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Nitrogen fertilization reduces diversity and alters community structure of active fungi in boreal ecosystems

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Abstract

Nitrogen (N) availability is increasing in many ecosystems due to anthropogenic disturbance. We used a nucleotide analog technique and sequencing of ribosomal RNA genes to test whether N fertilization altered active fungal communities in two boreal ecosystems. In decaying litter from a recently burned spruce forest, Shannon diversity decreased significantly with N fertilization, and taxonomic richness declined from 44 to 33 operational taxonomic units (OTUs). In soils from a mature spruce forest, richness also declined with N fertilization, from 67 to 52 OTUs. Fungal community structure in litter differed significantly with N fertilization, primarily because fungi of the order Ceratobasidiales increased in abundance. We observed similar changes in fungal diversity and community structure with starch addition to litter, suggesting that N fertilization may affect fungal communities by altering plant carbon inputs. These changes could have important consequences for ecosystem processes such as decomposition and nutrient mineralization.

Keywords: Fungi; Nitrogen; Litter quality; Fertilization; Boreal forest; Diversity; Community structure; Nucleotide analog; Ribosomal genes; Carbon

1. Introduction

Human activities have more than doubled the amount of fixed nitrogen (N) that enters ecosystems annually (Vitousek et al., 1997). Increased N deposition can have dramatic impacts on ecosystem processes and biological communities. For example, N deposition increases N leaching and trace gas losses, alters soil carbon (C) stocks, and changes plant community composition (Hall and Matson, 1999; Mack et al., 2004; Waldrop et al., 2004; Suding et al., 2005). When N availability exceeds biological demand in forested ecosystems, N saturation may occur, leading to changes in soil pH, forest decline, and massive N losses (Aber et al., 1998).

In boreal forest and tundra ecosystems, anthropogenic N deposition is low compared to the rest of the globe (but see Lilleskov et al., 2001). However, climate change may lead to changes in N availability in these ecosystems. The IPCC predicts that 21st century warming due to greenhouse gas emissions will be most intense at high latitudes (Houghton et al., 2001). This warming could increase decomposition rates, nutrient mineralization, and N availability in high-latitude ecosystems (Van Cleve et al., 1990).

Especially in arctic and boreal ecosystems, fungi play a major role in the decomposition and mineralization of organic matter. Many soils in these systems are acidic and receive recalcitrant litter inputs, conditions that often favor fungi over bacteria (Hobbie and Gough, 2004). Therefore, predicting N deposition and/or climate warming effects on ecosystem processes depends on understanding fungal responses to increasing N availability. There is evidence that N deposition decreases the diversity of ectomycorrhizal fungi in white spruce forests of Alaska (Lilleskov et al., 2001, 2002), and a recent meta-analysis indicated that N fertilization reduces mycorrhizal abundance by 15% (Treseder, 2004). However, mycorrhizal fungi are not believed to play a major role in organic carbon decomposition (Read, 1991; Dighton, 2003), and the responses of decomposer fungi to N deposition remain largely untested.

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Despite this lack of evidence, studies of C cycle responses to N addition frequently hypothesize that shifts in fungal community structure are driving changes in C cycling (Fog, 1988; Carreiro et al., 2000; Waldrop et al., 2004). In particular, declines in the abundance or function of Basidiomycete fungi under N deposition could lead to slower C turnover if decomposition depends on powerful oxidative enzymes that they produce (Fog, 1988). Declines in fungal diversity could also reduce decomposition rates if a diverse community of fungal decomposers is required to degrade the range of C compounds within soils.

Recent advances in molecular techniques permit detailed studies of microbial community structure in response to environmental drivers such as N deposition (Anderson and Cairney, 2004). Microbial DNA can be extracted from environmental samples, amplified with polymerase chain reaction (PCR), cloned into bacteria, and sequenced in high-throughput facilities (Bridge and Spooner, 2001). It is also possible to isolate DNA from the active members of the soil microbial community using approaches such as nucleotide analog labeling. This approach was pioneered by Borneman (1999), and relies on the incorporation of a thymidine analog into newly synthesized DNA.

Our objective was to determine how fungal communities in Alaskan boreal ecosystems respond to N deposition. We used molecular techniques to extract and clone ribosomal DNA from active fungi in decomposing litter and soil, and then sequenced clone libraries to test for changes in fungal diversity and community structure. Based on studies with ectomycorrhizal fungi, we hypothesized that N deposition would decrease fungal diversity. We also hypothesized that N deposition would alter fungal community structure, and would suppress Basidiomycete fungi in particular. Because N deposition increases the quantity and quality of plant C inputs in our sites, we predicted that fungal community responses to C addition would parallel responses to N deposition in decomposing litter.

2. Materials and methods

2.1. Site description

We collected samples from two sites in east-central Alaska, USA (63°55′N, 145°44′W) described in detail by Treseder et al. (2004). The first site is a boreal spruce forest that burned in 1999 and is now dominated by shrubs, herbaceous perennials, and small deciduous trees. The second site has not burned for >80 years and is dominated by mature black spruce (*Picea mariana*) with an understory of shrubs, mosses, and lichens. Organic matter concentrations in the top 10 cm of soil were ~3-fold higher in the mature forest site than in the 1999 burn site.

