition of a suite of 25 organic substrates.

The activity of β-glucosidase, a cellulose-degrading enzyme, was significantly higher in the high N compared to low N and control plots in the hardwood stand ($P = 0.014$; Fig. 2). Conversely, phenol oxidase activity, an indicator of lignin-degrading potential, was significantly reduced by 28–48% in the N treatments of both stands. Microbial extracellular enzyme activity has been correlated with litter mass loss rates (Sinsabaugh and Linkins, 1993) and shifts in enzyme activities have been observed in response to simulated N deposition at other sites (Carreiro et al., 2000; Saiya-Cork et al., 2002; Sinsabaugh et al., 2002). β-Glucosidase activity was significantly stimulated by

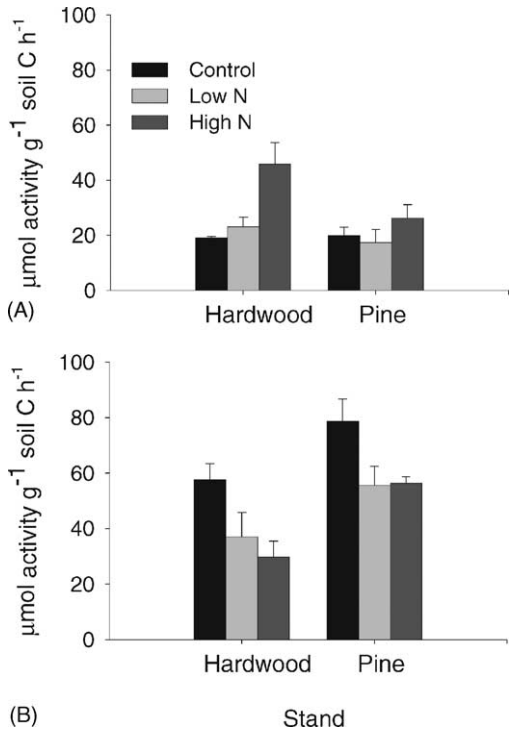


Fig. 2. Activity of the cellulolytic enzyme, β-1,4-glucosidase (A), and the lignin-degrading enzyme, phenol oxidase (B), for soil collected from control, low N, and high N plots in the hardwood and pine stands at the Chronic Nitrogen Amendment Study at Harvard Forest. Each bar represents the mean of four replicates and the error bars are one standard error.

added N during decay of dogwood, maple, and oak leaf litters in a mixed deciduous forest in New York (Carreiro et al., 2000), and in both litter and soil collected from a Michigan hardwood forest stand (Saiya-Cork et al., 2002). Phenol oxidase activity was suppressed on decomposing oak litter (Carreiro et al., 2000; Sinsabaugh et al., 2002) and forest soil (Saiya-Cork et al., 2002) in N-fertilized plots. Carreiro et al. (2000) observed that these differential enzyme activities corresponded to increased litter mass loss for dogwood, decreased decay for oak, and no change in decomposition of maple litter in response to N additions. Our enzyme results at Harvard Forest correspond to the lower litter decay rates and higher lignin contents noted above for this site (Magill and Aber, 1998) and suggest that suppression of lignin degradation outweighs any stimulation of cellulose-degrading enzymes following N enrichment.

The overall respiratory response following addition to soil of 25 organic substrates was significantly affected by N treatment and stand ($P < 0.001$). The microbial communities in the fertilized plots exhibited a reduced response in comparison to the control for a majority of the substrates (Fig. 3); this could be attributed largely to lower microbial biomass with N addition. There was a significant correlation between active microbial biomass and respiration for all but six substrates (data not shown). To normalize the data for differences in microbial biomass, the response for each substrate was divided by the average response for each stand–treatment–replicate combination (Degens, 1998). Normalized patterns of substrate use differed significantly across stands and treatments, indicating shifts in the potential enzyme spectrum of the microbial community, which could reflect changes in microbial community structure and/or N-induced shifts in enzyme production (Figs. 4 and 5). In the hardwood stand, the high N treatment showed a different utilization pattern than the low N and control soils, due primarily to differential use of carbohydrates and carboxylic acids. Overall, hardwood high N soils had significantly lower relative utilization of carbohydrates and carboxylic acids compared to the control ($P < 0.001$), with the exception that citric acid and malonic acid (substrates 18 and 20 in Fig. 5) were utilized to a significantly greater degree. The high N-treated plot had a low carbohydrate utilization response despite the fact that β-glucosidase activity was high in these same soils (Fig. 2). Labile C was also 20–30% lower in the N-treated plots than in the control plot (Table 2). These results suggest that labile C may be limiting in these soils and that microbes are allocating relatively more resources towards its acquisition under the high N treatment.

