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Microbial activity and soil respiration under nitrogen addition in Alaskan boreal forest

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Abstract

Climate warming could increase rates of soil organic matter turnover and nutrient mineralization, particularly in northern high-latitude ecosystems. However, the effects of increasing nutrient availability on microbial processes in these ecosystems are poorly understood. To determine how soil microbes respond to nutrient enrichment, we measured microbial biomass, extracellular enzyme activities, soil respiration, and the community composition of active fungi in nitrogen (N) fertilized soils of a boreal forest in central Alaska. We predicted that N addition would suppress fungal activity relative to bacteria, but stimulate carbon (C)-degrading enzyme activities and soil respiration. Instead, we found no evidence for a suppression of fungal activity, although fungal sporocarp production declined significantly, and the relative abundance of two fungal taxa changed dramatically with N fertilization. Microbial biomass as measured by chloroform fumigation did not respond to fertilization, nor did the ratio of fungi : bacteria as measured by quantitative polymerase chain reaction. However, microbial biomass C : N ratios narrowed significantly from 16.0 ± 1.4 to 5.2 ± 0.3 with fertilization. N fertilization significantly increased the activity of a cellulose-degrading enzyme and suppressed the activities of protein- and chitin-degrading enzymes but had no effect on soil respiration rates or ^{14}C signatures. These results indicate that N fertilization alters microbial community composition and allocation to extracellular enzyme production without affecting soil respiration. Thus, our results do not provide evidence for strong microbial feedbacks to the boreal C cycle under climate warming or N addition. However, organic N cycling may decline due to a reduction in the activity of enzymes that target nitrogenous compounds.

Keywords: Alaska, bacteria, boreal forest, carbon cycle, ectomycorrhizal fungi, extracellular enzyme, microbial biomass, nitrogen fertilization, nucleotide analog, soil respiration

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Introduction

Nitrogen (N) is an essential element that can limit the growth of living organisms across a wide range of ecosystems (Vitousek & Howarth, 1991). However, human activities have more than doubled the input of N that enters ecosystems globally (Vitousek *et al*., 1997). This increased N availability can have serious consequences, including altered species composition (Ostertag & Verville, 2002), changes in biological diversity (Suding *et al*., 2005), N saturation (Aber *et al*., 1989), and increased N losses (Hall & Matson, 1999).

Even in ecosystems where anthropogenic N inputs are low, climate warming may increase the availability of N by increasing rates of nutrient release from soil organic matter (Hobbie *et al*., 2002). This effect is a particular concern in northern high-latitude ecosystems where N inputs are low and permafrost and waterlogging limit decomposition and the release of available N from soil organic matter (Preston *et al*., 2006). Together, boreal forest and tundra ecosystems contain 10–20% of global soil carbon (C) (Gorham, 1991; Jobbagy & Jackson, 2000) and may warm by up to 8 °C over the next century (Houghton *et al*., 2001). If large amounts of N are released from this organic

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matter, the increase in N availability could feed back to affect C cycling by altering rates of decomposition or plant growth (Hobbie *et al*., 2002; Strömgren & Linder, 2002; Mack *et al*., 2004; Olsson *et al*., 2005).

Because N availability is low in many boreal soils, these ecosystems are highly sensitive to N deposition (Kellner & Mårshagen, 1991; Strengbom *et al*., 2001). In particular, soil fungi show strong and typically negative responses to N deposition and fertilization (Boxman *et al*., 1998; Brandrud & Timmermann, 1998; Lilleskov *et al*., 2001, 2002a). This pattern is critical because fungi are believed to be the most important regulators of soil C and nutrient cycling in ecosystems with low soil pH, such as boreal forests (Fierer & Jackson, 2006; Högberg *et al*., 2007). In these systems, fungi control the breakdown of complex organic matter (e.g. wood) and form mycorrhizal symbioses that facilitate nutrient acquisition by plants (Robinson, 2002; Read *et al*., 2004).

Many groups of fungi are known to produce hydrolases and oxidases that degrade complex polymers in soil and convert organically bound nutrients into forms that are available to plants and microbes (Dighton, 2003). The activities of these enzymes may change if N addition alters microbial allocation to enzyme production or shifts the abundance of fungi that produce specific enzymes (Sinsabaugh & Moorhead, 1994). For example, N addition could cause microbes to produce more C-degrading enzymes relative to N-degrading enzymes, thereby increasing rates of C cycling (Allison & Vitousek, 2005). Given these potential consequences for C and nutrient cycling, surprisingly few studies have investigated how soil enzyme activities respond to N addition in boreal ecosystems.

Mycorrhizal fungi are especially sensitive to N addition because plants are thought to reduce C allocation to mycorrhizal symbionts when N availability is high (Högberg *et al*., 2003). N addition often suppresses mycorrhizal fungi (Arnolds, 1991; Lilleskov *et al*., 2001, 2002a), and may reduce organic N cycling, CO_2 respiration, and the sequestration of C in mycorrhizal pools. Ericoid and ectomycorrhizal fungi in particular produce extracellular proteases and other enzymes that break down organic polymers in soil (Read & Perez-Moreno, 2003); reduced allocation of C resources to these fungi could therefore affect enzyme production and the turnover of soil C pools.

In an earlier study, we found that N addition in Alaskan boreal forest reduced fungal diversity, but had relatively little effect on the community structure of soil fungi (Allison *et al*., 2007). However, that study was conducted early in the growing season, before the peak activity of ectomycorrhizal host plants. The objective of the current study was to expand on our prior work by assessing fungal responses to N at the peak of the boreal growing season and measuring functional characteristics of the microbial community such as soil respiration and extracellular enzyme activity. We hypothesized that N addition would increase bacterial abundance and suppress fungal abundance, particularly ectomycorrhizae. Based on allocation theory, we predicted that N fertilization would increase the activity of C-degrading enzymes but suppress the activity of N-releasing enzymes. Because of the increase in C-degrading enzyme activity, we hypothesized that soil respiration would also increase with N addition. However, we expected that this effect might be partially offset by reduced CO_2 production from ectomycorrhizae.