In 2002, we began adding N and C to plots at each of the sites. Each site contained four $10 \times 10\,m^2$ control plots and four plots that received 200 kg N ha^{-1} $year^{-1}$ as $NH_4^+NO_3^-$ in 2002 and 2003 and 100 kg N ha^{-1} $year^{-1}$ in 2004. We used these high levels of N addition to ensure that biological processes such as plant and microbial growth were not limited by N. C addition plots were $1 \times 1\,m^2$ and received 200 kg C ha^{-1} $year^{-1}$ as starch in all 3 years. This rate of addition is equal to ~15% of net primary productivity (Michelle Mack, unpublished data), and is a reasonable approximation of the increase in productivity expected under N fertilization. We chose to add starch because its availability to microbes is representative of the C compounds present in herbaceous plant litter at this site. C and N were applied annually during the first-half of June, and all sampling was completed before fertilizer was applied in 2005.

2.2. Litter sampling

Samples of intact, surface litter from the 1999 burn site were collected on July 28, 2004 from five random points within the control and N plots, and from the entire surface of the C plots. All litter was combined within a plot, shipped to the University of California, Irvine (UCI) on ice, and stored at −20 °C. Before analysis, a 2–6 g sample of litter was thawed and air dried in a weigh boat under a plastic cover for 24 h. This sample processing may have favored disturbance-tolerant fungi over slow-growing filamentous fungi, although freezing and drying of the litter layer are common natural disturbances in this site. To label the DNA of active microbes, we added 120 µl of 7.69 mM sterile bromodeoxyuridine (BrdU) solution per g litter, mixed the BrdU solution and litter thoroughly in a plastic bag, and incubated the BrdU + litter for 24 h in the dark. BrdU is a thymidine analog that is incorporated preferentially into the DNA of microbes that are actively growing (Borneman, 1999). The labeled litter was ground with liquid N_2 in a mortar and pestle and stored for several months at −80 °C.

2.3. Soil sampling

To avoid disruption of the soil structure and fungal hyphae, we labeled soil microbial DNA with BrdU in the field on May 29, 2005. We conducted this experiment in the unburned site because its high fungal diversity (based on preliminary mushroom surveys) and well-developed, organic-rich soils provide a contrasting set of conditions in which to test our hypotheses. In each control or N-fertilized plot, we used a sterile needle to inject 10 ml of 2.5 mM BrdU solution at each of 5 points arranged in an "X" pattern 30 cm wide. BrdU was added at least 1 m from the edge of the plot, and mosses, lichens, and litter were removed from the soil surface before injection. During injection, the needle was inserted and withdrawn several times to distribute the solution evenly between 0 and 5 cm depth. 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.

2.4. DNA extraction

For DNA extraction from litter, we vortexed 0.1 g BrdU-labeled litter with 0.1 mm zirconia/silica beads for 3.5 min at high speed and separated the DNA using a silica isolation procedure according to Boom et al. (1990). The DNA was further purified with the Wizard DNA Cleanup System (Promega). For soil samples, cores were combined within each plot and homogenized briefly (~10 s) in a blender prior to DNA extraction. For each sample, we extracted total DNA from a 0.25 g subsample of homogenized soil using the PowerSoil DNA kit (MoBio Laboratories) with 10 min of disruption on a reciprocating shaker.

Following extraction of total DNA, we used the method of Yin et al. (2004) to separate BrdU-labeled DNA from unlabeled DNA. For each sample, we combined 9 μl of 6.25 mg ml^{-1} denatured herring sperm DNA (Promega) with 1 μl anti-BrdU antibody (0.1 μg μl^{-1}, Roche) and incubated the mixture under slow rotation (~10 rev min^{-1}) for 30 min at 23 °C. We then combined 8 μl total DNA extract with 2 μl phosphate buffered saline (PBS), pH 7.4, and denatured the DNA. This DNA solution was then combined with the herring sperm DNA/anti-BrdU solution and incubated for 30 min under slow rotation. Following incubation, the mixture was added to 3.13 μl washed Dynabeads M-450 sheep anti-mouse IgG (Dynal) in PBS–bovine serum albumin solution (PBS–BSA) and incubated again under slow rotation for 30 min. This step forms a complex containing the BrdU-labeled DNA, the anti-BrdU antibody, and the Dynabeads, which can be isolated by using a magnet to trap the iron-containing Dynabeads. The Dynabead complex was washed with 100 μl PBS–BSA three times by adding the wash solution, centrifuging briefly, and trapping the complex with a magnet as the wash solution was removed. BrdU-labeled DNA was released from the washed Dynabeads by adding 10 μl 1.7 mM BrdU in PBS–BSA and incubating under slow rotation for 30 min. The BrdU-DNA was separated from the Dynabeads by using the magnet, and was then reserved for amplification by PCR.

2.5. DNA amplification

For BrdU-labeled DNA from litter samples, we amplified ~700 bp that included the internal transcribed spacer-1 (ITS1), ITS2, and the 5.8S region of the ribosomal RNA gene complex. We used the Basidiomycete-specific primers ITS1-F (CTTGGTCATTTAGAGGAAGTAA) and ITS4-Basidio (CAGGAGACTTGTACACGGTCCAG) (Gardes and Bruns, 1993). To determine if N addition affected the fungal community in the soil samples, we used variants of the general fungal primers nu-SSU-0817-5′ (GGAGACAUTTAGCATGGAATAATRRAATAGGA) and nu-SSU-1536-3′ (GGGAAAGUATTGCAATGCYCTATCCCCA) to amplify a ~760 bp region of the 18S ribosomal gene from BrdU-labeled soil DNA (Borneman and Hartin, 2000). The first 8 nucleotides of these primers are not part of the 18S gene, but enabled compatibility with additional cloning kits.