In the pine stand, both N addition plots had different substrate use patterns than the control. Carbohydrates and carboxylic acids, as for the hardwood stand, showed a reduced respiratory response relative to the control ($P < 0.001$). Again citric and malonic acids showed the opposite trend. Relative use of the amines and amino acids was not significantly different across treatments in either stand, supporting the results of Compton et al. (this volume) showing no significant effect of N additions on utilization of all but 2 of 95 N-containing substrates. We found catabolic evenness,

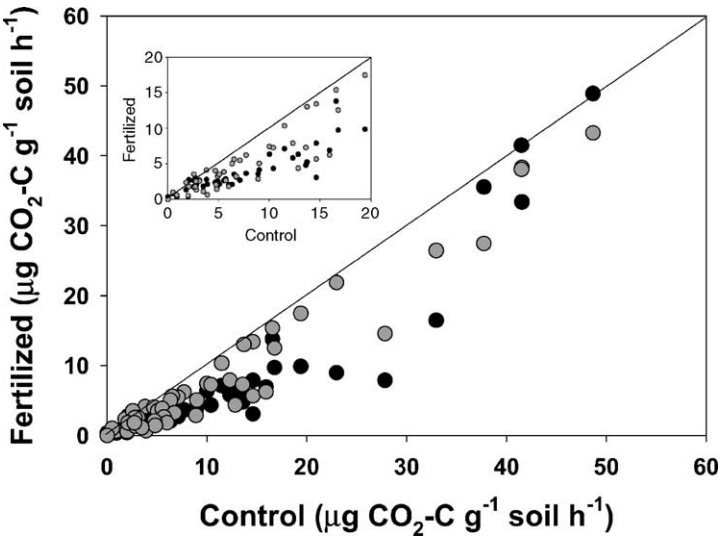


Fig. 3. Relationship between the microbial respiratory response (μg $CO_2$-C $g^{-1}$ soil $h^{-1}$) following substrate addition to soil collected from control compared to fertilized plots. Each point represents the average of three replicates for one of 25 organic substrates. Values falling on the 1:1 line indicate a similar respiratory response for control versus fertilized plots, whereas, points above or below this line indicate an increase or decrease in respiration, respectively. Gray and black circles represent samples collected from low and high N addition plots, respectively. Inset: Those substrates that elicited a response below 20 μg $CO_2$-C $g^{-1}$ soil $h^{-1}$.

a measure of variability in substrate use (Degens et al., 2000), to be significantly lower in the hardwood high N plot and both pine N addition plots (Table 1). Taken as a whole, the catabolic response profile data indicate that overall substrate use was more variable and that there was a differential utilization pattern and rate of use of specific substrates in N-fertilized soils. Catabolic response profiles differ between microbial