Materials and methods

Site description

We collected samples from a boreal forest site in central Alaska, USA (63°55′N, 145°44′W), described in detail by Treseder *et al*. (2004). The site is dominated by mature black spruce [*Picea mariana* (P. MILL) B.S.P.] with an understory of shrubs, mosses, and lichens. The growing season lasts ~4 months, with bud break occurring in mid-May and leaf senescence in mid-September. Background N deposition in the region is $\sim 1\,kg\,ha^{-1}\,yr^{-1}$ (EPA, 2006). In 2002, we established a fully factorial N × phosphorus (P) fertilization experiment with 10 m × 10 m control, N, P, and N + P plots in a randomized block design ($n = 4$). N was added at a rate of $200\,kg\,N\,ha^{-1}\,yr^{-1}$ as $NH_4^+NO_3^-$ in 2002–2003 and $100\,kg\,N\,ha^{-1}\,yr^{-1}$, thereafter. Rates of P addition were half those of N. These high rates of addition were chosen to ensure that nutrients were no longer limiting to any biological processes, such as plant or microbial growth. Fertilizer was applied annually during the first half of June. Soil pH was determined in a 2 : 1 (w : w) water : soil slurry in May 2007, and did not differ significantly among control and N-fertilized plots ($P = 0.10$, *t*-test, Table 1).

Nutrient availability

We used resin bags to measure the availability of ammonium and nitrate in the fertilized and control plots. Nylon mesh bags were filled with 5 g cation or anion exchange resin, soaked in 0.5 M HCl for 20 min, rinsed in deionized water (DI) water, and washed with 2 M NaCl until the pH of the wash solution was >5. On May 28, 2005, we placed four cation and four anion bags in each plot at ~5 cm depth. We retrieved the bags on July 27, 2005, and replaced them with new bags that remained in the soil until September 15, 2005. In 2006,

Table 1 Soil pH, nutrient availability, microbial biomass parameters, and ^{14}C-CO_2 signatures in control and nitrogen (N)-fertilized soils

		Control (Mean ± SE)	Nitrogen (Mean ± SE)
Soil pH	2007	4.9 ± 0.2	5.5 ± 0.1
Resin NO_3^- ($\mu g\,N\,g^{-1}$ resin day^{-1})	2005†	0.011 ± 0.004	24 ± 8***
	2006	0.028 ± 0.005	35 ± 12***
Resin NH_4^+ ($\mu g\,N\,g^{-1}$ resin day^{-1})	2005†	0.036 ± 0.017	17 ± 4***
	2006	0.026 ± 0.009	21 ± 3***
Microbial biomass (CFDE) ($\mu g\,g^{-1}$ dry soil)	C	1234 ± 150	1073 ± 280
	N	79 ± 12	204 ± 46*
	C : N	16.0 ± 1.4	5.2 ± 0.3**
Bacterial relative abundance‡ (qPCR)	May 2005	1.00 ± 0.09	1.22 ± 0.59
	August 2005	0.80 ± 0.17	0.84 ± 0.28
Fungal relative abundance‡ (qPCR)	May 2005	1.00 ± 0.12	0.76 ± 0.35
	August 2005	1.79 ± 0.35	1.95 ± 0.48
$\Delta^{14}C$-CO_2 (‰)		97 ± 4	93 ± 4

*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$ for comparison of control vs. nitrogen.
†Represents average of two sampling dates in 2005.
‡Values scaled to DNA quantity in May 2005 samples.

bags were placed in the soil on May 14 and retrieved on September 17.

Retrieved bags were rinsed briefly to remove soil particles and extracted in 100 mL 0.1 M HCl/2.0 M NaCl for 1 h with shaking at 120 rpm. The cation extracts were analyzed for ammonium concentrations using a modified Berthelot-salicylate method (Weatherburn, 1967) adapted for 96-well microplates. Absorbance was measured spectrophotometrically at 650 nm. Nitrate concentrations in anion extracts were determined spectrophotometrically at 540 nm using the vanadium method of Doane & Horwath (2003) modified for microplate assays. Blanks were included to account for background N in the reagents. We expressed ammonium-N and nitrate-N availability as $\mu g\,N\,g^{-1}$ resin day^{-1}.

Sporocarp sampling

On August 18, 2005, we collected all sporocarps present in control plots and plots that had been fertilized with N, P and N + P for a total of 16 10 m × 10 m plots. We included sporocarp biomass data from P plots to increase our sample size; because there was no significant P effect on sporocarp biomass, we pooled data into −N and + N treatments. Although sporocarps may appear throughout the growing season, preliminary surveys indicated that the vast majority of sporocarp production occurs in early to mid-August, just before our sampling time. Collected sporocarps were frozen within 4 h, shipped to the University of California, Irvine (UCI) frozen, and stored at −20 °C. Using a sterile scalpel, we excised a ~100 mg tissue sample from each sporocarp for DNA analysis and determined the dry weight of the remaining material (65 °C).

Microbial biomass

We measured microbial biomass C and N using the chloroform fumigation-direct extraction technique (Brookes *et al*., 1985; Vance *et al*., 1987). On July 2, 2006, we collected five 2 cm × 5 cm cores from each experimental plot and shipped them to UCI at 4 °C for analysis within 1 week. For each plot, the five cores were homogenized by hand and subsampled (10 g) to determine soil water content. We calculated microbial biomass as the difference in K_2SO_4-extractable C or N concentration between fumigated and unfumigated soils, divided by 0.45 for C (Vance *et al*., 1987) and 0.54 for N (Brookes *et al*., 1985).