The PCR reaction for the litter samples contained 2.5 mM $MgSO_4$, 2.0 μg μl^{-1} BSA, 250 μM of each dNTP, 400 nM of each primer, 0.04 U μl^{-1} Platinum Taq (High Fidelity, Invitrogen), 1X Platinum Taq buffer, and 0.1 μl template DNA μl^{-1} reaction mixture. PCR was carried out on an iCycler thermocycler (BioRad) with a 3 min initial denaturation step at 94 °C; 35 cycles of 1 min denaturation at 94 °C, 1 min primer annealing at 50 °C, and 3 min elongation at 72 °C; and a final 10 min elongation step at 72 °C. For soil samples, the PCR reaction contained 2.5 mM $MgSO_4$, 0.5 μg μl^{-1} BSA, 250 μM of each dNTP, 400 nM of each primer, 0.04 U μl^{-1} Platinum Taq, 1X 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 30 s denaturation at 94 °C, 30 s primer annealing at 56 °C, and 2 min elongation at 72 °C; and a final 2 min elongation step at 72 °C.

2.6. Cloning

PCR products were run out on 1.5% agarose mini-gels, and the target amplicon was cut out and purified with a Qiaquick gel extraction kit (Qiagen). Where necessary, we combined gel bands from multiple PCR runs to increase the concentration of DNA used in the cloning reaction. We used the TOPO TA Cloning Kit for Sequencing (Invitrogen) to create clone libraries for each litter or soil treatment (i.e. control, C, N). We carried out one ligation reaction for each experimental plot by incubating 3.5 μl (litter) or 4 μl (soil) gel-extracted PCR product with 1 μl pCR4-TOPO vector and 1 μl TOPO salt solution for 20 min. This mixture was then added to 1 vial of DH5α-T1 Chemically Competent *Escherichia coli* cells (Invitrogen) and incubated on ice for 20 min. The cells were heat-shocked for 45 s at 42 °C and immediately placed on ice. We then added 250 μl SOC medium (Invitrogen) to the cell suspension and incubated for 45 min at 37 °C with shaking (60 rev min^{-1}). The cell suspension was spread on agar plates containing LB media and 100 μg ml^{-1} ampicillin, and the plates were incubated for 16 h at 37 °C to produce clone libraries.

Using sterile toothpicks, we picked colonies at random from the agar plates into 96-well plates containing 160 μl 10% glycerol-LB media and 30 μg ml^{-1} kanamycin. The plates were incubated for 16 h at 37 °C, sealed, and frozen at −80 °C until sequencing. Clones were sequenced by SymBio Corp., Menlo Park, CA, using MegaBACE 4000 automated sequencers. The number of sequences obtained per plot ranged from 36 to 85 for litter and 33 to 120 for soil; this unbalanced distribution could bias community structure toward more well-sampled plots.

In theory, the number of clones with a given DNA sequence is proportional to the abundance of active fungi

containing that sequence in the original sample. In reality, varying copy numbers of ribosomal genes and biases in DNA extraction and PCR amplification probably alter this proportionality (Borneman et al., 2007). Also, we did not determine the amount of BrdU-free DNA that passed through the immunocapture procedure, so some DNA sequences from non-labeled fungi may also appear in the clone libraries. However, we assume that inactive fungi are unlikely to respond to the treatments and therefore any observed differences in community structure are mainly due to fungi that have been active since fertilization began. Since DNA contamination from inactive fungi should reduce our ability to detect changes in the active fungal community, we view our tests for treatment responses to be conservative.

2.7. Sequence analysis

We edited sequences from the ITS and 18S regions using BioEdit (Hall, 1999), and aligned each region using ClustalW (Chenna et al., 2003) with default parameters. Because the ITS region is highly variable, we used a ~250 bp region that included ~100 bp of ITS2 and ~150 bp of the well-conserved 5.8S gene to facilitate alignment. Still, much of the ITS2 region could not be aligned reliably, and thus we did not attempt to infer phylogenetic relationships from the alignment. For the 18S sequences from soil, we limited our analyses to ~230 bp in the center of the amplified region because sequencing errors were common at the ends. Substantial variability useful for dividing fungi into taxonomic groups remained in this region, although again we did not attempt to construct phylogenies. We assumed that the number of chimeric sequences was negligible because of the short length of the region. The alignments were used to generate distance matrices in the Phylip program DNADIST (Felsenstein, 2005) with an F84 evolutionary model and a transition:-transversion ratio of 1.0. The distance matrices provided the input data for analyses to test for N or C effects on fungal communities.

We used BLAST searches to match our DNA sequences from litter and soil with known organisms in the National Center for Biotechnology Information database (Appendix A, available as supplementary material on the Soil Biology & Biochemistry web site, or upon request from the authors). We identified each sequence to order according to the first BLAST hit unless it was an uncultured organism. In this case, we used the first identified organism within the top five BLAST hits, or left the identity as "uncultured" if none of the top five hits produced a reliable match. All DNA sequences were submitted to GenBank (accession numbers EF380394 to EF381740).