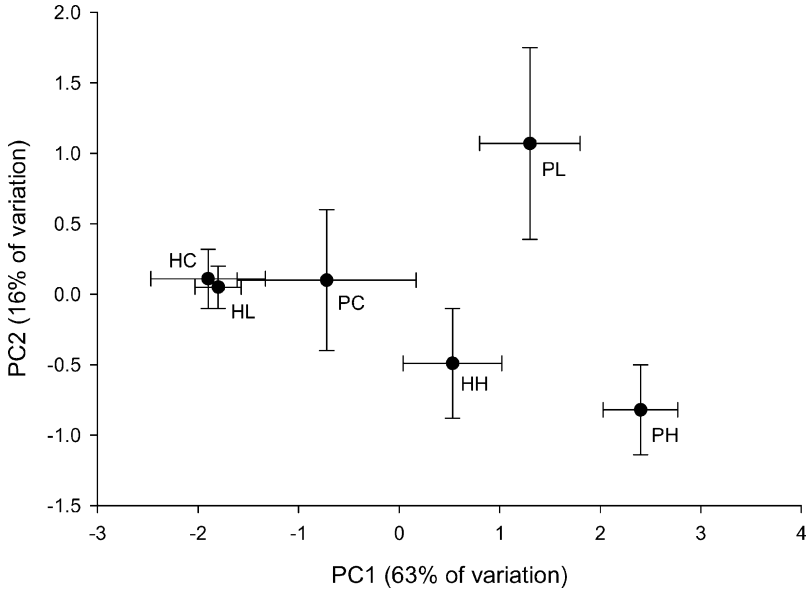


Fig. 4. Principal components analysis of the catabolic response profiles for soil collected from control (C), low N (L), and high N (H) plots in the hardwood (H) and pine (P) stands.