Nucleotide analog labeling of active fungal communities

Also on August 18, 2005, we used the method of Borneman (1999) and Allison *et al*. (2007) to label the DNA of active soil microbes with the nucleotide analog 3-bromo-deoxyuridine (BrdU). Briefly, we used a sterile needle to inject 10 mL of 2.5 mM BrdU solution at five points arranged in an 'X' pattern 30 cm wide in each plot. The needle was inserted and withdrawn to distribute the BrdU solution evenly throughout the top 5 cm of organic horizon (mean O-horizon depth is 9.8 cm). Where present, litter and living moss were removed from the soil surface before injection. In one pair of adjacent control and N plots, we carried out a

second set of injections ~5 m from the first injection site to assess within-plot heterogeneity in fungal community structure. The BrdU solution was allowed to incubate in the field for 24 h before removing 2 cm × 5 cm cores from each injection site. The cores were frozen within 1 h, shipped to UCI frozen, and stored at −80 °C.

DNA extraction and amplification

The five soil cores from within each plot were combined and homogenized briefly (~10 s) in a blender before DNA extraction. Because we wanted to examine the entire active fungal community, roots and ectomycorrhizal root tips were not removed from the soil. For each sample, we extracted total DNA from a 0.12 g subsample of homogenized soil using the MoBio PowerSoil DNA kit (MO BIO Laboratories, Carlsbad, CA, USA). To extract sporocarp DNA, we used the MoBio UltraClean Plant DNA kit.

Following extraction of total soil DNA, we used the immunocapture procedure of Yin *et al*. (2004) and Allison *et al*. (2007) to separate BrdU-labeled DNA from unlabeled DNA. This method was also applied to a comparable soil DNA sample from the same site that was not labeled with BrdU to determine if any unlabeled DNA passed through the separation procedure.

We used the general fungal primers nu-SSU-0817-5′ and nu-SSU-1536-3′ to amplify a ~760 bp region of the 18S ribosomal RNA gene from BrdU-labeled soil DNA (Borneman & Hartin, 2000). Sequence similarity is relatively conserved in this region, allowing us to identify sequences at the family to phylum level (Anderson & Cairney, 2004). Targeting the internal transcribed spacer (ITS) region of the rRNA gene complex would have allowed for greater taxonomic resolution, but unknown ITS sequences cannot be reliably assigned to broad taxonomic groups. Therefore, we only sequenced the 18S rRNA gene in our soil DNA samples. For sporocarp DNA, we used the primers nu-SSU-0817-5′ and ITS4 (Gardes & Bruns, 1993) to amplify ~2000 bp that included the 18S region, as well as the ITS1, ITS2, and 5.8S region of the ribosomal RNA gene complex.

The polymerase chain reaction (PCR) conditions for the soil samples are described in Allison *et al*. (2007). For sporocarp samples, the PCR contained 3.3 mM $MgSO_4$, 0.5 μg μL^{-1} BSA, 250 μM of each dNTP, 333 nM of each primer, 0.03 U μL^{-1} Platinum Taq, 1 × Platinum Taq buffer, and 0.1 μL template DNA μL^{-1} reaction mixture. The reactions were run with a 3 min initial denaturation step at 94 °C; 32 cycles of 1 min denaturation at 94 °C, 1.5–3 min primer annealing at 54.0–55.5 °C, and 2–5 min elongation at 72 °C; and a final 10 min elongation step at 72 °C.

Cloning and DNA sequencing

We used the PCR products from the control and N treatments to create clone libraries (Allison *et al*., 2007). The plot is the unit of replication for subsequent analyses. The number of sequences obtained per plot ranged from 76 to 93, except for the two plots in which we conducted two sets of BrdU injections where the number of sequences was 171–181. The no-BrdU control sample produced a very weak PCR product that we also subjected to the cloning procedure.

Sporocarp DNA samples were sequenced using the primers nu-SSU-1536-3′ and ITS1 (Gardes & Bruns, 1993). Thus, we obtained two sequence reads per sporocarp, one with ~760 bp of the 18S region and another with ~650 bp of the ITS region.

Sequence analysis

We edited 18S and ITS sequences using BioEdit (Hall, 1999), and aligned ~653 bp of the 18S sequences with CLUSTALW (Chenna *et al*., 2003). The quality of the alignment was verified by careful visual examination and manual adjustment. Bad sequence reads, one nonfungal sequence, and two potential chimeras were removed. The alignments were input to the Phylip program DNADIST (Felsenstein, 2005) to generate distance matrices for subsequent analyses of N effects on fungal communities.

We grouped fungal DNA sequences into operational taxonomic units (OTUs) and analyzed community structure using the approaches described in Allison *et al*. (2007). The software program DOTUR (Schloss & Handelsman, 2005) was used to assign 18S sequences into OTUs defined by ≥99% sequence similarity. Within each treatment (excluding sporocarps), we calculated the relative abundance of each OTU in each plot. Using these abundance distributions, we calculated a matrix of community distances between plots (SAS PROC DISTANCE, NONMETRIC option) (SAS, Version 9.0, 2004, SAS Institute Inc., Cary, NC, USA). OTUs were identified to the family or ordinal level using BLAST searches of the National Center for Biotechnology Information database (see Supplementary material Appendix SA1). For ITS sporocarp sequences, identifications could be made at the genus or species level. Sequences generated from this study are available in GenBank under the accession numbers EU222021–EU223006.

To test for significant N effects on fungal community structure, we used multiresponse permutational procedures (MRPP) (McCune & Grace, 2002) in the statistical package R (R Development Core Team, 2006, *R: A Language and Environment for Statistical Computing*, R Foundation for Statistical Computing, Vienna, Austria). We also tested for treatment effects on the relative frequencies of

individual OTUs using nonparametric Kruskal–Wallis tests. The level of significance was set at $P < 0.05$.