2.8. Statistical analyses

We used the software program DOTUR (Schloss and Handelsman, 2005) to group sequences into operational taxonomic units (OTUs). Matrices of genetic distance from DNADIST were input to DOTUR and used to generate OTUs containing sequences with $\geqslant$70%, $\geqslant$80%, $\geqslant$90%, $\geqslant$95%, or $\geqslant$97% similarity based on a furthest-neighbor algorithm. For each treatment (control, N, C) and level of sequence similarity, we used DOTUR to calculate rarefaction curves of OTU richness, Chao1 estimates of OTU richness (Chao, 1984), and Shannon diversity (Magurran, 1988) as a function of sequences sampled. We also generated rank abundance curves for each treatment. Rarefaction curves were generated by sampling all available sequences at random without replacement and plotting observed OTU richness as a function of the sequence number. This process was repeated 1000 times to obtain average OTU richness and 95% confidence intervals (CIs). Chao1 estimates of OTU richness are useful for statistical comparison of the actual richness of different microbial communities (Hughes et al., 2001). The Shannon index of diversity accounts for evenness as well as richness of each community (Magurran, 1988). We tested for significant treatment effects on these parameters by checking for non-overlapping 95% CIs.

We examined fungal community structure using multivariate tests on relative abundances of OTUs from DOTUR. OTU assignments were based on a distance matrix that included all sequences from either litter or soil. Using this approach, we obtained slightly different OTU groupings than when control and fertilized matrices were analyzed individually (as above), although patterns in OTU richness with fertilization remained the same. We used OTUs from DOTUR rather than taxonomic assignments based on BLAST analyses because not all sequences matched a known sequence in the database, and using sequence similarities avoids uncertainties associated with fungal taxonomy and classification. Also, we found that OTUs from DOTUR often matched taxonomic divisions based on BLAST identification (see Appendix A).

Within each treatment, we calculated the relative abundance of each OTU in each plot. Using these abundance distributions and a Bray–Curtis coefficient, we calculated a matrix of community distances between plots (SAS PROC DISTANCE, NONMETRIC option). Distance matrices were used as the basis for ordination by non-metric multidimensional scaling (NMS) and statistical tests of treatment effects by multi-response permutational procedures (MRPP) (McCune and Grace, 2002). NMS is an ordination technique that represents the distances between communities in low-dimensional space. For each level of sequence similarity, we used SAS PROC MDS to generate ordination graphs of distances between communities in control, N, and C plots from PROC DISTANCE. This procedure configures the communities so as to minimize the badness-of-fit ("stress") of the ordinated distances to the distances in the original matrix. In other words, it uses a minimization algorithm to draw a "map" of the communities from the distance matrix.

PROC MDS used the input data to generate a starting point for the minimization algorithm and carried out iterations until the stress value stabilized (convergence criterion >0.01). We determined the dimensionality of the data by adding dimensions (starting with 1) until the final stress did not change by more than 0.05. This approach resulted in ordinations with either 2 or 3 dimensions. Final stress values ranged from 0.04 to 0.10 for litter ordinations and 0.01–0.06 for soil ordinations.

We used MRPP in the statistical package *R* (R Development Core Team, 2006) to test for significant effects of treatments on fungal community structure at each level of sequence similarity. This procedure uses the same distance matrices as NMS to calculate the mean distance between communities within a group, where groups correspond to treatments (control, N, or C). Communities are then assigned to groups at random to calculate the probability of obtaining the observed mean distance by chance. The procedure is analogous to a non-parametric ANOVA on multivariate community data. If the overall MRPP was significant ($P<0.05$) for litter data, we also conducted MRPP for each pair of treatments.

These analyses were conducted on OTUs defined by $\geqslant$70% (litter only), $\geqslant$80%, $\geqslant$90%, $\geqslant$95%, and $\geqslant$97% sequence similarity. We also tested for treatment effects on the relative frequencies of individual OTUs using non-parametric Kruskal–Wallis tests. The level of significance was set at $P<0.05$.

3. Results

3.1. Taxonomic richness

We obtained 258 sequences from control litter, 259 from N-fertilized litter, and 258 from C-fertilized litter. Taxonomic richness at the 97% similarity level declined from 44 OTUs in control litter to 33 and 28 under N and C fertilization, respectively. Rarefaction curves diverged after sampling 154 sequences in the N treatment and 87 in the C treatment (Fig. 1A), suggesting that observed richness in the two treatments differed from the control litter. N and C treatments did not differ in OTU richness until 226 sequences had been sampled, suggesting that OTU richness does not differ strongly among the two treatments. The pattern of lower taxonomic richness in the N and C treatments was also evident at the $\geqslant$95% and $\geqslant$90% similarity levels (data not shown), indicating that negative effects of N and C apply to broad taxonomic groups.

In the soil communities, we obtained 245 sequences from control plots and 327 from N-fertilized plots. Rarefaction curves (Fig. 1B) indicated a decline in taxonomic richness in response to N addition at $\geqslant$97% sequence similarity. The 95% CI for the N rarefaction curve (52 ± 5 OTUs) does not include the observed richness of 67 OTUs in the control soils after sampling 245 sequences, and the curves first diverge after 198 sequences (Fig. 1B). This pattern was also evident at $\geqslant$95% sequence similarity, but not at lower similarities (data not shown).