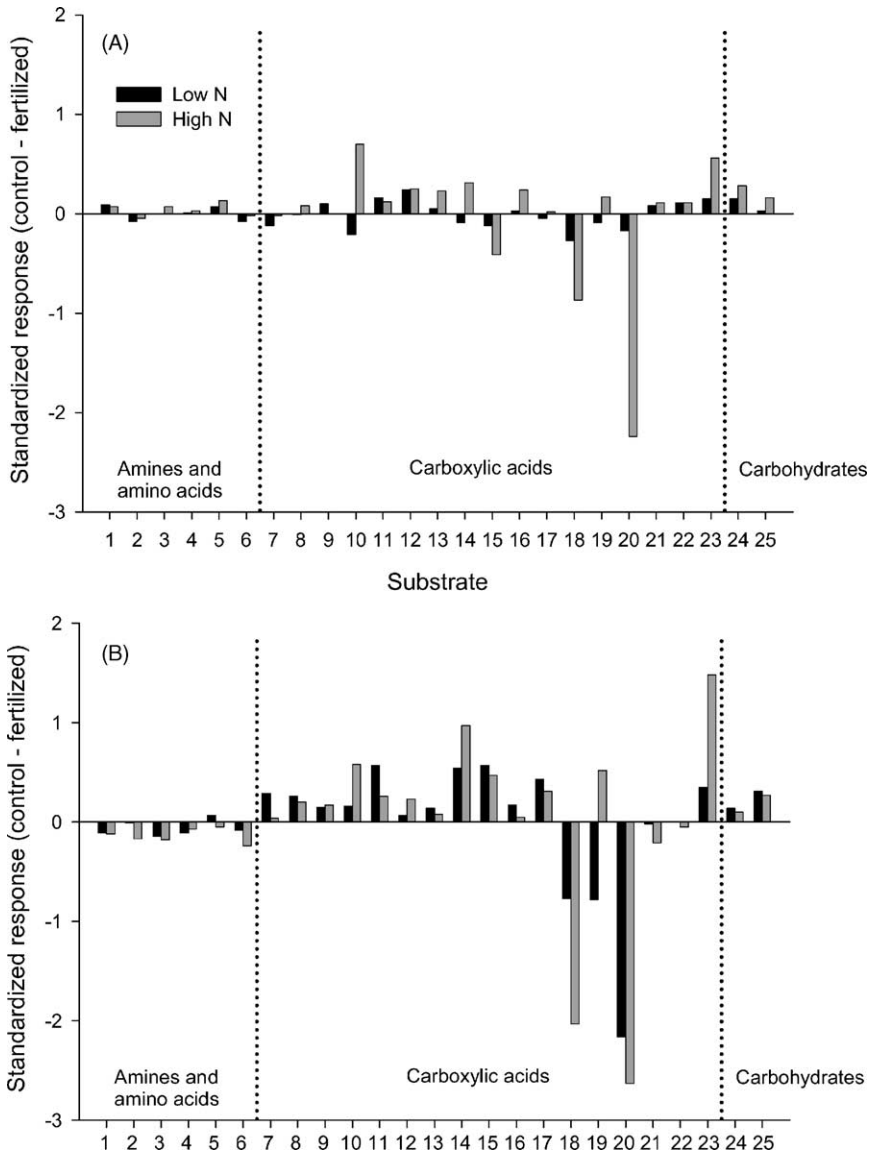


Fig. 5. Relative respiratory response (control minus fertilized) for soil collected from the hardwood (A) and pine (B) stands. Soil samples were incubated for 4 h following addition of 25 different organic substrates, bars from left to right: (1) L-lysine, (2) L-histidine, (3) serine, (4) D-(+)-glucosamine, (5) lysine, (6) L-asparagine, (7) L-glutamine, (8) L-glutamic acid; 15 carboxylic acids: (9) L-gluconic acid, (10) α-ketobutyric acid, (11) malic acid, (12) α-ketoglutaric acid, (13) uric acid, (14) fumaric acid, (15) succinic acid, (16) D-pantothenic acid, (17) quinic acid, (18) citric acid, (19) L-ascorbic acid, (20) malonic acid, (21) L-tartaric acid, (22) urocanic acid, (23) α-ketovaleric acid; and two carbohydrates: (24) D-glucose, (25) D-mannose.

species grown in the same soil, but also differ substantially for the same species grown in different soils (Degens, 1999). Microbial community composition and soil conditions thus interact to determine substrate use patterns, making it difficult to discern whether changes in catabolic response profiles are indicative of a change in species composition or a change in microbial function in response to altered soil conditions. In any case, a reduced range and rate of substrate use in the N amended soils correlated with

lower relative fungal biomass and phenol oxidase activity.

In summary, long-term N additions have impacted microbial community composition and function by decreasing active fungal biomass, fungal:bacterial biomass ratios, ectomycorrhizal fungal species diversity, lignin-degrading activity, and catabolic evenness. Mycorrhizal community composition and overall patterns of substrate use have also been altered. Concomitant with these changes has been a trend toward increasing accumulation of fungal-derived glucosamine and glomalin. Most studies examining the effects of N deposition on the microbial community have focused on mycorrhizal fungi (e.g., Arnolds, 1991; Dighton and Jansen, 1991; Egerton-Warburton and Allen, 2000; Treseder and Allen, 2000; Lilleskov et al., 2002a,b). Our results, together with several recent studies (Carreiro et al., 2000; Saiya-Cork et al., 2002; Sinsabaugh et al., 2002), indicate that saprotrophic fungi may be as susceptible to N deposition as are mycorrhizal fungi.

## Acknowledgements

We thank John Aber and Alison Magill for access to the Chronic Nitrogen Amendment Plots at Harvard Forest. Rachel Thiet, Kirsten Lloyd, Erica Cate, Tiffany Black, Lindsay Scott, and Mary Baillargeon assisted with sample collection and analysis. William Morris assisted with the statistical analysis of the EMF community data. John Aber, Bill McDowell, Margaret Carreiro, and Erik Hobbie provided helpful comments that improved the quality of the manuscript. This work was funded by grants from the National Science Foundation (DEB-0224917; MCB-0084207), and the University of New Hampshire Agricultural Experiment Station (MS-48). This is scientific contribution number 2220 from the New Hampshire Agricultural Experiment Station.

## References

Aber, J.D., 1992. Nitrogen cycling and nitrogen saturation in temperate forest ecosystems. Trends Ecol. Evol. 7, 220–223.

Aber, J.D., Magill, A., Boone, R., Melillo, J.M., Steudler, P., Bowden, R., 1993. Plant and soil responses to chronic nitrogen additions at the Harvard Forest. Massachusetts Ecol. Appl. 3, 156–166.

Aber, J., McDowell, W., Nadelhoffer, K., Magill, A., Berntson, G., Kamakea, M., McNulty, S., Currie, W., Rustad, L., Fernandez, I., 1998. Nitrogen saturation in temperate forest ecosystems. BioScience 48 (11), 921–934.