Quantitative PCR (qPCR)

We used qPCR to measure the abundance of fungal 18S and bacterial 16S DNA in control and N-fertilized soils. On May 30, 2005, and August 18, 2005, we collected soil cores and extracted total DNA using the procedures described above. Assays were carried out in 96-well plates with a BioRad MyiQ single color real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA, USA). For fungal abundances, each 30 μL reaction contained 15 μL iQSYBR Green PCR Super Mix, 3 μL DNA template, and 0.6 μL (10 μM) of each of the primers nu-SSU-0817-5′ and nu-SSU-1196-3′ (Borneman & Hartin, 2000). qPCR amplifications were run as follows: 95 °C initial denaturation step for 15 min followed by 37 cycles of 15 s denaturation at 94 °C, 30 s annealing at 55.5 °C, and 30 s elongation at 72 °C. Each sample extract was amplified in triplicate, and bulk DNA extracted from the study soils was used to construct standard curves. A melting curve analysis was performed after each analysis to confirm the specificity of the qPCR. Fungal biomass is expressed as relative abundance, scaled to the quantity of fungal DNA observed on May 30, 2005 in control plots. We used the same method to determine bacterial relative abundance, but with the primers Eub338 and Eub518 (Fierer *et al*., 2005) and PCR parameters as follows: 95 °C initial denaturation step for 15 min followed by 37 cycles of 15 s denaturation at 94 °C, 30 s annealing at 56 °C, and 30 s elongation at 72 °C.

Enzyme activities

Starting in May 2005, we collected soils for enzyme analyses approximately monthly during the growing seasons of 2005–2006. From each plot, we collected at least three 2 cm × 5 cm cores and processed them in the same manner as the microbial biomass samples (above). We assayed the activities of four extracellular enzymes involved with the breakdown of complex organic polymers in soil: β-glucosidase (BG) catalyzes one of the final steps in cellulose breakdown, polyphenol oxidase (PPO) is a nonspecific enzyme that breaks down recalcitrant polymers such as lignin and humic acids, *N*-acetyl-glucosaminidase (NAG) aids in chitin breakdown, and glycine aminopeptidase (GAP) is involved in protein degradation.

Enzyme activities were determined according to the procedures of Allison & Jastrow (2006), modified for 96-well microplates. Briefly, 50 μL of homogenized soil slurry in 50 mM pH 5.0 acetate buffer was combined with 150 μL substrate solution and incubated for 1–5 h at 21 °C with constant shaking. Substrate solutions were 5 mM *p*-nitrophenyl-β-glucopyranoside for BG; 50 mM pyrogallol, 50 mM EDTA for PPO; 2 mM *p*-nitrophenyl-β-*N*-acetylglucosaminide for NAG; and 5 mM glycine *p*-nitroanilide for GAP, all in acetate buffer. After incubation, 100 μL of the slurry-substrate supernatant (without soil particles) was carefully transferred to another microplate for colorimetric determination of product concentrations. Eight analytical replicates were run per sample, and controls were included to account for background absorbance by the substrate solution and soil slurry. Activities are expressed as μmol product formed g^{-1} dry soil h^{-1}, except PPO, which is presented as μmol substrate consumed g^{-1} dry soil h^{-1}.

Soil respiration

Soil CO_2 fluxes were determined using flux chambers and a PP Systems EGM-4 infrared gas analyzer. Two chamber bases were inserted ~2 cm into the soil surface at random locations in each plot on May 25, 2005. We did not remove grasses and shrubs because they were not abundant within the chambers. Because mosses cannot be removed without severely disrupting the soil surface, they were also left in the chambers if present. Fifty percent of the moss and litter volume was subtracted from the total chamber volume. Measurements were made approximately monthly during the growing seasons of 2005 and 2006, starting in July 2005. Fluxes are expressed as mg CO_2-C m^{-2} h^{-1} (Czimczik *et al*., 2006).

We also measured the ^{14}C isotopic composition of soil-respired CO_2 in order to determine if N addition altered the C source of soil respiration. In the 1950s and 1960s, atmospheric weapons testing nearly doubled the concentration of ^{14}C in the atmosphere, and this 'bomb spike' has declined over time due to mixing with oceanic and biospheric C reservoirs and dilution by fossil fuel-derived CO_2. Thus, a shift in the source of soil respiration from recently fixed plant C to 40–50-year-old soil organic C would result in a greater $\Delta^{14}C$ value. We expected that such a shift might occur if N fertilization suppressed the activity of mycorrhizal fungi that respire recently fixed plant C. Because ^{14}C samples were collected in mid-September when plants were senescent, we expected root respiration to be relatively low.

We measured the ^{14}C signature of chamber CO_2 on September 13, 2005, by closing the tubing on the chamber lid and allowing the CO_2 to accumulate for ≥30 min. CO_2 was trapped and analyzed for ^{14}C content according to Czimczik *et al*. (2006). Before CO_2 accumulation, we circulated chamber air through soda lime traps to reduce the amount of atmospheric CO_2 present in our chamber samples. However, the samples still contained some CO_2 from the atmosphere.

We assumed that the ^{13}C value of soil respiration was −23‰ and used a mixing model to calculate the fraction of chamber CO_2 derived from air vs. soil respiration (Czimczik *et al*., 2006). The ^{14}C signature of soil respiration was calculated based on this fraction and the ^{14}C signature of ambient air sampled from a well-aerated area adjacent to the sampling site (Czimczik *et al*., 2006). ^{13}C signatures of saprotrophic and ectomycorrhizal sporocarps did not differ significantly at the site (unpublished data), so changes in fungal community structure are unlikely to affect the assumed ^{13}C value of respired CO_2.

Statistical analyses

To test for nutrient effects on sporocarp biomass, we conducted ANOVAs with SAS PROC MIXED (SAS Institute, 2004). Block was included as a random effect, and data were log-transformed before analysis to achieve normality. We analyzed resin nutrients, enzyme activities, and soil respiration using repeated measures ANOVAs in SAS PROC MIXED, with a compound symmetry covariance structure and block as a random effect. Where date × fertilization effects were significant, we used the SLICE option in SAS to test for significant fertilization effects by date. Nutrient data, BG activities, NAG activities, and CO_2 fluxes were log-transformed before analysis to improve normality. PPO and GAP activities were square-root transformed. We used *t*-tests to discern significant fertilization effects on microbial biomass C and N, qPCR data, and ^{14}C signatures. The level of significance was set at $\alpha = 0.05$.