Chao1 estimated richness provides an estimate of actual rather than observed OTU richness in each treatment, and is more sensitive to changes in richness that occur with increased sampling. In the litter, Chao1 estimated richness was higher in the controls, but the 95% CIs overlapped with the N and C treatments across all sequences sampled at the 97% similarity level (Fig. 2A). In contrast, Chao1 estimated richness was significantly lower in N-fertilized soil (82 OTUs, 95% CI of 63–130) than in control soil (361 OTUs, 95% CI of 179–839) after sampling 245 sequences (Fig. 2B). Chao1 estimated richness in soil was also significantly lower under N fertilization at $\geqslant$95% similarity (data not shown).

OTU diversity as measured by the Shannon index largely mirrored the rarefaction analysis. Nitrogen addition reduced the index significantly to 2.44 ± 0.16 ($\pm$95% CI) as compared to 2.94 ± 0.15 in the control litter (Table 1). Significant differences were evident down to at least the $\geqslant$90% level of sequence similarity. Shannon diversity in the C treatment declined to 1.93 ± 0.19 and remained significantly lower than the control and N treatment at all levels of sequence similarity down to $\geqslant$80% (Table 1). In

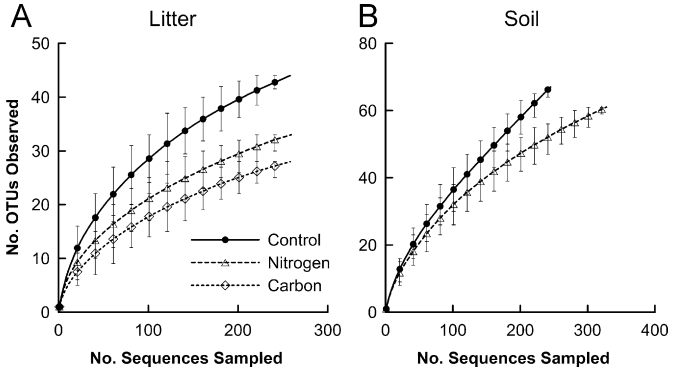

Fig. 1. Rarefaction curves for (A) litter and (B) soil for operational taxonomic units defined by $\geqslant$97% sequence similarity. Error bars represent 95% confidence intervals.

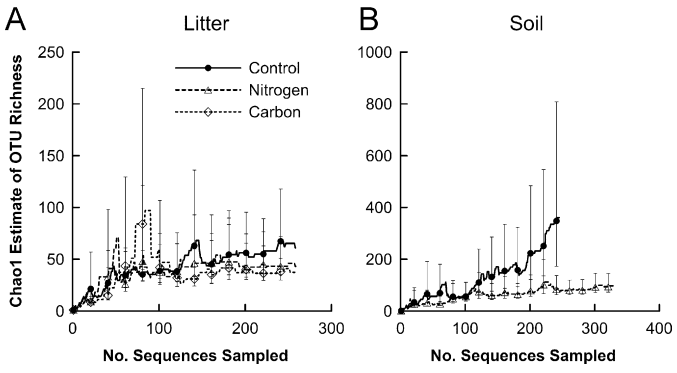

Fig. 2. Chao1 estimates of operational taxonomic unit richness for (A) litter and (B) soil at $\geqslant$97% sequence similarity. Error bars represent 95% confidence intervals.

Table 1
Shannon diversity indices (±95% CI) for observed DNA sequences at increasing levels of sequence similarity in litter and soil

	Sequence similarity				
	⩾70%	⩾80%	⩾90%	⩾95%	⩾97%
Litter					
Control	0.99±0.13	1.74±0.13	2.48±0.13	2.81±0.14	2.94±0.15
Nitrogen	1.05±0.11	1.55±0.15	1.67±0.17	2.24±0.14	2.44±0.16
Carbon	1.05±0.11	0.89±0.14	1.07±0.17	1.51±0.20	1.93±0.19
Soil					
Control		0.66±0.10	1.68±0.15	2.69±0.17	3.21±0.18
Nitrogen		0.67±0.02	1.87±0.10	2.66±0.14	3.01±0.16

soil, the N effect on Shannon diversity at ⩾97% sequence similarity was smaller, as evidenced by a non-significant decline from 3.21±0.18 in control soil to 3.01±0.16 under N fertilization.

3.2. Community structure

As expected from using Basidiomycete-specific primers, litter clone libraries were dominated by Basidiomycota (according to BLAST searches), although up to 3% of the sequences were identified as Ascomycota and 15–46% were uncultured (Table 2). Litter Basidiomycota were heavily dominated by fungi belonging to the order Ceratobasidiales, which comprised 40–74% of the total community. The other major orders were the Polyporales and Agaricales, but none represented more than 7% of the total community in a given treatment (Table 2).

Basidiomycota also dominated the soil fungal community, comprising 77% of sequences in control plots and 64% of sequences under N-fertilization (Table 2). The N treatment contained 29% Ascomycota, compared to 16% in the control. The largest group of Basidiomycota belonged to the order Agaricales (47% of sequences in the control, 32% under N fertilization), but the order Cantharellales also represented a substantial fraction of the community and increased in relative abundance from 15% to 22% under N fertilization. Most of the Ascomycota belonged to the order Dothideales (Table 2).