Anderson, J.P.E., Domsch, K.H., 1975. Measurement of bacterial and fungal contributions to respiration of selected agricultural and forest soils. Can. J. Microbiol. 21, 314–322.

Arnolds, E., 1991. Decline of ectomycorrhizal fungi in Europe. Agric. Ecosyst. Environ. 35, 209–244.

Bardgett, R.D., Hobbs, P.J., Frostegard, A., 1996. Changes in soil fungal:bacterial ratios following reductions in the intensity of management of an upland grassland. Biol. Fert. Soils 22, 261–264.

Bardgett, R.D., Lovell, R.D., Hobbs, P.J., Jarvis, S.C., 1999. Seasonal changes in soil microbial communities along a fertility gradient of temperate grasslands. Soil Biol. Biochem. 31, 1021–1030.

Bardgett, R.D., McAlister, E., 1999. The measurement of soil fungal:bacterial ratios as an indicator of ecosystem self-regulation in temperate meadow grasslands. Biol. Fert. Soils 29, 282–290.

Beare, M.H., 1997. Fungal and bacterial pathways of organic matter decomposition and nitrogen mineralization in arable soil. In: Brussaard, L., Ferrera-Cerrato, R. (Eds.), Soil Ecology in Sustainable Agricultural Systems. Lewis Publications, CRC Press, Boca Raton, FL, pp. 37–70.

Beare, M.H., Neely, C.L., Coleman, D.C., Hargrove, W.L., 1991. Characterization of a substrate-induced respiration method for measuring fungal, bacterial and total microbial biomass on plant residues. Agric. Ecosyst. Environ. 34, 65–73.

Bossuyt, H., Denef, K., Six, J., Frey, S.D., Merckx, R., Paustian, K., 2001. Influence of microbial populations and residue quality on aggregate stability. Appl. Soil Ecol. 16, 195–208.

Brock, T.D., Madigan, M.T., 1988. Biology of Microorganisms, 5th ed. Prentice-Hall, Englewood Cliffs, NJ.

Carreiro, M.M., Sinsabaugh, R.L., Repert, D.A., Parkhurst, D.F., 2000. Microbial enzyme shifts explain litter decay responses to simulated nitrogen deposition. Ecology 81, 2359–2365.

Chantigny, M.H., Angers, D.A., Prevost, D., Vezina, L., Chalifour, F., 1997. Soil aggregation and fungal and bacterial biomass under annual and perennial cropping systems. Soil Sci. Soc. Am. J. 61, 262–267.

Degens, B.P., 1998. Decreases in microbial functional diversity do not result in corresponding changes in decomposition under different moisture conditions. Soil Biol. Biochem. 30, 1989–2000.

Degens, B.P., 1999. Catabolic response profiles differ between microorganisms grown in soils. Soil Biol. Biochem. 31, 475–477.

Degens, B.P., Harris, J.A., 1997. Development of a physiological approach to measuring the catabolic diversity of soil microbial communities. Soil Biol. Biochem. 29, 1309–1320.

Degens, B.P., Schipper, L.A., Sparling, G.P., Duncan, L.C., 2001. Is the microbial community in a soil with reduced catabolic

diversity less resistant to stress or disturbance? Soil Biol. Biochem. 33, 1143–1153.

Dighton, J., Jansen, A., 1991. Atmospheric pollutants and ectomycorrhizas: more questions than answers. Environ. Poll. 73, 179–204.

Egerton-Warburton, L.M., Allen, E.B., 2000. Shifts in arbuscular mycorrhizal communities along an anthropogenic nitrogen deposition gradient. Ecol. Monogr. 10, 484–496.

Fransson, P.M.A., Taylor, A.F.S., Finlay, R.D., 2001. Elevated atmospheric $CO_2$ alters root symbiont community structure in forest trees. New Phytol. 152, 431–442.

Frey, S.D., Elliott, E.T., Paustian, K., 1999. Bacterial and fungal abundance and biomass in conventional and no-tillage agroecosystems along two climatic gradients. Soil Biol. Biochem. 31, 573–585.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specificity for basidiomycetes: application to the identification of mycorrhizae and rusts. Molec. Ecol. 2, 113–118.