Results

Nutrient availability

Resin NO_3^- availability increased significantly with N fertilization from 0.011 ± 0.004 to $24 \pm 8\,\mu g\,N\,g^{-1}\,resin\,day^{-1}$ in 2005 and from 0.028 ± 0.005 to $35 \pm 12\,\mu g\,N\,g^{-1}$ resin day^{-1} in 2006 (Table 1). NH_4^+ availability also increased significantly in both years, from 0.03 ± 0.02 to $17 \pm 4\,\mu g\,N\,g^{-1}\,resin\,day^{-1}$ in 2005 and 0.026 ± 0.009 to $21 \pm 3\,\mu g\,N\,g^{-1}\,resin\,day^{-1}$ in 2006.

Sporocarp biomass

N addition significantly reduced mean sporocarp biomass from $0.64 \pm 0.25\,g\,m^{-2}$ to $0.08 \pm 0.03\,g\,m^{-2}$ (Fig. 1, $P = 0.018$). We obtained usable DNA sequences from 57 sporocarps, which were assigned to 13 OTUs (≥99% similarity for 18S region) by DOTUR. Nine of these OTUs representing 42 sporocarp sequences were also observed in the soil clone libraries (Supplementary material Appendix SA1). Thus, a majority of the Basidiomycetes that produced sporocarps may have also been present in our soil molecular survey. Most of the identified sporocarp biomass belonged to the genera *Lactarius*, *Tricholoma*, and *Hydnellum* (Fig. 1).

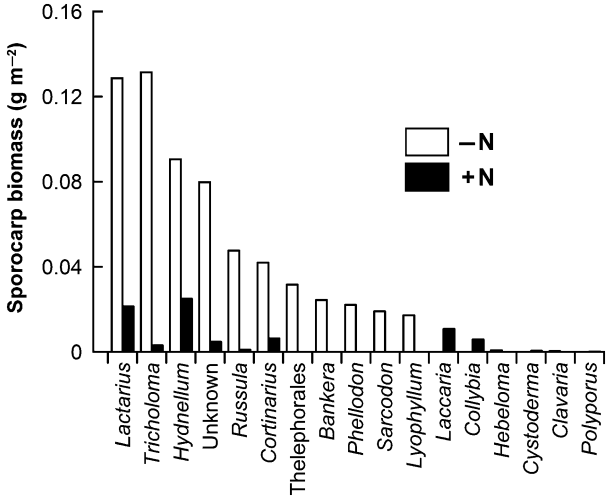

Fig. 1 Sporocarp biomass in control and nitrogen (N)-fertilized plots by taxon based on BLAST matches to 18S ribosomal and internal transcribed spacer DNA sequences. $P < 0.018$ for N effect on total sporocarp biomass across taxa ($n = 8$).

Microbial biomass

Microbial biomass C as measured by chloroform fumigation-direct extraction was 1073 to $1234\,\mu g\,C\,g^{-1}$ dry soil and did not change significantly with N fertilization (Table 1). However, microbial biomass N increased significantly ($P < 0.05$) from 79 ± 12 to $204 \pm 46\,\mu g\,N\,g^{-1}$ dry soil. This change resulted in a significant reduction in the microbial biomass C : N ratio from 16.0 to 5.2 under N fertilization (Table 1). The reduction in C : N ratio was not associated with a shift in the dominance of bacteria vs. fungi, because neither bacterial abundance nor fungal abundance as measured by qPCR varied significantly with N addition (Table 1).

Active fungal communities under N addition

We obtained 874 active fungal DNA sequences from control and N-fertilized plots. DOTUR assigned these sequences to 59 OTUs using a cutoff of ≥99% sequence similarity, with 34 OTUs in control plots and 36 in N plots. Consistent with these values, there were no statistically significant differences in diversity indices between the two treatments (data not shown). We assume that contamination by unlabeled DNA is minimal in the clone libraries because we obtained

Table 2 Relative abundances (%) of major taxa in control and nitrogen-fertilized clone libraries

Fungal class	Control ± SE	Nitrogen ± SE
Basidiomycetes	87.9 ± 2.3	85.8 ± 5.1
Ascomycetes	7.7 ± 1.3	6.1 ± 1.2
Zygomycetes	0.7 ± 0.4	3.1 ± 2.2
Urediniomycetes	2.3 ± 1.3	1.3 ± 1.0
Unknown	1.4 ± 0.8	1.8 ± 0.9
Ustilaginomycetes	0.0	1.1 ± 1.4
Chytridiomycetes	0.0	0.4 ± 0.6
Saccharomycetes	0.0	0.2 ± 0.3

OTU designation†

Number	Class	Order	Family	Control ± SE	Nitrogen ± SE
4*	Basidiomycetes	Agaricales		6.5 ± 2.9	43.4 ± 12.1
5*	Basidiomycetes	Agaricales	Cortinariaceae	32.4 ± 10.2	0.4 ± 0.6
12	Basidiomycetes	Agaricales	Cortinariaceae	17.5 ± 14.9	0.4 ± 0.3
7	Basidiomycetes	Cantharellales	Hydnaceae	5.4 ± 4.5	10.1 ± 6.2
1	Basidiomycetes	Russulales	Russulaceae	3.5 ± 4.6	8.8 ± 11.0
24	Basidiomycetes	Phallales		8.9 ± 5.6	0.0
3	Ascomycetes			4.2 ± 0.9	4.0 ± 0.9
15	Basidiomycetes	Agaricales	Cortinariaceae	0.0	6.5 ± 8.2
13	Basidiomycetes	Russulales	Russulaceae	1.6 ± 1.0	3.8 ± 2.3
18	Basidiomycetes	Tremellales	Exidiaceae	2.3 ± 1.3	1.8 ± 0.3
9	Basidiomycetes	Agaricales	Clavariaceae	4.0 ± 2.5	0.0
8	Urediniomycetes			2.3 ± 1.3	0.7 ± 0.5
16	Basidiomycetes	Thelephorales		0.2 ± 0.1	2.5 ± 1.9
25	Basidiomycetes	Agaricales		0.0	2.7 ± 1.3
34	Basidiomycetes	Boletales	Boletaceae	2.8 ± 1.6	0.0

*$P < 0.05$ for Kruskal–Wallis test of control vs. nitrogen.
†Operational Taxonomic Unit from DOTUR analysis, followed by taxonomic designation based on BLAST matches to GenBank sequences.