3.3. Community structure analyses

In the litter, NMS and MRPP tests showed that fungal communities in C plots differed from control plots at sequence similarities of 70–90% (Fig. 3A–C). A similar pattern occurred with N fertilization, except that the differences were only marginally significant for 80–90% sequence similarity. C and N treatments did not differ from each other.

We used Kruskal–Wallis tests to determine which fungal OTUs were driving community responses to N and C treatments. In litter, two main groups were responsible: a

Table 2
Relative abundance (% of sequences in clone library) of dominant fungal classes and orders by treatment (N = nitrogen, C = carbon) in litter and soil fungal communities based on NCBI BLAST matches

	Control	N	C		Control	N	C
Litter							
Basidiomycota	54	60	83	Ceratobasidiales	40	49	74
				Polyporales	7	3	3
				Agaricales	5	4	3
Ascomycota	0	3	2	Dothideales	0	3	0
Uncultured	46	37	15				
Soil							
Basidiomycota	77	64		Agaricales	47	32	
				Cantharellales	15	22	
				Polyporales	6	4	
Ascomycota	16	29		Dothideales	11	11	
				Saccharomycetales	0	5	
				Helotiales	1	4	

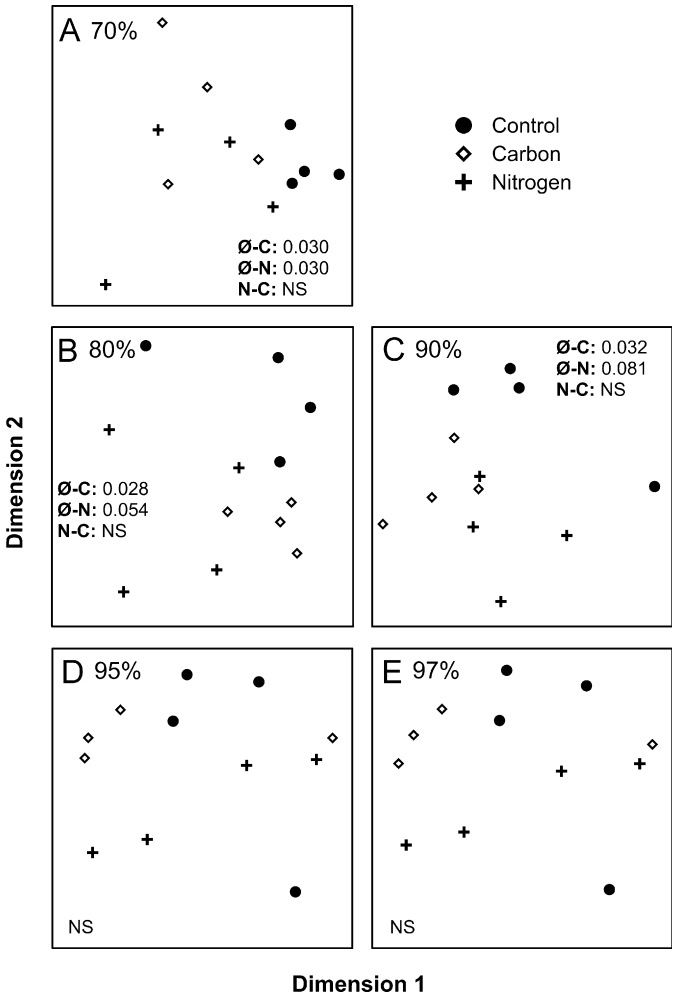

Fig. 3. Non-metric multidimensional scaling plots at (A) ⩾70%, (B) ⩾80%, (C) ⩾90%, (D) ⩾95%, and (E) ⩾97% sequence similarity for active fungal communities from litter. Ordinations represented 85–95% of the variance in the input distance matrix. *P*-values for differences among treatments were determined by multi-response permutational procedures. NS = not significant. Ø = control, N = nitrogen, C = carbon.

Table 3
Names and relative abundance (% of sequences in clone library) of taxa showing significant differences in abundance under nitrogen and/or carbon fertilization in litter and soil. Relative abundances with the same letter within a row are not significantly different ($P > 0.05$) according to Kruskal–Wallis tests ($n = 4$)

Taxon*	Similarity group (OTU†)	Control	Nitrogen	Carbon
Litter				
Ceratobasidiales	⩾70% (4)	41.5[a]	54.4[b]	74.0[b]
	⩾80% (12)	41.5[a]	54.1[b]	74.0[b]
	⩾90% (21)	36.8[a]	51.7[b]	73.2[b]
Uncultured	⩾80% (17)	0[a]	6.9[b]	0[a]
	⩾90% (31)	0[a]	6.9[b]	0[a]
	⩾95% (53)	0[a]	6.9[b]	0[a]
	⩾97% (69)	0[a]	6.9[b]	0[a]
Soil				
Hysteriales	⩾90% (13)	0[a]	1.8[b]	
Polyporales	⩾95% (48)	0[a]	4.3[b]	
	⩾97% (71)	0[a]	2.8[b]	
Polyporales	⩾95% (69)	6.1[a]	0[b]	
Filobasidiales	⩾97% (45)	0[a]	1.5[b]	

*Represents the dominant order (based on NCBI BLAST matches) within the operational taxonomic unit (OTU)
†See Appendix A for OTU designations.

taxon dominated by the order Ceratobasidiales comprising ~57% of all sequences, and an Uncultured taxon representing ~2% of all sequences (Table 3). Both of these taxa increased significantly under N fertilization, with Ceratobasidiales also increasing under C addition. The Ceratobasidiales group responded to N and C at broad taxonomic levels, but the response was not detectable at higher taxonomic resolution due to smaller sample sizes (Table 3). However, the response of the Uncultured group to N was well defined at 80–97% sequence similarity.