Guggenberger, G., Frey, S.D., Six, J., Paustian, K., Elliott, E.T., 1999. Bacterial and fungal cell-wall residues in conventional and no-tillage agroecosystems. Soil Sci. Soc. Am. J. 63, 1188–1198.

Gundersen, P., Emmet, B.A., Kjonaas, O.J., Koopmans, C.J., Tietema, A., 1998. Impact of nitrogen deposition on nitrogen cycling in forests: a synthesis of NITREX data. For. Ecol. Manage. 101, 37–56.

Hobbie, S.E., 2000. Interactions between litter lignin and soil nitrogen availability during leaf litter decomposition in a Hawaiian montane forest. Ecosystem 3, 484–494.

Kårén, O., Nylund, J.E., 1997. Effects of ammonium sulphate on the community structure and biomass of ectomycorrhizal fungi in a Norway spruce stand in southwestern. Sweden Can. J. Bot. 75, 1628–1642.

Lilleskov, E.A., Fahey, T.J., Lovett, G.M., 2001. Ectomycorrhizal fungal aboveground community change over an atmospheric nitrogen deposition gradient. Ecol. Appl. 11, 397–410.

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

Lilleskov, E.A., Hobbie, E.A., Fahey, T.J., 2002b. Ectomycorrhizal fungal taxa differing in response to nitrogen deposition also differ in pure culture organic nitrogen use and natural abundance of nitrogen isotopes. New Phytol. 154, 219–231.

Magill, A.H., Aber, J.D., 1998. Long-term effects of experimental nitrogen additions on foliar litter decay and humus formation in forest ecosystems. Plant Soil 203, 301–311.

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

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

Robinson, D.P., 2002. Glomalin: a potential sink for nitrogen and possible contributor to dissolved organic carbon and nitrogen within the organic soils of the Harvard Forest. MS Thesis, University of New Hampshire, 62 pp.

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 Biol. Biochem. 34, 1309–1315.

Sinsabaugh, R.L., Carreiro, M.M., Repert, D.A., 2002. Allocation of extracellular enzymatic activity in relation to litter composition, N deposition, and mass loss. Biogeochemistry 60, 1–24.

Sinsabaugh, R.L., Linkins, A.E., 1993. Statistical modeling of litter decomposition from integrated cellulase activity. Ecology 74, 1594–1597.

Simpson, R.T., Frey, S.D., Six, J., Thiet, R.K., in press. Preferential stabilization of microbial-derived carbon in microaggregates contained within macroaggregates of no-tillage soils. Soil Sci. Soc. Am. J.

Tietema, A., 1998. Microbial carbon and nitrogen dynamics in coniferous forest floor material collected along a European nitrogen deposition gradient. For. Ecol. Manage. 101, 29–36.

Treseder, K.K., Allen, M.F., 2000. Mycorrhizal fungi have a potential role in soil carbon storage under elevated $CO_2$ and nitrogen deposition. New Phytol. 147, 189–200.

Vitousek, P.M., Aber, J.D., Howarth, R.W., Likens, G.E., Matson, P.A., Schindler, D.W., Schlesinger, W.H., Tilman, D.G., 1997. Human alteration of the global nitrogen cycle: sources and consequences. Ecol. Appl. 7, 737–750.

Walker, J.F., Parrent, J.L., in press. Molecular phylogenetic evidence for the mycorrhizal status of *Tremellodendron* (Sebacinaceae). Memoirs of the New York Botanical Garden.

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

White, T.J., Bruns, T.D., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, NY, USA, pp. 315–322.

Zhang, X., Amelung, W., 1996. Gas chromatographic determination of muramic acid, glucosamine, mannosamine, and galactosamine in soils. Soil Biol. Biochem. 28, 1201–1206.

Zolan, M.E., Pukkila, P.J., 1986. Inheritance of DNA methylation in *Coprinus cinereus*. Molec. Cell. Biol. 6, 195–200.