~10-fold fewer clones from the no-BrdU control sample compared with the labeled samples. We were able to obtain 66 fungal sequences from the no-BrdU control, of which 62% belonged to the Hydnaceae, 9% belonged to the Sistotremataceae, and 8% belonged to a Zygomycete taxon, according to BLAST searches. Therefore, some of the Hydnaceae sequences observed in the BrdU-labeled library may be derived from nonactive fungi (OTU 7, Table 2).

In contrast to the diversity measures, there were strong N effects on fungal community structure at the ≥99% sequence similarity level ($P = 0.029$ for control vs. N, MRPP). Both treatments were dominated by Basidiomycete fungi (>85% relative abundance), but specific taxa of Basidiomycetes shifted dramatically with N addition (Table 2). Two groups of fungi in the family Cortinariaceae accounted for nearly 50% of the sequences in control plots, but <1% in N plots (OTUs 5 and 12, Table 2). These taxa were largely replaced by OTU 4, which increased significantly from 6.5 ± 2.9% to 43.4 ± 12.1% relative abundance under N fertilization (Table 2). OTU 4 contains fungi of the order Agaricales, and includes sporocarp sequences belonging to the genus *Laccaria*.

Enzyme activities

The activity of the cellulose-degrading enzyme BG was greater under N fertilization (repeated-measures ANOVA, $P < 0.05$, Fig. 2a). In contrast, the activity of the oxidative enzyme PPO did not change significantly with N fertilization, although there was a trend toward lower activity at some time points (Fig. 2b). Activity of the chitin-degrading enzyme NAG was significantly lower with N addition on May 15, 2006 and August 22, 2006 (significant date × fertilizer interaction, Fig. 2c). The activity of the protein-degrading enzyme GAP declined strongly and consistently with fertilization ($P < 0.05$, Fig. 2d). All enzymes showed significant variation with date (Fig. 2), although there were no striking patterns to this variation.

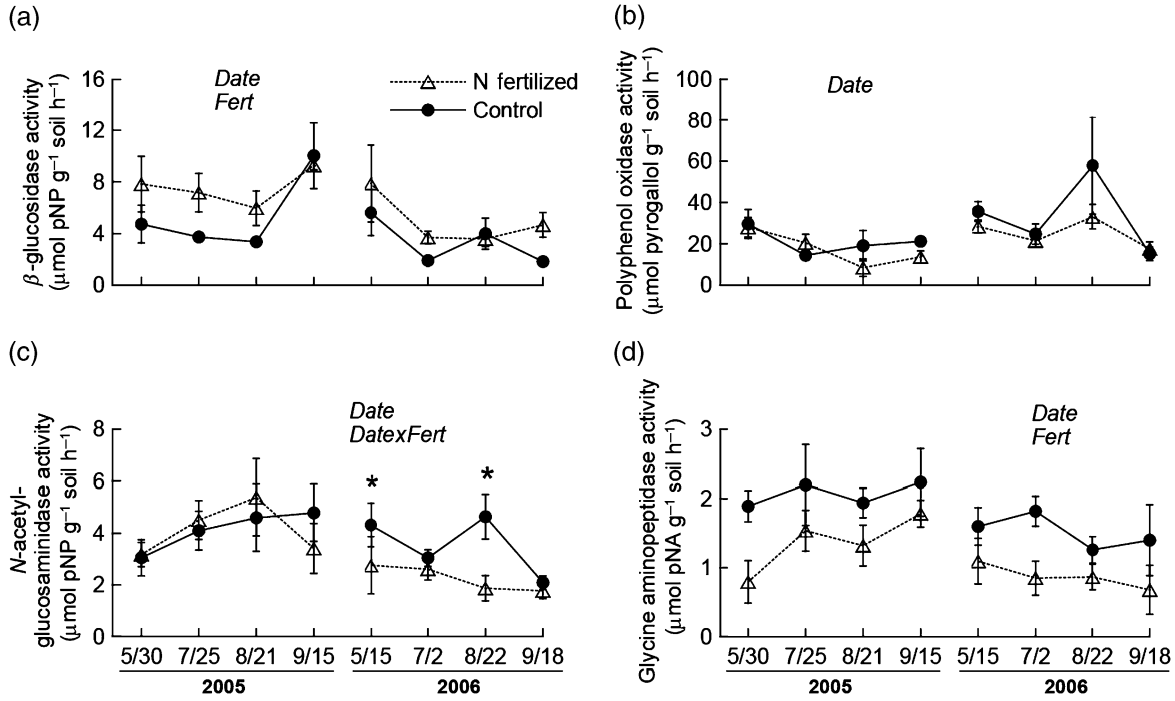

Fig. 2 Activities of (a) β-glucosidase, (b) polyphenol oxidase, (c) *N*-acetyl-glucosaminidase, and (d) glycine aminopeptidase across the 2005–2006 growing seasons. Significant effects ($P<0.05$) from repeated-measures ANOVA are shown in italicized text. (*) $P<0.05$ for fertilizer effect within date where date × fertilizer effect is significant.

Soil respiration

Soil CO_2 fluxes showed no significant response to N fertilization, although there was a slight decline at the end of the 2006 growing season (Fig. 3). Similarly, we did not observe any N effects on the ^{14}C signature of soil respiration, which ranged from 93‰ to 97‰. This range corresponds to the ^{14}C-CO_2 signature of the atmosphere in 1998–1999.