In contrast, the structure of the soil fungal community did not respond significantly to N fertilization at any level of sequence similarity. Nonetheless, there were several taxonomic groups that did show significant responses to N according to Kruskal–Wallis tests, especially at higher levels of taxonomic resolution. Groups dominated by Hysteriales, Polyporales (OTU 48), and Filobasidiales were significantly more abundant (and only found) under N fertilization, while another group of Polyporales (OTU 69) was strongly suppressed (Table 3). However, each of these groups represented <3% of the total sequences, which may explain why the multivariate analyses of community response to N were not significant.

According to a rank-abundance analysis, dominance by the Ceratobasidiales and/or Uncultured groups came at the expense of common taxa; the fraction of rare OTUs (abundance of 1 or 2 sequences within a treatment at ⩾97% similarity) increased from 59% to 67% and 79% under N and C addition, respectively. In contrast to the litter results, the fraction of rare OTUs at ⩾97% similarity decreased from 78% to 67% with N addition in soil.

4. Discussion

Overall, we obtained 83 OTUs at ⩾97% similarity from litter (44 in control plots) and 118 OTUs in soil (67 in control plots). These levels of fungal diversity are consistent with other studies. Viaud et al. (2000) obtained 58 RFLP groups from 118 fungal cultures and environmental clones from a French agricultural soil using the ITS region. Recently, O'Brien et al. (2005) sequenced >800 ITS clones from temperate forest soils and found 412 OTUs (⩾97% similarity). They may have observed higher richness because they sampled two different forests and multiple soil horizons, or because their sequences included both dormant and active fungi. Our approach identifies only fungi that are active at the time of sampling, so may have missed members of the community active at other times during the year. Also, the small size of our sampling area (0.3–1 m diameter) may have reduced the observed diversity of fungi, although the declining slopes of the rarefaction curves (Fig. 1) suggest that the communities were sampled adequately.

The overall composition of our fungal communities is also reasonable compared to prior studies. Like O'Brien et al. (2005), we found a mixture of Ascomycetes and Basidiomycetes in the soil community, with the Agaricales representing the largest order of Basidiomycetes. Landeweert (2003) sequenced 318 clones from boreal forest soils using Basidiomycete-specific primers for the ITS region and found 29 OTUs (each defined as a cluster on a phylogenetic tree produced by ClustalW). Twenty-five of the OTUs were analyzed with BLAST, and 12 of those were found to match ectomycorrhizal species or genera. A substantial fraction of our soil sequences may also be ectomycorrhizal; in particular, members of the abundant order Cantharellales form mycorrhizal associations (Agerer et al., 1996; Alexopoulos et al., 1996).

Although we conducted BLAST searches for all of our sequences, the resulting species identifications were not always reliable. Multiple organisms with varying ecological characteristics may produce a match, making it difficult to infer the function of an organism based on its DNA sequence. For example, sequences that clustered in the Ceratobasidiales group produced BLAST hits corresponding to widely divergent organisms, including Ascomycetes and Basidiomycetes (Appendix A). However, examination of the detailed database entries revealed that the "divergent" organisms are probably Ceratobasidiales that have been mis-identified. Conversely, sequences in the same taxonomic group according to BLAST were not always in the same OTU as defined by sequence similarity. For instance, soil-derived sequences producing good (⩾97% identity) matches to the genus *Candida* actually belonged to three different OTUs at 90% similarity. These discrepancies could be addressed by sequencing additional genes or more base pairs, and by increasing the number of reliable entries in the public databases.

As predicted, we found good evidence that N deposition reduces Basidiomycete diversity in litter and total fungal diversity in soil. The vast majority of information about fungal responses to N deposition focuses on mycorrhizal (especially ectomycorrhizal) fungi (Treseder, 2004). In general, studies show that ectomycorrhizal diversity declines with N deposition (Lilleskov et al., 2001, 2002; Frey et al., 2004), and our data indicate that this pattern may be generalizable to broader groups of fungi. Although diversity studies on non-mycorrhizal taxa are rare, there is evidence that wood-decaying fungi compete poorly under high-N conditions (Fog, 1988). A reduction in the diversity of decomposer fungi with N addition would be consistent with this pattern.

In addition to lower diversity, litter analyses showed evidence for altered fungal community structure with fertilization. Changes in OTU richness and structure under N and C addition appeared to result from competitive dominance by the Ceratobasidiales group. Parallel responses by the Ceratobasidiales group in the N and C addition treatments could have resulted from parallel changes in C availability. Adding N at the litter study site increases growth and litter inputs of annual grasses (pers. obs.), which is likely to increase the quality and quantity of C available in litter (Yarie and Van Cleve, 1996). Adding starch in the C addition plots probably caused an analogous change in C availability. However, the treatments did not have identical effects, since the Uncultured group responded positively to N but not C addition. Still, these results suggest that the direct effects of N on fungal communities could be outweighed by indirect effects mediated through the plant community.