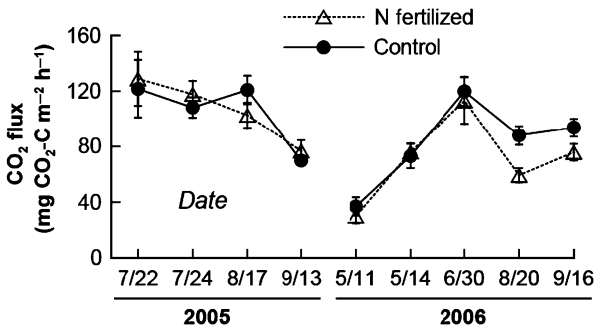

Fig. 3 Soil CO_2 fluxes across the 2005–2006 growing seasons. Only the date effect was significant ($P<0.05$) in a repeated-measures ANOVA.

Discussion

Sporocarp biomass

N fertilization strongly suppressed sporocarp biomass (Fig. 1), even though we did not detect any change in fungal relative abundance by qPCR. Sporocarp biomass in 2005 control plots was high relative to other years, such as 2002 when biomass was $0.45 \pm 0.10\,g\,m^{-2}$ (K. K. Treseder, unpublished data). The strong response to N addition that we observed in a relatively productive year suggests that N effects on sporocarp production can be substantial. Negative responses of sporocarp biomass to N fertilization or deposition are widespread (Wallenda & Kottke, 1998), having been observed in boreal forests (Lilleskov *et al.*, 2001), temperate oak savannah (Avis *et al.*, 2003), and across much of Europe (Arnolds, 1991). For some taxa in our study, such as *Cortinarius*, the reduction in sporocarp output corresponded to a decline in belowground relative abundance as indicated by the molecular analyses (i.e. Cortinariaceae). If N availability increases in soils due to N deposition or climate change, changes in sporocarp output could further alter the structure of fungal communities by reducing the spore dispersal and genetic recombination of N-sensitive taxa. These fungi may be replaced by nitrophilic taxa such as Agaricales OTU 4, which responded to N addition with increased belowground activity (Table 2) and in some cases greater sporocarp production (e.g. *Laccaria*, Fig. 1).

Microbial biomass

Contrary to our initial hypothesis, fungal and bacterial abundances were surprisingly resistant to N addition. In particular, there was no decline in fungal abundance with N, and no change in overall microbial biomass. However, some other investigators have found similar results. There is emerging evidence that ectomycorrhizal biomass may increase in tundra ecosystems with N addition (Clemmensen *et al*., 2006), and in other boreal systems, fungal and microbial biomass do not always respond to N (Boxman *et al*., 1998; Brenner *et al*., 2005). At our site, other indicators of fungal biomass such as hyphal counts and mycorrhizal colonization also do not decline with N addition, and may even increase slightly (Treseder *et al*., 2007). Thus fungal biomass in boreal soils may not be as sensitive to N deposition as other parameters, such as sporocarp production or fungal community structure.

Community structure of active fungi

Both control and N treatments were heavily dominated by Basidiomycete fungi, particularly the Cortinariaceae and Agaricales OTU 4. Based on DNA sequence similarity with sporocarp samples, it is possible that these taxa represent the ectomycorrhizal genera *Cortinarius* and *Laccaria*, respectively. However, Agaricales OTU 4 may also include saprotrophic taxa that did not produce sporocarps. N addition caused a near-complete replacement of Cortinariaceae by Agaricales OTU 4, similar to a pattern observed by Lilleskov *et al*. (2002a) along an N deposition gradient in a forest dominated by white spruce. This pattern likely occurs because members of the Cortinariaceae grow preferentially on organic forms of N, while members of Agaricales OTU 4, such as *Laccaria*, prefer inorganic N (Lilleskov *et al*., 2002b). Some saprotrophic fungi also respond positively to increasing N availability (Trudell & Edmonds, 2004), and may have contributed to increased relative abundance of Agaricales OTU 4.

Within the Basidiomycetes, fungi belonging to the order Agaricales comprised a large fraction of the community (Table 2). Using molecular surveys, O'Brien *et al*. (2005) also found that Agaricales dominated the Basidiomycete communities of surface soils in a forested ecosystem. Fungi of the family Hydnaceae were moderately abundant in our system, representing 7.7% of the total sequences, and probably correspond to ectomycorrhizal species. Taken together, the high relative abundance of ectomycorrhizal taxa within the Agaricales, Hydnaceae, and Russulales suggests that ectomycorrhizae dominate the active fungal community in this system. Using molecular approaches, Lindahl *et al*. (2007) also found that ectomycorrhizal fungi dominated organic and mineral soil in a Swedish boreal forest. These results may imply that heterotrophic decomposition of soil organic matter is conducted by a relatively small fraction of the active fungal community, or that mycorrhizal fungi also contribute to this ecosystem process. However, more work is needed to determine the biomass of saprotrophic fungi that would be required to sustain decomposition at steady state in boreal soils.

The fungal community response to N fertilization may vary considerably over the course of the growing season. In a prior study, we found that fungal diversity declined while overall community structure varied little with N addition early in the growing season (Allison *et al*., 2007). By the peak of the growing season, diversity was similar but community structure had changed significantly in the N addition plots. This reversal may have been due to the presence of different soil fungi in August, perhaps driven by increased plant activity and belowground C allocation. Although fungal communities at both times were dominated by Basidiomycetes, the Agaricales had increased in abundance relative to the Cantharellales by August. Alternatively, we may have increased our ability to detect changes in community structure in August because we analyzed a longer region of the 18S rRNA gene.

The shifts in active fungal communities that we observed are based on the relative abundances of 18S rRNA gene sequences from clone libraries. Although we believe the results are robust, they represent only a first step toward quantification of fungal community structure. Because of potential biases in DNA extraction and PCR amplification, changes in relative abundance from clone libraries should be confirmed with additional approaches, such as qPCR (Anderson & Cairney, 2004; Borneman *et al*., 2007). Nonetheless, there is evidence that clone libraries can provide a reasonable approximation of the results obtained from qPCR (Landeweert *et al*., 2003).