In the soil, fungal responses to N were limited, and involved a small fraction of the community. We did observe a significant decline in Chao1 estimated richness, probably because rare taxa were less common in soil with added N. Also, three minor groups increased under N addition, while one group declined significantly (Table 3). However, no changes occurred among dominant groups, which is consistent with the non-significant community structure and Shannon diversity results. We note, though, that greater diversity in the soil versus the litter fungal community would make changes in the relative abundances of different OTUs more difficult to detect due to reduced statistical power.

The soil community analyses did not support the hypothesis that N deposition suppresses Basidiomycete fungi. Although there was a trend toward lower relative abundance of Basidiomycetes with fertilization (Table 2), there were no statistically significant differences in community structure at broad levels of taxonomic resolution (80–90% similarity). It is still possible that the absolute biomass of Basidiomycete fungi declined under N addition, but there is no evidence that Basidiomycetes compete poorly relative to other fungi in the soil. Notably, the Cantharellales (which mainly represent the ectomycorrhizal genus *Hydnum*) showed a trend toward increased relative abundance with N addition.

Conducting community analyses at varying levels of sequence similarity provides insight into the taxonomic level at which microbes respond to N. In the litter community, the N response was driven by broad groups of fungi with sequence similarities $\leqslant 90\%$. In the soil, N effects on the fungal community were only evident at higher levels of sequence similarity ($\geqslant 90\%$, Table 3). Some of this difference may be due to the use of the 18S rRNA gene for the soil analyses, which is more conserved than the ITS region used for the litter analyses. Nonetheless, both sets of analyses suggest that fungal responses to N availability can be detected at low levels of taxonomic resolution. Although some responses could be missed, approaches that monitor only broad taxonomic groups may be sufficient to detect fungal responses to environmental changes.

The differences in fungal community structure observed with fertilization emphasize the need to discern the functions of different soil microbes. In particular, the importance of N-responsive taxa for decomposition processes must be determined in order to attribute changes in C cycling to shifts in fungal community structure (Carreiro et al., 2000). Our approach dictates that the DNA sequences we obtained belonged mainly to fungi that were active at the time of sampling. Beyond that, we have little information about the ecology, physiology, or metabolism of the fungi that responded to fertilization. However, the abundance and community structure data will be useful for selecting fungi for more detailed ecophysiological studies.

Our data enabled us to conduct meaningful comparisons of community structure, but what are the ecological consequences of such changes? Reductions in diversity and changes in community structure in other systems can result in reduced or altered ecological function (Hooper and Vitousek, 1997; Tilman et al., 1997). This relationship is especially common when the initial diversity of the community is low, or when particular functions are confined to a small number of taxa (“narrow” functions) (Schimel, 1995; Hooper et al., 2005). For example, Setälä and McLean (2004) found that increasing fungal diversity only affected CO_2 respiration when <12 species were present. Narrow functions include the degradation of organic compounds that require specialized enzymes, such as cellulose (Lynd et al., 2002). However, the degree to which the ecological functions of organic matter degradation and nutrient mineralization are narrowly distributed among fungal taxa is not currently known.

Equally plausible is the possibility that many taxa are functionally redundant, and that changes in community structure have little impact on ecosystem processes. Even under N fertilization, the fungal community still contained at least 33 fungal OTUs in litter and 61 OTUs in soil, values that are well above the saturation cutoffs for richness effects in most fungal studies (Setälä and McLean,

2004; Dang et al., 2005). Thus, the changes in community structure that we observed are not necessarily sufficient to alter ecosystem processes. However, even functionally redundant taxonomic groups may enhance ecosystem stability and serve as "insurance" if some taxa decline in response to disturbance (Chapin et al., 1997; Hooper et al., 2005).

5. Conclusion

Using novel molecular techniques, we found that N fertilization reduces fungal taxonomic richness and alters community structure. These responses occurred in fungal communities that were active at the time of sampling, and applied to both litter and soil environments despite differences in the region that was sequenced (ITS vs. 18S) and the taxonomic breadth of the analysis (mainly Basidiomycetes vs. all fungi). Community changes in the litter were probably driven by shifts in plant inputs associated with N fertilization. Similar responses of fungal communities to N and C fertilization support this conclusion.

Our results highlight several challenges involved in the analysis of microbial community data. Since different analyses often produced slightly different results, we recommend using a range of approaches to assess community change. Due to the possibility of PCR biases and other potential artifacts inherent to molecular techniques, broad surveys of rRNA genes (such as ours) represent only a first step toward identifying members of the microbial community that respond to environmental stimuli (Borneman et al., 2007). Such responses should be confirmed with independent measures of microbial abundance, such as quantitative PCR with primers that target specific taxa. Finally, our results also emphasize the need for more taxonomic and functional data on soil fungi. Declines in fungal diversity and changes in community structure could have important ecosystem consequences, but this question will remain unresolved until more is known about the ecological functions of N-sensitive fungal taxa.

6. Supplementary data

The file "AllisonSBB2007Appendix A.xls" contains a spreadsheet with supplementary Appendix A, which shows the top NCBI BLAST matches for DNA sequences in the (A) litter and (B) soil clone libraries.

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.soilbio.2007.02.001.

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