Differences in BrdU uptake rates among fungal taxa could also bias our results. However, we constructed a small clone library with total DNA from one of the control plots and also found dominance by the Cortinariaceae (data not shown). This similarity suggests that active fungi are a representative subset of the total fungal community, and that BrdU is probably not taken up disproportionately by particular fungi. Our earlier study at this site also supports these assertions (Allison *et al*., 2007).

Because most of the sporocarps and active fungal DNA belonged to ectomycorrhizal taxa, we speculate that N effects were mainly due to altered belowground C allocation by host plants. If N is highly available in

mineral forms, host plants have less incentive to allocate C to ectomycorrhizae for N acquisition (Wallenda *et al*., 1996; Hampp *et al*., 1999; Högberg *et al*., 2007). Reduced C supply from host plants may limit the ability of fungi to allocate C resources to reproductive structures (Kuikka *et al*., 2003). However, our data indicate that the tendency of plant hosts to withhold C may vary for different ectomycorrhizal symbionts. The marked decline in Cortinariaceae relative abundance and sporocarp production under N addition suggests that plant hosts can easily reduce C allocation to this taxon. In contrast, there was no evidence that C supply to Agaricales OTU 4 declined under N addition, while other taxa showed some symptoms of C deficiency (reduced sporocarp production, but still abundant in clone libraries).

Enzyme activities

The changes in enzyme activity that we observed are consistent with our predictions from allocation theory. Adding N to the soil not only provides one of the essential building blocks for enzyme construction, but may also increase microbial demand for C (Allison & Vitousek, 2005). Saprotrophic microbes could meet this demand by allocating resources to synthesize enzymes that acquire C, such as BG. Other studies have found that N addition increases cellulose-degrading enzymes in temperate forests (Carreiro *et al*., 2000; Frey *et al*., 2004; Gallo *et al*., 2005), and a shift from recalcitrant phenolic metabolism to polysaccharide hydrolysis under N deposition may be a general phenomenon in these ecosystems (Sinsabaugh *et al*., 2002). Our boreal soils showed a similar response, with PPO activity staying the same or declining while BG activity increased with N addition. If saprotrophic fungi are present in Agaricales OTU 4, increased abundance of this fungal group may have contributed to this increase in BG activity.

Activities of the N-releasing enzymes GAP and NAG also responded as expected based on allocation theory. If mineral N is abundant, then microbes should reduce their allocation to enzymes that acquire complex N (Sinsabaugh & Moorhead, 1994). In our boreal soils, declines in N-releasing enzyme activities were consistent with this prediction and probably associated with changes in the structure of the ectomycorrhizal fungal community. Ectomycorrhizae are known to produce extracellular enzymes, particularly proteases, that facilitate organic N scavenging (Read & Perez-Moreno, 2003; Read *et al*., 2004). In culture, *Cortinarius* spp. grow preferentially on protein-N sources that require enzymatic breakdown (Lilleskov *et al*., 2002b). Thus reduced GAP and NAG activities are probably driven by declines in specific ectomycorrhizal taxa, such as Cortinariaceae OTUs 5 and 12.

Soil respiration

Despite shifts in fungal community structure and C-degrading enzyme activity, we did not observe major changes in soil respiration with N addition. One potential explanation for this pattern is that soil respiration is closely related to microbial biomass, which did not respond to fertilization. Although N addition caused the replacement of one fungal taxon by another, this shift would not necessarily drive a change in CO_2 production if both taxa have similar respiration rates.

Soil respiration includes autotrophic and heterotrophic components, both of which could be affected by N fertilization. However, our ^{14}C results do not provide evidence for shifts in the source of soil respiration with N addition, although it would be difficult to distinguish root/ectomycorrhizal ^{14}C values from recent litter ^{14}C values. Nonetheless, an unchanging source of soil respiration is consistent with data on root biomass from the site, which remained constant with N addition (Treseder *et al*., 2007). Typically, nutrient additions are thought to cause reductions in root and fungal biomass (Wallander & Nylund, 1992; Cannell & Dewar, 1994; Treseder, 2004). At our site, N addition may cause secondary phosphorus limitation to plants and microbes, thereby preventing a decline in belowground biomass.

Conclusions

Taken together, our results indicate that microbial biomass and ecosystem C cycling in this boreal forest ecosystem are relatively resistant to N enrichment, despite changes in fungal community structure. Earlier studies have shown that ectomycorrhizal communities in boreal forest ecosystems are particularly sensitive to N deposition (Arnolds, 1991; Lilleskov *et al*., 2001, 2002a, b). Like these studies, our results show that certain fungal taxa, such as the Cortinariaceae and Agaricales OTU 4, have predictable responses to N availability that can be observed at the community level and explained by physiological preferences for different N resources. Despite changes in fungal community composition and soil enzyme activities, we found that the large-scale process of soil respiration remained invariant with N addition. Under N fertilization, reductions in belowground C allocation by plants may have affected fungal community composition and sporocarp production, but not C fluxes. Based on these results, we predict that soil C pools at this site have also remained stable in the face of N addition, unlike other sites where N has dramatic impacts on soil C balance (Mack *et al*., 2004; Waldrop *et al*., 2004).

In contrast to the resistance of the C cycle, our results suggest that N addition will affect organic N cycling in

these soils. Declines in GAP and NAG activity with N fertilization indicate a reduced potential for breakdown of protein and chitin, which are abundant organic N sources in boreal soils. Such a suppression of N-releasing enzymes could drive a negative feedback to increasing N availability with climate warming or N deposition.

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Supplementary material

The following material is available for this article online:

Appendix SA1. Contains operational taxonomic units, BLAST hits, and taxonomic designations of ribosomal gene sequences from soil (18S) and sporocarps (18S and ITS).

This material is available as part of the online article from http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2486.2008.01549.x

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